Phosphorylation activates Chk1 and is required for checkpoint-mediated cell cycle arrest

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Summary

In the fission yeast *Schizosaccharomyces pombe*, the protein kinase Chk1 has an essential role in transducing a delay signal to the cell cycle machinery in the presence of DNA damage. Fission yeast cells lacking the *chk1* gene do not delay progression of the cell cycle in response to damage and are thus sensitive to DNA damaging agents. We have previously shown that Chk1 is phosphorylated following DNA damage induced by a variety of agents and that this is dependent on the integrity of the DNA damage checkpoint pathway, including Rad3, the ATR homolog. Through a combination of mutagenesis and phospho-specific antibodies, we have shown that serine at position 345 (S345) is phosphorylated in vivo in response to DNA damage, and that S345 phosphorylation is required for an intact checkpoint response. We have developed a kinase assay for Chk1, and have shown that basal Chk1 kinase activity is increased in response to DNA damage and that this increase, but not the basal activity, is dependent on S345. Furthermore, we show that S345 phosphorylation is required for Chk1 to associate with Rad24, a 14-3-3 protein, upon DNA damage. These results are consistent with a model whereby Chk1 phosphorylation results in increased Chk1 kinase activity that is necessary for both checkpoint delay and cellular survival following damage to the genome. These data are similar to observations made in mammalian cells and *Xenopus* oocyte extracts, suggesting that mechanisms leading to Chk1 activation have been conserved in evolution.

Key words: Chk1, DNA damage, Checkpoint, Cell cycle

Introduction

The DNA damage checkpoint is a signal transduction pathway that delays entry into mitosis following DNA damage (Hartwell and Weinert, 1989). Many of the proteins of this pathway have been conserved throughout evolution. In the fission yeast *Schizosaccharomyces pombe*, the protein kinase Chk1 has an essential role in transducing the delay signal to the cell cycle machinery. Fission yeast cells lacking the *chk1* gene do not delay progression of the cell cycle in response to damage and are thus hypersensitive to DNA damaging agents (Walworth et al., 1993; al-Khodairy et al., 1994). Chk1 kinase phosphorylates specific components of the cell cycle machinery that may lead to blockage of the entry into mitosis until genomic integrity is restored (O'Connell et al., 2000).

Cell cycle progression in eukaryotes is mediated by the activation of a highly conserved family of protein kinases, the cyclin-dependent kinases (CDKs) (Pines, 1995). Much has been learned in recent years regarding the interaction between Chk1 and the core regulators of CDKs (O'Connell et al., 2000). Chk1 from fission yeast and *Xenopus* can phosphorylate the proteins Wee1 and Cdc25 in vitro (O'Connell et al., 1997; Peng et al., 1997; Sanchez et al., 1997; Zeng et al., 1998; Baber-Furnari et al., 2000). These proteins regulate phosphorylation of critical residues of Cdc2, the cdk that controls entry into mitosis (MacNeill and Nurse, 1997). The Wee1 tyrosine kinases (Wee1 and Mik1 in fission yeast, Wee1 in higher eukaryotes) phosphorylate Cdc2 at tyrosine 15 (Y15), and the Cdc25 phosphatases dephosphorylate this residue (Tang et al., 1993; Lundgren et al., 1991; Millar et al., 1991). In higher eukaryotes, an additional kinase, Myt1, additionally phosphorylates Cdc2 on threonine 14 (T14), though recent studies suggest that phosphorylation of this residue is not involved in checkpoint control (Fletcher et al., 2002). During G2, the phase of the cell cycle between S phase (DNA replication) and mitosis, Cdc2 is regulated by cyclin B association and phosphorylation at the negative regulatory sites, T14 and Y15 in vertebrates and Y15 alone in fission yeast. Cdc2/cyclin B complexes become fully active when these residues are dephosphorylated, allowing cells to progress into mitosis (MacNeill and Nurse, 1997). In response to DNA damage, phosphorylation of Y15 of fission yeast Cdc2 is maintained, probably through the activities of Wee1 and Mik1 (Kharbanda et al., 1994; O'Connell et al., 1997; Rhind et al., 1997), suggesting that regulators of this phosphorylation may be targets of the DNA damage checkpoint pathway in fission yeast. One role of Chk1 may be to cause cell cycle arrest in

response to DNA damage by regulating the activity of Wee1 and Mik1 proteins via phosphorylation (O'Connell et al., 1997; Baber-Furnari et al., 2000). Additionally, Chk1 may act by inhibiting the function of the Cdc25 phosphatase (Peng et al., 1997; Sanchez et al., 1997).

We have previously shown that a significant fraction of Chk1 is phosphorylated following DNA damage induced by a variety of agents including UV light, ionizing radiation (IR), reduced DNA ligase activity, and camptothecin (CPT) (Wan et al., 1999; Walworth and Bernards, 1996). Phosphorylation results in a species of Chk1 with decreased mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This decrease in mobility is dependent on several components of the DNA damage checkpoint pathway including Rad1, Rad3, Rad9, Rad17, Rad26, Hus1 and Crb2 (Walworth and Bernards, 1996; Saka et al., 1997). As a presumed kinase-inactive mutant of Chk1 was shown to lack this mobility shift, we initially proposed that the shift might result from autophosphorylation (Walworth and Bernards, 1996). However, a different kinase inactive mutant does undergo the mobility shift suggesting that the phosphorylation event that causes it is likely to be carried out by a distinct kinase [(Wan and Walworth, 2001) and see below].

Vertebrate Chk1 is phosphorylated by ATR, the ATM- and Rad3-related protein kinase (Zhao and Piwnica-Worms, 2001; Liu et al., 2000). ATM is the gene that is mutated in the human genetic disorder Ataxia Telangiectasia (AT), which is characterized by hypersensitivity to IR, neuronal degeneration, immune dysfunction, cancer predisposition, and premature aging (Savitsky et al., 1995; Lavin and Shiloh, 1997). Cell lines from AT patients have a defective checkpoint response to IR (Kastan and Lim, 2000). ATR is an ATM-related protein that also has a role in the IR-induced DNA damage checkpoint. Unlike ATM, however, ATR also has a role in the UV and hydroxyurea (HU)-induced damage checkpoints (Cimprich et al., 1996). ATR, but not ATM, has been shown to be an essential gene for mouse embryogenesis, as has Chk1 (Barlow et al., 1996; Xu and Baltimore, 1996; Brown and Baltimore, 2000; Liu et al., 2000), though expression of dominant negative mutants of ATR or Chk1 in cell lines interferes with checkpoint responses, but not cellular viability (Koniarias et al., 2001). However, a recent study suggests that conditional homozygous knockout of ATR is not compatible with long-term somatic cell viability (Cortez et al., 2001).

