Interaction of 14-3-3 protein with Chk1 affects localization and checkpoint function

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Summary

The protein kinase Chk1 is required for proper arrest of the cell cycle in response to DNA damage. We have previously shown in *Schizosaccharomyces pombe*, that upon DNA damage, phosphorylation of Chk1 correlates with checkpoint activation and that phosphorylated Chk1 is capable of interacting with the 14-3-3 proteins, Rad24 and Rad25. The interaction between Rad24 and Chk1 is stimulated tenfold after exposure to DNA damaging agents and we postulate that it is an important event in the DNA damage checkpoint response pathway in fission yeast. We identified a stretch of leucine residues as the domain in Chk1 that mediates the interaction with 14-3-3 proteins. Substitution of leucine residues with alanine disrupts the interaction with Rad24 and also prevents Chk1 from becoming phosphorylated in response to DNA damaging agents. Cells expressing the mutants are sensitive to UV radiation. In this study, we also show that Chk1 accumulates in the nucleus in response to DNA damage and this behavior is dependent on Rad24. Interestingly, the 14-3-3 binding domain mutants also fail to localize to the nucleus prompting a search for localization sequences within Chk1. Our investigations have identified the presence of both functional nuclear import and nuclear export sequences encoded in *S. pombe* Chk1 that, in conjunction with 14-3-3 proteins, may play a prominent role in regulating Chk1 localization and function.

Key words: Chk1, Checkpoint, 14-3-3 protein, DNA damage, Rad24, Cell cycle

Introduction

The DNA damage checkpoint is defined as a signal transduction pathway that delays entry into mitosis in the presence of damaged DNA to preserve genomic integrity (Hartwell and Weinert, 1989). Many of the proteins involved in this pathway have been identified genetically and are well conserved throughout evolution. Mutation or deletion of these proteins leads to a failure of cells to arrest cell cycle progression in the presence of DNA damage and manifests itself as a profound loss of cell viability upon exposure to DNA damaging agents (al-Khodairy and Carr, 1992; Enoch et al., 1992; Ford et al., 1994; Rowley et al., 1992; Walworth et al., 1993; Weinert and Hartwell, 1988; Weinert et al., 1994). In the fission yeast *Schizosaccharomyces pombe*, the Chk1 protein kinase is an essential component of the DNA damage checkpoint response pathway. Chk1 is phosphorylated in response to DNA damage and this event is dependent on several components of the pathway, such as Rad3 (Walworth and Bernards, 1996). Rad3 is a fission yeast orthologue of the ATM family of phosphoinositol 3-kinases (Bentley et al., 1996; Seaton et al., 1992; Shiloh, 2003). The founding member of this family, ATM, when mutated in humans leads to the progressive neurodegenerative disorder ataxia telangiectasia characterized by severe hypersensitivity to DNA damaging agents (Savitsky et al., 1995). Chk1 can phosphorylate key regulators of the cell cycle in vitro providing an attractive link between the DNA damage checkpoint pathway and the cell cycle machinery (O'Connell et al., 1997; O'Connell et al., 2000; Sanchez et al., 1997).

In eukaryotes, the cell cycle is controlled by the periodic activation and inactivation of the highly conserved cyclin-dependent kinases (CDKs) (Pines, 1995). The major CDK that controls mitotic entry and progression in fission yeast is Cdc2 (MacNeill and Nurse, 1997). Cdc2 is phosphorylated during interphase of the cell cycle at an inhibitory tyrosine residue that becomes dephosphorylated, allowing activation and subsequent mitotic entry (MacNeill and Nurse, 1997). The phosphorylation of this residue is thought to be controlled by the Wee1 and Mik1 kinases, whereas dephosphorylation is carried out by the Cdc25 phosphatase (Lundgren et al., 1991; Millar et al., 1991). When DNA damage is incurred, the tyrosine-phosphorylated form of Cdc2 is present (Rhind et al., 1997). Chk1 is thought to control the functions of Wee1 and Cdc25 in response to DNA damage based on its ability to phosphorylate these targets, suggesting that there may be a direct link between the DNA damage checkpoint pathway and cell cycle progression (O'Connell et al., 1997; Peng et al., 1997; Sanchez et al., 1997).