Both ATM and ATR prefer to phosphorylate serine (S) or threonine (T) residues that are followed by a glutamine (Q) (Kim et al., 1999). The Chk1 amino acid sequence has several conserved SQ/TQ sites. In *Xenopus* oocyte extracts, Chk1 phosphorylation in response to aphidicolin is dependent on ATR. This phosphorylation is also dependent on the integrity of the conserved SQ/TQ sites. A phosphoantibody was used to identify S344 as one of the phosphorylated sites (Guo et al., 2000). Human Chk1 is phosphorylated by ATR at the analogous site, S345, and another site, S317, in response to UV, IR, and HU (Zhao and Piwnica-Worms, 2001). In fission yeast, Rad3 and Chk1 can co-immunoprecipitate following moderate overexpression, even in the absence of DNA damage, suggesting that Rad3, like its human homolog ATR, may phosphorylate Chk1 in response to DNA damage (Martinho et al., 1998). Rad3 can phosphorylate a fragment of fission yeast Chk1 in vitro (Lopez-Girona et al., 2001).

The in vivo consequences of S317 or S345 phosphorylation are not yet clear. Phosphorylated fission yeast Chk1 co-immunoprecipitates with 14-3-3 proteins, small highly conserved proteins that may be involved in regulation of intracellular localization and facilitation of protein-protein interactions (Chen et al., 1999). Due to conflicting results, it is unclear whether Chk1 phosphorylated in response to DNA damage has increased activity. Two groups claim that human Chk1 kinase activity is increased following treatment with UV, HU, or BNP 1350, a topoisomerase I inhibitor (Zhao and Piwnica-Worms, 2001; Mailand et al., 2000); whilst one other group claims no change in activity following genotoxic stress (Kaneko et al., 1999).

To determine which phosphorylation site(s) in fission yeast Chk1 is important for the function of Chk1 in the DNA damage checkpoint pathway, we constructed mutants in which serines or threonines within the SQ/TQ motifs of *S. pombe* Chk1 were substituted with alanine or aspartic acid. Consistent with the results of others (Lopez-Girona et al., 2001), we demonstrate that mutation of serine 345 to alanine abolishes the mobility shift caused by exposure to DNA damaging agents. However, it is clear that mutation of a variety of other residues in Chk1 also abolishes the mobility shift [(Wan and Walworth, 2001) and see below], and that this largely correlates with a failure to localize to the nucleus. The serine 345 to alanine mutant, however, is nuclear, eliminating mislocalization as a reason for the failure to shift. More significantly, using antibody directed toward a peptide containing phospho-serine 345, we report that serine 345 of fission yeast Chk1 is phosphorylated in vivo in response to treatment with UV light or CPT, a topoisomerase I poison. Furthermore, S345 phosphorylation is necessary for an increase of basal Chk1 kinase activity in response to DNA damage that is critical to the integrity of the DNA damage checkpoint pathway. DNA damage-induced activation of Chk1 is impaired in a strain lacking Rad3 function. In sum, these data provide important in vivo evidence to support the model of Chk1 activation by Rad3-mediated phosphorylation.

Materials and Methods

Plasmids, strains, media, and growth conditions

Site-directed mutagenesis of *S. pombe chk1* was performed using the QuickChange Site-Directed Mutagenesis Kit from Stratagene. Primers were synthesized as instructed by the manufacturer to anneal to approximately 30 bases surrounding the target codons. The templates for PCR were cDNA *chk1* with three copies of the HA epitope (Walworth and Bernards, 1996) cloned into pBluescript or pRep1 (Basi et al., 1993; Maundrell, 1993; Maundrell, 1990) at the *Nde*I site or a genomic fragment of *chk1* with three copies of the HA epitope (Walworth and Bernards, 1996) cloned into pSP1 (Cottarel et al., 1993) at the *Hin*dIII site or a modified version of pSP1, pSP1-chk1epΔAH (Wan and Walworth, 2001). The entire coding region of each mutant *chk1* allele was sequenced. Mutant cDNA *chk1* alleles generated in pBluescript were cloned into the *Nde*I site of pRep1. cDNA alleles were fused to green fluorescent protein by cloning a *Not*I fragment of GFP (Tatebe et al., 2001) into the *Not*I site of pRep1/*chk1:ep*. Plasmids were transformed into an *S. pombe chk1::ura4* strain, NW 158 (Walworth and Bernards, 1996), using the LiAc transformation method as described (Moreno et al., 1991). *Hin*dIII or *Eco*RV fragments of genomic *chk1* alleles generated in pSP1 or pSP1-chk1epΔAH, respectively, were integrated into the *S. pombe* genome by gene replacement of the *chk1::ura4* allele as described previously for integration of the *chk1:ep* allele (Walworth

and Bernards, 1996). Integrations were confirmed by southern blot analysis. The *S. pombe* strains used in this study are listed in Table I. Cells were grown in YEA medium (Moreno et al., 1991) at 30°C, except in experiments utilizing temperature-sensitive mutants, which were grown at 25°C. Cells harboring plasmids were grown in PM medium made from Edinburgh minimal medium (EMM) (Bio-101) prepared according to the manufacturers instructions, with the appropriate supplements added at 75 μg/ml. In experiments utilizing thiamine repressible promoters, 20 μM thiamine was added to the medium to repress protein overexpression.

Treatment with camptothecin and UV light

Camptothecin lactone (CPT) was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Cells were treated with 40 μM CPT for 2 hours as described (Wan et al., 1999). To determine the state of Chk1 phosphorylation after UV treatment, cells were grown overnight to mid-log phase and 1×10^8 cells were spread on agar plates. Cells were exposed to a dose of 100 J/m^2 of UV light and harvested for lysate preparation after 20 minutes. Survival following UV treatment was determined as described (Walworth et al., 1993) or by spotting 5 μl 10-fold dilutions of a 1×10^7 cells/ml culture on agar plates and exposing the cells to 100 J/m^2 of UV light. Plates were analyzed after 3 days. The ability of *chk1⁻:ep* cells to arrest the cell cycle in response to UV light treatment was determined by measuring the percentage of cells from a synchronized population of cells exposed to UV light that have passed mitosis at 20 minute intervals following treatment, as described (Wan and Walworth, 2001).

Lysate preparation, immunoprecipitation and immunoblotting

For lysate preparation, cells were harvested by centrifugation and lysed using glass beads and a FastPrep (Bio 101) vortexing machine. Lysate was centrifuged at 735 ***g*** for 1 minute and the supernatant was collected for protein determination using the Bradford assay method (Bio-Rad). For western blot analysis of total cell extracts, cells were lysed in Phosphate Buffered Saline (PBS) + 1% Triton-X 100 + Complete Protease Inhibitor (Roche Diagnostics), aliquots were run on 8% SDS-PAGE, transferred to nitrocellulose (BA83, Schleicher and Schuell), and probed with 12CA5 antibody. Blocking of the membranes and all antibody incubations and washes were done with 1% milk and 0.05% Tween-20 in PBS. 12CA5 antibody to the HA epitope was used at a 1:500 dilution. A peroxidase-coupled secondary antibody (Boehringer Mannheim) and the enhanced chemiluminescence detection system from NEN-Dupont (Renaissance) were used to detect the immune complexes. Filters were exposed to Kodak BioMax film for 2-60 minutes.

For Chk1 immunoprecipitation, cells were lysed in IP buffer (10 mM $NaPO_4$ pH 7, 0.15 M NaCl, 1% NP-40, 10 mM EDTA, 50 mM NaF, 2 mM DTT, 50 mM PMSF, and Complete Protease Inhibitor (Roche Diagnostics) and 100 μl of F-7 anti-HA antibody (Santa Cruz) was added to 7-10 mg of protein. The IP volume was brought up to 500 μl using IP buffer and the IPs were incubated at 4°C on a rotator for 1-16 hours. 30 μl of recombinant protein A sepharose beads were added and the IPs were incubated for 1-2 hours. The IPs were then washed three times with IP buffer. 120 μl of 2× Laemmli sample buffer was added to the dry beads and boiled for 5 minutes. The IPs were split into 55 μl aliquots, run on 8% SDS-PAGE, transferred to nitrocellulose, and probed with SN252-1, antibody to *S. pombe* Chk1 phosphorylated on serine 345, at 1:4000 or Y-11 anti-HA antibody (Santa Cruz) at 1:1000. The rabbit polyclonal antibody, SN252-1, was raised against a phospho-serine 345 peptide, VEVYGALS(PO3)QPVQLNK by SynPep Corporation. Attempts to raise an antibody against a phospho-serine 367 peptide, DPSLS(PO3)QPVQLNK, that was useful for western blot analysis were unsuccessful. Blocking of the membranes and all antibody incubations and washes were done as described above for the 12CA5 antibody.

For co-immunoprecipitation of Chk1 with Rad24, 500 ml of mid-log phase cells were or were not treated with 40 μM CPT for two hours, harvested, and lysates were prepared by grinding in liquid nitrogen based on a method described by Ansari et al. (Ansari et al., 1999), as described (Chen et al., 1999). Immunoprecipitations were performed with anti-Rad24 antibody (UMDNJ 55) as described (Chen et al., 1999). The IPs were washed 3 times using lysing buffer and 60 μl 2× Laemmli sample buffer was added to the dry beads. 55 μl of each IP was run on 8% SDS-PAGE, transferred to nitrocellulose, and probed with 12CA5 antibody as described above.

Localization of SQ/TQ mutants in *S. pombe*

To determine the localization of Chk1, Chk1D155A, Chk1S345A, Chk1S345D, Chk1S367A and Chk1S367D, *chk1::ura4* cells were transformed with pRep1 harboring *chk1⁺* or *chk1⁻* cDNA fused to the gene encoding GFP (Tatebe et al., 2001). Cells were grown to mid-log phase in PMA containing 20 μM thiamine and analyzed using a wildtype GFP filter in a Zeiss microscope equipped with a Zeiss Axiocam and OpenLab software. The localization of Chk1K38A, Chk1G108S, Chk1N142D, Chk1L144P and Chk1D155G were determined by visualizing those proteins fused in genomic constructs to GFP and expressed from the Chk1 promoter in the plasmid pSP1 (Cottarel et al., 1993) in a *chk1::ura4* background. Cells were visualized with an Olympus IX70 microscope and images captured with a MicroMax CCD camera from Princeton Instruments using IP Lab Software.

Synthetic lethality of *chk1* SQ/TQ mutants with *cdc17-K42*

chk1⁻ cdc17-k42 double mutants were obtained using random spore analysis. Cells were grown to mid-log phase at 25°C and resuspended in YEA to 1×10^7 cells/ml. 5 μl of 10-fold dilutions were spotted onto YEA agar plates and incubated at 25°C, 32°C, or 36°C for 3 days. Pictures were analyzed to determine if colonies were formed by the double mutant strains.

Kinase assay

Optimal assay conditions for Chk1 were determined by kinetic analysis of recombinant protein, and defined conditions that were linear over time (U.J. and M.O., data not shown). *Chk1⁺:ep* or *chk1⁻:ep* cells were grown to mid-log phase, were or were not treated with CPT, and harvested. Cells were lysed in IP buffer as described above. Chk1 was immunoprecipitated from 2-3 mg of protein, in triplicate, using F-7 anti-HA antibody and 20 μl of recombinant protein A sepharose beads as described above. The IPs were washed 3 times with IP buffer and 3 times with 2× kinase buffer (50 mM Tris pH 7, 1 mM DTT, 5 mM $MgCl_2$, 0.4 mM $MnCl_2$, 25% SIGMA glycerol, 0.1% Triton X-100, 100 μM ATP). 24 μl of kinase reaction (kinase buffer, 8-12 μg peptide (RIARAASMAAALARK), and [γ-^{32}P]ATP (3000 Ci/mmole)) was then added to each IP, incubated at 30°C for 10 minutes, and immediately placed on ice. 20 μl of kinase reaction was spotted onto P81 phosphocellulose paper (Whatman), washed three times in 5% phosphoric acid, rinsed with ethanol, dried, placed in a scintillation vial with scintillation fluid, and counted for 30 seconds in a liquid scintillation counter.

Results

Mutation of serine 345 to alanine abolishes the Chk1 electrophoretic mobility shift in response to UV light and camptothecin and confers sensitivity to UV light

The Chk1 protein contains several SQ/TQ motifs that are consensus sites for phosphorylation by the ATM family of

protein kinases (Kim et al., 1999). Three of these consensus sites, T323, S345, and S367, can be aligned in the human, mouse, *Xenopus*, and fission yeast homologs (Fig. 1A). Phosphorylation of Chk1 in response to DNA damage can be observed as a decrease in mobility on SDS-PAGE (Walworth and Bernards, 1996). To determine if phosphorylation of one or more of the conserved SQ/TQ motifs might be required for this mobility shift, we mutated codons within genomic *chk1* to change the relevant amino acid residues from serine or threonine to alanine. The mutant *chk1* alleles were integrated into the genome of a *S. pombe* strain lacking *chk1* and expressed from the endogenous *chk1* promoter (see Materials and Methods). Chk1 is phosphorylated in response to DNA damage caused by ionizing radiation, UV light, inactivation of DNA ligase, and treatment with CPT, a topoisomerase I inhibitor (Wan et al., 1999; Walworth and Bernards, 1996). We exposed the cells to CPT or UV light and used western blot analysis to monitor the mobility of the mutant proteins. The mobility shift in response to CPT or UV light was abolished in Chk1S345A (Fig. 1B,C), suggesting that S345 is necessary for the mobility shift of Chk1 in response to DNA damage. The Chk1S367A protein undergoes a mobility shift equivalent to that of wild-type Chk1, while the Chk1T323A mutant protein appears to shift less efficiently (Fig. 1B,C). However, we see no phenotypic consequence of mutation at that site (Fig. 1D).

Cells lacking *chk1* have increased sensitivity to UV light as compared to wildtype cells due to an inability to prevent mitotic entry prior to completion of DNA repair (Walworth et al., 1993). To determine if the SQ/TQ motifs are required for cells to survive exposure to UV light, we inoculated decreasing concentrations of wildtype, *chk1::ura4*, or *chk1*$^{-}$ cells onto agar plates and exposed them to UV light (see Materials and Methods). Chk1S345A cells exhibited an increased sensitivity to UV light, similar to that of a *chk1* deletion strain; however, cells harboring the *chk1T323A* or *chk1S367A* mutant alleles remained wildtype for their UV response (Fig. 1D). Taken together, these results are consistent with the possibility that S345, but not T323 or S367, is a phosphorylation site critical for the mobility shift of Chk1 in response to DNA damage and for the function of Chk1 in the DNA damage checkpoint pathway.

Mutation of non-phosphorylatable residues abolishes mobility shift of Chk1

In a previous study, we isolated a number of DNA damage-

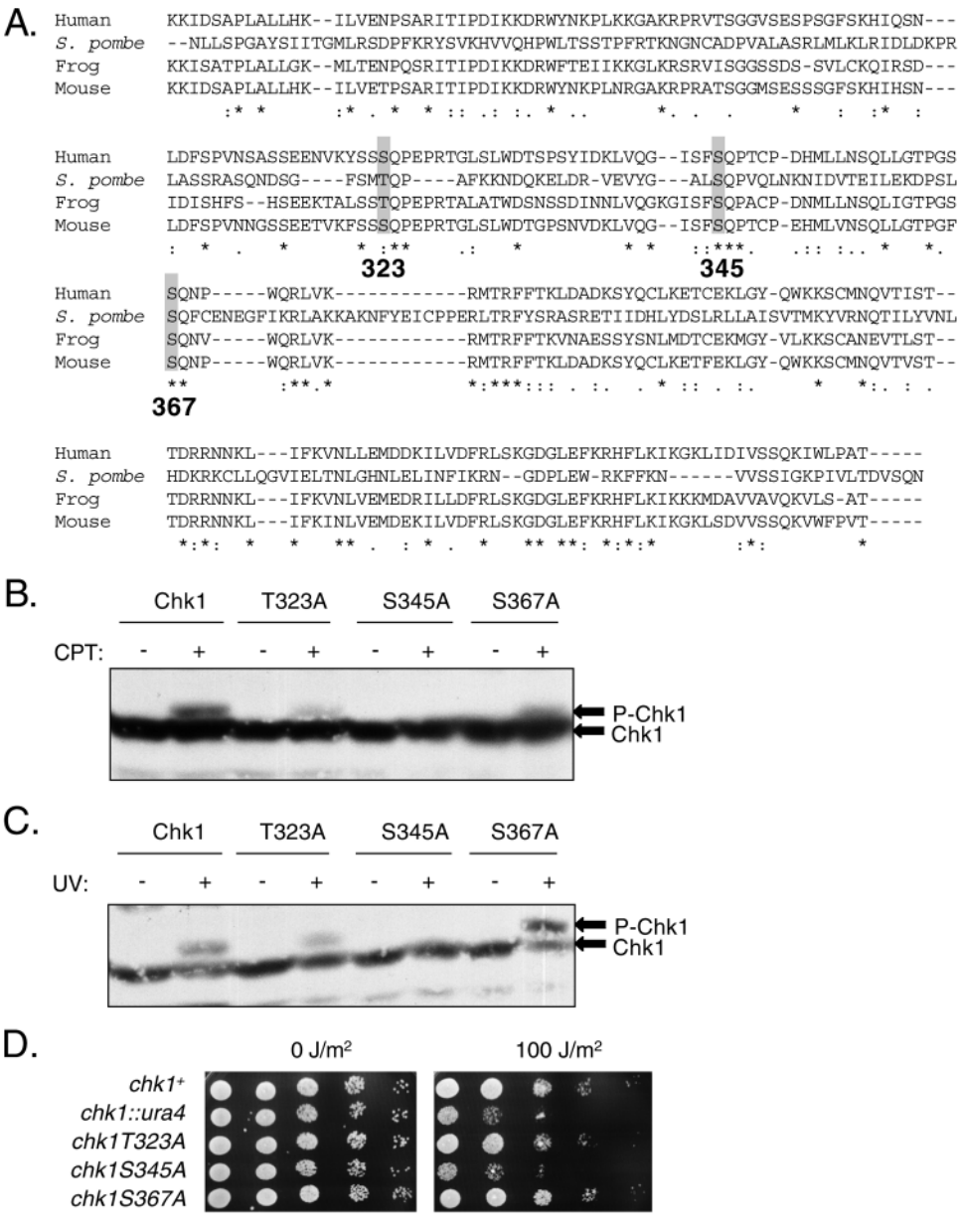

Fig. 1. Mutating serine 345 to alanine abolishes the change in Chk1 mobility in response to CPT and UV light and confers UV sensitivity. (A) CLUSTAL W alignment of the non-catalytic domains of human, fission yeast, frog and mouse Chk1. Numbers refer to amino acids in the fission yeast protein. (B) NW223 (Chk1), NW457 (T323A), NW444 (S345A) and NW481 (S367A) cells grown to mid-log phase were treated with CPT, lysed, subjected to SDS-PAGE and immunoblotted with 12CA5 anti-HA antibody. (C) Strains shown in B were grown to mid-log phase, spread on agar plates, treated with 100 J/m^2 UV light, lysed, subjected to SDS-PAGE, and immunoblotted with 12CA5 anti-HA antibody. (D) NW223 (*chk1*$^{+}$), NW158 (*chk1::ura4*), NW457 *(chk1T323A*), NW444 (*chk1S34A*), and NW481 (*chk1S36A*) cells were grown to mid-log phase, resuspended to 1×10^7 cells/ml, spotted onto agar plates, and incubated at 30°C for approximately 3 days.

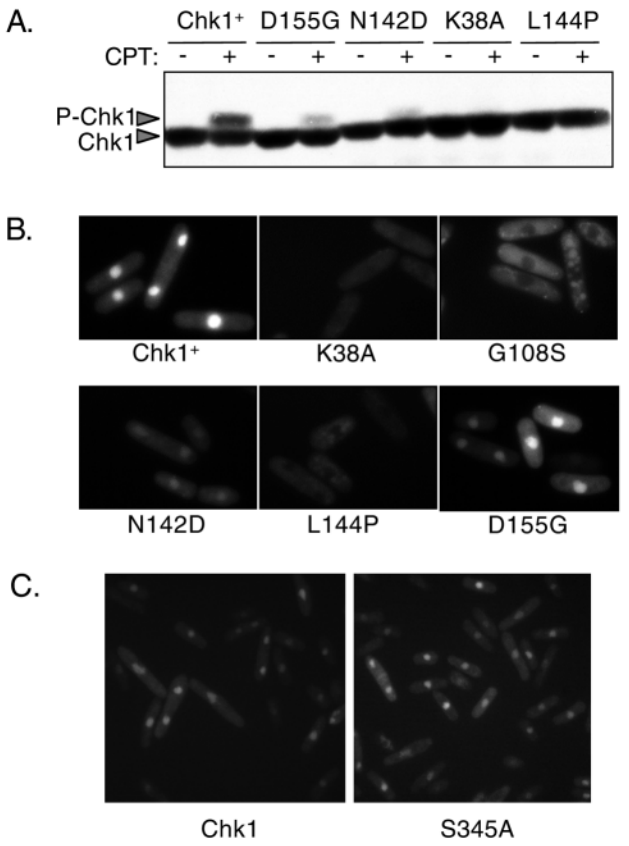

Fig. 2. Failure to undergo the DNA damage-induced mobility shift correlates with lack of nuclear localization. (A) Strains expressing the indicated catalytic domain mutants with three HA tags at the C-terminus were expressed from the chk1 promoter on the plasmid pSP1 in a *chk1::ura4* disruption strain (NW158). Cells in mid-log phase were exposed to 20 μM CPT for 2 hours. Lysates were prepared, separated by SDS-PAGE and examined by western blot analysis for the HA tag on Chk1. Data is not shown for Chk1G108S, but it shows no evidence of a mobility shift in response to CPT treatment. (B) Strains expressing the indicated catalytic domain mutants with GFP fused to the C-terminus were expressed in a *chk1::ura4* disruption strain (NW158) from the *chk1* promoter on the plasmid pSP1. Cells were visualized with a fluorescence microscope. $Chk1^+$ is wild-type Chk1-GFP. Alleles of Chk1 are indicated by the mutated amino acid number and substitution. (C) cDNAs encoding wild-type Chk1 or Chk1S345A fused to GFP were expressed from the *nmt1* promoter on the pREP1 vector under repressing conditions in a *chk1::ura4* disruption strain (NW158). Live cells were visualized by fluorescence microscopy.

sensitive alleles of Chk1 with mutations in the catalytic domain that conferred checkpoint sensitivity (Wan and Walworth, 2001). Several of these mutants did not undergo the mobility shift normally observed for the wild-type protein, yet none of the mutations were found to be in serine, threonine or tyrosine residues (Wan and Walworth, 2001). Thus, the failure of the S345 mutant to undergo the mobility shift is not necessarily attributable to its being a site of phosphorylation. Analysis of the localization of the mutants identified in the aforementioned study indicates that failure to undergo the mobility shift correlates with a failure to localize efficiently to the nucleus (Fig. 2A,B and data not shown). To determine whether mutation of S345 affects nuclear localization, the Chk1S345A protein was fused to GFP and examined by microscopy. As shown in Fig. 2C, the Chk1S345A protein localizes to the nucleus in a manner that is indistinguishable from wild-type Chk1.

A serine 345 phosphoantibody recognizes only phosphorylated Chk1

Failure of Chk1 to undergo the mobility shift is clearly not a suitable assay for concluding that a residue is a phosphorylation site; therefore, we produced antibodies against a serine 345 phosphopeptide (see Materials and Methods) to determine if this antibody would recognize Chk1 that is phosphorylated in response to the DNA damage produced upon CPT treatment. To this end, we immunoprecipitated Chk1 or Chk1S345A and performed immunoblot analysis of SDS-PAGE with antibody to the phosphopeptide (see Materials and Methods). The serine 345 phosphoantibody strongly recognized Chk1 only upon treatment with CPT and did not recognize Chk1S345A with or without CPT treatment (Fig. 3A). We conclude that serine 345 in fission yeast Chk1 is a site that is phosphorylated in response to DNA damage and is important for the function of Chk1 in the DNA damage checkpoint pathway.

Chk1S345A and Chk1S345D retain a basal level of kinase activity, but this activity is not activated by DNA damage

To determine the role of S345 phosphorylation in regulating the activity of Chk1, we performed a kinase assay using protein immunoprecipitated from CPT-treated or untreated cells and a peptide substrate (see Materials and Methods). The kinase activity of Chk1 was increased approximately two-fold in response to CPT treatment. Activity associated with a presumed catalytically inactive allele of Chk1, Chk1D155A, was minimal. To determine what role the phosphorylation state of serine 345 might play in regulating Chk1 activity, we assayed the kinase activity of the Chk1S345A mutant. In addition, we constructed an allele of Chk1 in which S345 was changed to aspartic acid in an attempt to mimic the negatively charged, phosphorylated state (Li et al., 2000; Huang and Erikson, 1994), and assayed its kinase activity as well. As shown in Fig. 3B, the kinase activity of Chk1S345A and Chk1S345D from treated or untreated cells was equivalent to that of Chk1 from untreated cells, suggesting that Chk1S345A and Chk1S345D possess a basal level of kinase activity that cannot be activated in response to CPT (Fig. 3B). Since the activity of the Chk1S345D mutant protein resembles that of the Chk1S345A mutant, we assume that aspartic acid is not a good substitute for phosphorylated serine in the context of Chk1.

Overexpression of Chk1, Chk1S345A, and Chk1S345D, but not Chk1D155A, causes cell elongation [(Rhind et al., 1997; Ford et al., 1994) and data not shown], providing further evidence that the serine 345 mutants retain a basal level of kinase activity that can generate a cell cycle arrest when expressed at high levels. Chk1D155A possesses a mutation at position 155 of Chk1, which is within subdomain VII of the catalytic domain. Structural data for several kinases suggest this conserved residue helps to orient the γ-phosphate of ATP

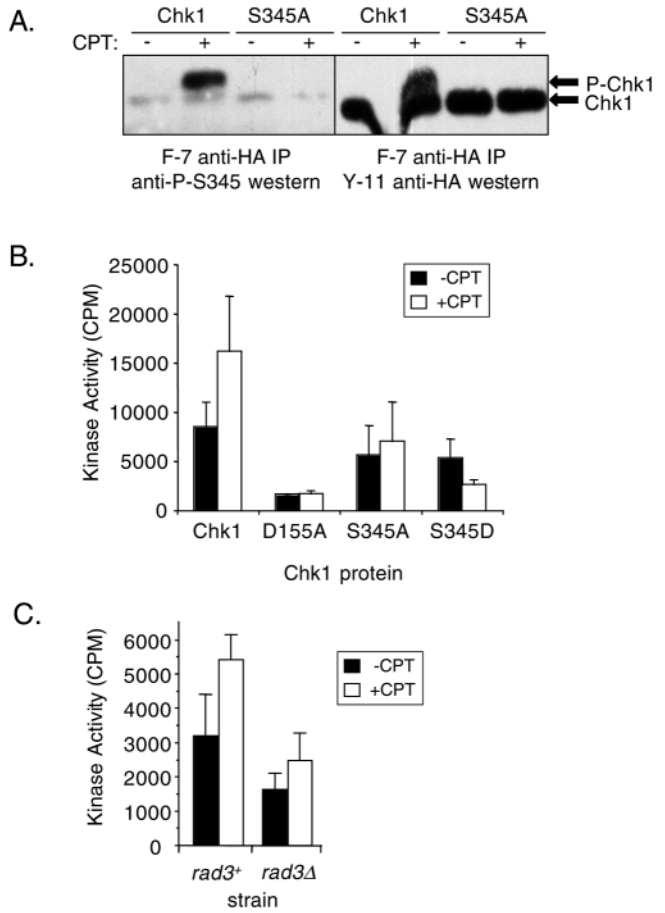

Fig. 3. Serine 345 is phosphorylated and required for activation of Chk1 kinase activity in response to DNA damage. (A) A serine 345 phosphoantibody recognized Chk1 but not Chk1S345A after exposure to CPT. NW556 (Chk1) and NW492 (S345A) were grown to mid-log phase, treated with CPT and lysed. Lysate was incubated with F-7 anti-HA antibody, subjected to SDS-PAGE, and immunoblotted with S345 phosphoantibody or Y-11 anti-HA antibody. (B) Serine 345 phosphorylation is required for activation of Chk1 kinase activity in response to CPT. Chk1S345D is not constitutively activated. Immunoprecipitated Chk1, Chk1D155A, Chk1S345A and Chk1S345D were assayed for their ability to phosphorylate a peptide substrate. All values are from triplicate immunoprecipitations except Chk1+CPT and S345D+CPT, which are derived from two immunoprecipitations. (C) Full activation of Chk1 requires the checkpoint pathway protein Rad3. Chk1 was prepared as in B from a wild-type strain, NW223, and from a strain with a deletion of the rad3 protein, NW246, and assayed as above. All values are from triplicate immunoprecipitations; the data shown represents the mean, with error bars showing the standard deviation.

by chelating the activating Mg^{2+} ions that bridge the β- and γ-phosphates (Hanks and Hunter, 1995). This mutation, therefore, is expected to compromise catalytic activity, an expectation that is supported by its minimal level of kinase activity in this assay (Fig. 3B). These results suggest that Chk1 kinase activity is increased in response to CPT and that this increase is dependent on the phosphorylation of serine 345.

Phosphorylation of Chk1 requires the kinase Rad3 (Walworth and Bernards, 1996). To determine whether Rad3 function is required for activation of Chk1 kinase activity in response to DNA damage, Chk1 was prepared from cells that have a deletion of the *rad3* gene. As shown in Fig. 3C, full activation of Chk1 is impaired in cells lacking Rad3 function. In this experiment Chk1 activity prepared from wild-type cells increased nearly two-fold in response to CPT treatment, while an increase of ~50% was seen in the *rad3* deletion strain. The residual increase might simply reflect noise in the assay resulting from co-precipitation of contaminating kinases. Alternatively, the residual increase could reflect a Rad3-independent, but phosphorylation-dependent activation of Chk1.

Checkpoint function and the ability to overcome DNA damage is compromised in cells expressing Chk1S345A and Chk1S345D

To determine if the DNA damage checkpoint was intact in cells expressing Chk1S345A and Chk1S345D, we analyzed mitotic entry of a synchronous population of cells following exposure to UV light. The *chk1S345A* strain behaved in a manner similar to a *chk1::ura4* strain in that it entered mitosis without a UV-induced arrest, indicating that the checkpoint arrest is abolished in these cells. The *chk1S345D* strain delayed entry into mitosis 40 to 60 minutes longer than a *chk1::ura4* or *chk1S345A* strain, but entered mitosis 100 to 120 minutes earlier than a wildtype strain (Fig. 4A). We conclude that the mutation of serine 345 to alanine or aspartic acid results in the loss of the ability of cells to delay entry into mitosis for the length of time needed for cells to cope with DNA damaged by UV light.

Activation of the DNA damage checkpoint and the transient block to mitotic entry correlates with improved survival following DNA damage (al-Khodairy and Carr, 1992). Cells lacking *chk1* fail to arrest the cell cycle in response to DNA damage, enter mitosis with damaged DNA, and die (Walworth et al., 1993; al-Khodairy et al., 1994). To characterize the UV sensitivity of cells expressing Chk1S345A and Chk1S345D, we performed quantitative UV survival assays by plating 500 cells onto agar plates and exposing them to UV light (see Materials and Methods). As shown in Fig. 4B, cells expressing Chk1S345A and Chk1S345D have increased UV sensitivity equivalent to that of a *chk1* deletion strain.

Checkpoint function is also required for *S. pombe* cells to survive a reduction of DNA ligase activity, which among its essential functions is necessity to join Okazaki fragments following replication (Walworth et al., 1993; al-Khodairy and Carr, 1992). The *cdc17-K42* allele of the gene that encodes DNA ligase is completely inactive at its restrictive temperature of 36°C, but retains some activity at lower temperatures (Nasmyth, 1977). A *chk1::ura4 cdc17-K42* strain cannot survive at the semi-permissive temperature of 32°C, a temperature at which a *chk1*$^{+}$ *cdc17-K42* strain survives; however, it can survive at the permissive temperature of 25°C. *cdc17-K42* double mutant strains with both S345 *chk1*$^{-}$ alleles survived at 25°C, but were unable to survive at the semi-permissive temperature of 32°C (Fig. 4C), indicating that both S345 *chk1*$^{-}$ alleles abolish checkpoint function. Large colonies appeared on patches derived from both strains indicating the selection of suppressing mutations that rescue the viability of these strains. The identity of these suppressors is currently under investigation.

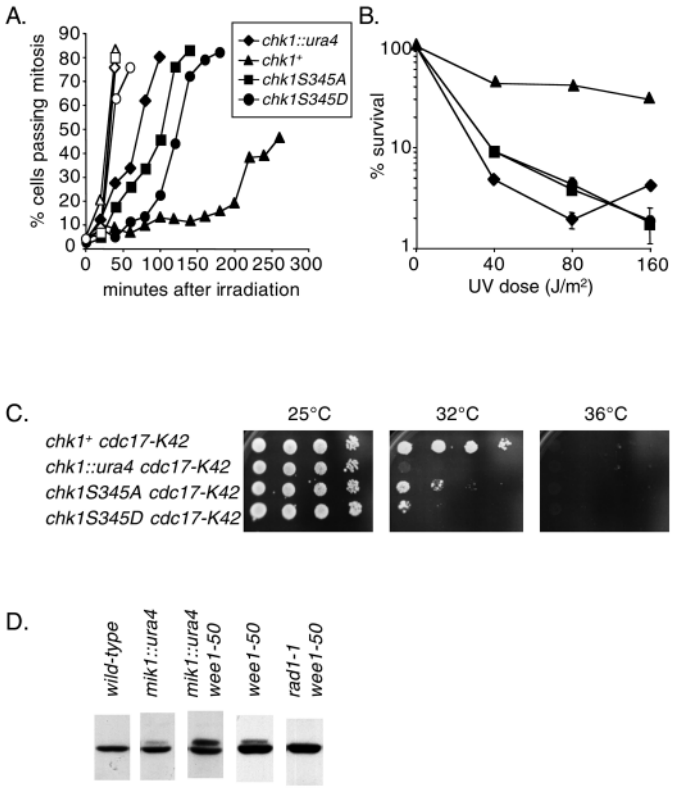

Fig. 4. Mutation of serine 345 to alanine or aspartic acid compromises checkpoint function and viability following exposure to DNA damage. (A) NW228 (*chk1*$^+$), NW107 *(chk1::ura4*), NW442 *(chk1S345A*) and NW477 (*chk1S345D*) cells harboring the *cdc25-22* temperature-sensitive mutant were grown to mid-log phase at 25°C, plated on agar plates, shifted to 36.5°C for 2 hours, treated with UV light, shifted back to 25°C, and monitored for entry into mitosis. Symbols for strains are shown; closed symbols, + UV; open symbols, – UV. (B) NW223 *(chk1*$^+$), NW158 *(chk1::ura4*), NW444 *(chk1S345A*) and NW463 (*chk1S345D*) cells were grown to mid-log phase, spread on agar plates, exposed to UV, and incubated for approximately 3 days. Colony number was determined and is expressed as a percentage of colonies appearing on untreated plates. Symbols for strains are as in A. (C) NW227 *(chk1*$^+$), NW157 (*chk1::ura4*), NW443 (*chk1S345A*) and NW479 (*chk1S345D*) cells harboring the *cdc17-K42* temperature-sensitive mutant were grown to mid-log phase, resuspended at 1×10^7 cells/ml, spotted onto agar plates at 10× dilutions, and incubated for approximately 3 days at 25°C, 32°C or 36°C. (D) Phosphorylation of Chk1 occurs in strains with the wee phenotype, which are dependent on *chk1* for survival. Strains NW223 (wild-type), NW252 (*mik1::ura4*), NW253 (*mik1::ura4 wee1-50*), NW230 (*wee1-50*) and NW239 (*rad1-1 wee1-50*) were grown at 25°C and lysed for analysis by immunoblot with 12CA5 anti-HA antibody.

Further evidence that phosphorylation of Chk1 is critical for Chk1 function in vivo stems from an examination of the status of Chk1 in strain backgrounds containing mutations in the Cdc2 tyrosine kinases Wee1 and Mik1. Cells with a temperature sensitive allele of *wee1*, *wee1-50*, advance mitosis such that they divide at a small cell size, but remain viable. However, simultaneous inactivation of *wee1* and a second tyrosine kinase in fission yeast, *mik1*, results in rapid accumulation of cells with a mitotic catastrophe phenotype (Lundgren et al., 1991), while inactivation of *wee1* in a checkpoint mutant background results in a gradual accumulation of cells in mitotic catastrophe (al-Khodairy and Carr, 1992; Walworth et al., 1993). Since Chk1 is essential for viability in cells with compromised Wee1 function, we examined the phosphorylation status of Chk1 in such cells. As shown in Fig. 4D, Chk1 is constitutively phosphorylated in cells with mutations in the Mik1 or Wee1 tyrosine kinases even at permissive temperature for the *wee1-50* mutant. Cells with mutations in both *mik1* and *wee1* enter mitosis at a smaller size than cells with mutations in either kinase alone and the fraction of phosphorylated Chk1 is highest in such a strain (*mik1::ura4 wee1-50*). Indeed, a *chk1::ura4 mik1::ura4 wee1-50* strain is barely viable even at 25°C (N.C.W., unpublished). As is the case for DNA damage-induced phosphorylation of Chk1 (Walworth and Bernards, 1996), phosphorylation of Chk1 in the wee mutants is dependent on *rad1*. Notably, a *rad1-1 wee1-50* strain is inviable when *wee1-50* is inactivated at 36°C (al-Khodairy and Carr, 1992).

Chk1S345A and Chk1S345D do not co-immunoprecipitate with Rad24

Phosphorylated Chk1 co-immunoprecipitates with Rad24, a 14-3-3 protein (Chen et al., 1999). This interaction may be essential for checkpoint function (Chen et al., 1999). To determine if Chk1S345D can constitutively co-immunoprecipitate with Rad24, we immunoprecipitated Rad24, performed SDS-PAGE, and probed for HA-tagged Chk1 using an anti-HA antibody. Chk1S345D exhibits a mobility similar to that of phosphorylated Chk1, but does not co-immunoprecipitate with Rad24 (Fig. 5). Chk1S345A also fails to co-immunoprecipitate with Rad24 (Fig. 5). These results suggest that S345 phosphorylation is required for Chk1 to associate with Rad24 and that aspartic acid at position 345 is not sufficient to induce this association.

Discussion

Chk1 is a protein kinase in the DNA damage checkpoint pathway that is conserved from yeast to humans. In response to many types of DNA damage, Chk1 becomes phosphorylated (Walworth and Bernards, 1996; Wan et al., 1999). Phosphorylation of fission yeast Chk1 is dependent on the activity of a number of checkpoint pathway proteins including Rad3 (Walworth and Bernards, 1996). Phosphorylation of vertebrate Chk1 in response to DNA damage is dependent on the Rad3 homologue ATR (Liu et al., 2000; Guo et al., 2000; Zhao and Piwnica-Worms, 2001). In fission yeast, Rad3 can coimmunoprecipitate with overexpressed Chk1 and can phosphorylate fission yeast Chk1 in vitro (Martinho et al., 1998). ATR contributes to checkpoint control in eukaryotes (Cimprich et al., 1996; Keegan et al., 1996) and Rad3 plays a pivotal role in checkpoint signaling in fission yeast (Bentley et al., 1996; O'Connell et al., 2000). Thus, it seems likely that Rad3-dependent phosphorylation of Chk1 plays an important role in the DNA damage checkpoint pathway, but this role has not yet been clearly defined. Previous work left unresolved whether sites that could be phosphorylated by Rad3 in vitro were in vivo sites of phosphorylation and whether phosphorylation affected Chk1 kinase activity (Lopez-Girona et al., 2001). In this study, we demonstrate that an identified in

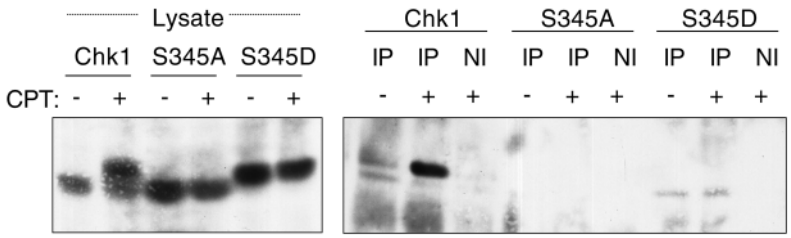

Fig. 5. Mutation of serine 345 to alanine or aspartic acid abolishes association of Chk1 with 14-3-3 upon DNA damage. NW223 (Chk1), NW444 (S345A) and NW463 (S345D) were grown to mid-log phase, treated with CPT and lysed. Lysates were incubated with antibody against Rad 24 (UMDNJ 55), subjected to SDS-PAGE and immunoblotted with 12CA5 anti-HA antibody (IP). Control precipitations (NI) with non-immune rabbit serum were processed exactly as anti-Rad24 IPs. Samples of the lysates, before immunoprecipitation are shown in the left panel.

vitro phosphorylation site is phosphorylated in vivo and that phosphorylation of this site is necessary for enhanced kinase activity of Chk1 following DNA damage.

Human Chk1 is phosphorylated in vivo at two SQ/TQ ATM or ATR consensus phosphorylation sites, S317 and S345, in an ATR-dependent manner (Zhao and Piwnica-Worms, 2001). Detection with a phosphospecific antibody indicates that S345 of mouse Chk1 is phosphorylated in an ATR-dependent manner in vivo (Liu et al., 2000). Four SQ/TQ consensus sites can be phosphorylated in vitro by *Xenopus* ATR and one, S344, is phosphorylated in vivo (Guo et al., 2000). During the preparation of this manuscript and consistent with our results, Lopez-Girona et al., reported that the S345A mutation of fission yeast Chk1 abolishes the DNA damage-induced mobility shift (Lopez-Girona et al., 2001). However, we have shown previously that mutation of amino acids that would never be phosphorylated can also abolish the DNA damage-induced phosphorylation of Chk1 (Wan and Walworth, 2001), making it impossible to determine from mutagenesis data alone that any particular amino acid is a site of phosphorylation. Therefore, we used a S345 phosphospecific antibody to identify S345 as a site that is phosphorylated in vivo in response to DNA damage (Fig. 3). Phosphorylation at this site is critical for checkpoint function and the association of Chk1 with the 14-3-3 protein Rad24 (Figs 4, 5). Consistent with this, Lopez-Girona et al., demonstrated that Rad3 can phosphorylate in vitro a 36 amino acid fragment of Chk1 encompassing this particular SQ site (Lopez-Girona et al., 2001).

There has been controversy over the effect of phosphorylation on the kinase activity of Chk1. It has recently been shown that human Chk1 kinase activity is elevated in response to hydroxyurea and that this elevation requires phosphorylation of serines 317 and 345 (Zhao and Piwnica-Worms, 2001). Mutation of either serine does not abolish a basal level of Chk1 kinase activity (Zhao and Piwnica-Worms, 2001). Unphosphorylated fission yeast Chk1 also maintains a basal level of kinase activity that does not appear to be abrogated when serine 345 is mutated to alanine (Fig. 3). Deletion of the C-terminus of human and *Xenopus* Chk1 activates its intrinsic kinase activity (Oe et al., 2001; Chen et al., 2000). Furthermore, the crystal structure of the catalytic domain of human Chk1 revealed an open kinase conformation that enables kinase activity without phosphorylation of the catalytic domain (Chen et al., 2000). This evidence suggests that the C-terminus of Chk1 acts to regulate kinase activity by suppressing activity when the kinase is in an unphosphorylated state. A change in conformation upon phosphorylation of the C-terminus may cause a derepression, or elevation, of kinase activity. In an attempt to construct a mutant of Chk1 that mimics its phosphorylated state (Huang and Erikson, 1994; Li et al., 2000), we mutated serine 345 to aspartic acid. Even though the mobility of this protein on SDS-PAGE is similar to phosphorylated Chk1, it does not seem to possess the properties of phosphorylated Chk1.

Fission yeast Chk1 kinase activity is elevated in response to DNA damage and this elevation requires serine 345 (Fig. 3). Mutating serine 345 to aspartic acid did not confer constitutively elevated Chk1 kinase activity as one might have expected. This mutant did, however, retain a basal level of kinase activity. A *chk1S345D* mutant behaved identically to *chk1S345A* except it seemed to initiate a checkpoint in response to UV light that could not be maintained (Fig. 4). Chk1S345D simply may not mimic phosphorylation of Chk1 at serine 345 or the cell may require both unphosphorylated and phosphorylated forms of Chk1 to maintain a checkpoint in response to DNA damage. However, expression of plasmid borne Chk1S345D in a *chk1S345A* background, or conversely, of Chk1S345A in a *chk1S345D* background, does not restore checkpoint function (H.C., H.R. and N.C.W., data not shown). It does not seem that the Chk1S345D mutation affects Chk1 function by causing a severe change in protein structure because the protein is stably expressed, localizes to the nucleus like wild-type Chk1 (Fig. 2) and is catalytically active (Fig. 3B).

We have shown previously that *chk1* is essential for the viability of wee mutants (Walworth et al., 1993), which are characterized by having a shortened G2 period. We show here that Chk1 is constitutively phosphorylated in these cells. Presumably, the phosphorylated and, hence active, Chk1 elicits a delay in these cells that is critical for preventing mitotic catastrophe when mitotic entry is not under normal size control. As Wee1 may be an effector of Chk1-mediated G2 arrest (O'Connell et al., 1997), the absence of functional Wee1 would explain why the phosphorylated, active Chk1 is unable to cause permanent G2 arrest in these cells. While it is not clear what signals Chk1 phosphorylation in cells with the wee phenotype, one might assume that it is an unusual DNA damage-like state or that it is the result of activation of Cdc2 in an inappropriate context. Indeed, a recent study indicates that premature activation of the Cdc2 homologue in *S. cerevisiae*, resulting from loss-of-function of a Cdc2 inhibitor, Sic1, causes genomic instability due to alterations in S phase progression (Lengronne and Schwob, 2002). Similarly, activation of Cdc2 in *S. pombe* by virtue of inactivation of the Wee1 or Mik1 kinases, may result in an inappropriate combination of events that leads to a demand for checkpoint function to ensure viability; hence, the phosphorylation of Chk1. The essential nature of Chk1 in wee mutants may be related to the embryonic lethality of *chk1* mutations in mice and *Drosophila*, where cells cycle rapidly and the major

challenge is to coordinate S- and M-phases (Fogarty et al., 1994; Fogarty et al., 1997; Takai et al., 2000; Liu et al., 2000).

Here we have determined using a combination of mutational studies and the use of phosphospecific antibodies that fission yeast Chk1 is, importantly, phosphorylated in vivo on S345 in response to DNA damage and that this is essential for the function of Chk1 in the DNA damage checkpoint pathway. We have also shown that mutation of S345 abolishes a DNA damage-induced elevation in Chk1 protein kinase activity and the association of Chk1 with 14-3-3 protein. Although the mechanism by which S345 phosphorylation activates Chk1 is not known, genetic analyses of the mutants described here may help in deciphering this. These findings highlight the conserved nature of the checkpoint pathway in eukaryotic organisms and confirm that studies in fission yeast are relevant to higher eukaryotes.

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