In addition to Chk1, a member of the 14-3-3 family of proteins, Rad24, is involved in the proper execution of cell

cycle arrest in response to DNA damage (Ford et al., 1994). The 14-3-3 proteins constitute a family of conserved multifunctional adapter proteins involved in a multitude of cellular functions, such as cell growth, cell signaling and cell death (van Hemert et al., 2001). These proteins are highly acidic dimeric proteins that mostly recognize and bind to target proteins with defined phosphoserine-containing motifs (RSXpSXP or RXXXpSxP) (Muslin et al., 1996; van Hemert et al., 2001; Yaffe et al., 1997). The precise sequence for the binding motifs has not yet been determined in yeast. Of the two fission yeast 14-3-3 proteins, Rad24 and Rad25, Rad24 appears to play the more important role in the DNA damage checkpoint response (Ford et al., 1994). Although cells lacking *rad24* are checkpoint deficient, cells lacking *rad25* are relatively checkpoint proficient, suggesting that Rad25 does not play as significant a role in the DNA damage response. Nonetheless, multi-copy expression of Rad25 can compensate for the loss of Rad24 function (Ford et al., 1994). Rad24 and Rad25 have been shown to interact with Chk1 and the association between these proteins is stimulated by DNA damage (Chen et al., 1999). Rad24 also binds to Cdc25 (Chen et al., 1999; Lopez-Girona et al., 1999) and is required for the DNA damage-induced relocation of Cdc25 to the cytoplasm of fission yeast cells in response to DNA damage (Lopez-Girona et al., 1999). A mutant allele of *cdc25* that cannot bind to 14-3-3 proteins remains nuclear and allows cells to enter mitosis despite treatment with agents that should activate the checkpoint delay to mitotic entry (Zeng and Piwnica-Worms, 1999).

Human Chk1 can phosphorylate Cdc25 on Ser216 (Peng et al., 1997; Sanchez et al., 1997), which creates a site for 14-3-3 protein binding. Several studies have examined the consequences of this interaction. Some suggest that this event inactivates the phosphatase activity of Cdc25 (Blasina et al., 1999; Kumagai et al., 1998). Other studies of mammalian and *Xenopus* Cdc25 suggest that binding of 14-3-3 proteins controls the cellular localization of Cdc25 (Dalal et al., 1999; Kumagai and Dunphy, 1999; Kumagai et al., 1998; Yang et al., 1999). Recently it has been shown that mammalian Chk1 can also phosphorylate Cdc25A and this promotes binding of 14-3-3 to Cdc25A and prevents functional interactions between Cdc25A and cyclinB1/Cdk1 (Chen et al., 2003). A role for 14-3-3 proteins interacting with mammalian Chk1 has also been described recently (Jiang et al., 2003).

In *S. pombe* the interaction of Chk1 with 14-3-3 proteins is stimulated tenfold in response to DNA damage and 14-3-3 proteins preferentially associate with phosphorylated Chk1 (Chen et al., 1999), which is the activated form (Capasso et al., 2002). Here we demonstrate that fission yeast Chk1 accumulates in the nucleus of cells treated with the drug camptothecin (CPT) that generates DNA damage via a topoisomerase I-dependent mechanism (Hsiang et al., 1989). This nuclear accumulation is Rad24 dependent. Additionally, we demonstrate that Chk1 contains nuclear localization and nuclear export sequences. Finally, we propose a model in which 14-3-3 protein association controls Chk1 localization and argue that this interaction is important for proper checkpoint function.

Materials and Methods

Yeast strains, media, and growth conditions

Yeast strains used in this study are listed in Table 1. The *chk1L→A* mutants were created by site-directed mutagenesis of *S. pombe* HA-tagged *chk1* cloned into the pSP1 vector (Cottarel et al., 1993), using the Quick Change Site-Directed Mutagenesis Kit (Stratagene). The vector was then digested with *Hin*dIII (New England Biolabs) and this fragment was used to perform gene replacement of *chk1L→A* mutants at the *chk*$^+$ genomic locus as described (Walworth and Bernards, 1996). The *chk1L→A+NLS* mutants were created by cloning the SV40 NLS from pSP1 containing wild-type *chk1*$^+$ and an SV40 NLS cloned at the C-terminus with *Avr*II and *Bgl*II. *S. pombe* strains were transformed using lithium acetate as described (Moreno et al., 1991). Strains were grown in pombe minimal (PM) medium with appropriate amino acid supplements at 75 μg/ml or in rich yeast extract (YE)

Table 1. Yeast strains used in this study

Strain	Genotype
SP80	*h*$^-$, *cdc17-K42*
NW158	*h*$^+$, *chk1::ura4 ura4-D18 leu1-32 ade6-216*
NW223	*h*$^+$, *chk1:ep leu1-32 ade6-216*
NW250	*h*$^+$, *chk1:ep rad24::ura4 ura4-D18 leu1-32 ade6-216*
NW653	*h*$^+$, *chk1::ura4 ura4-D18 cdc17-K42 leu1-32 ade6-216*
NW740	*h*$^+$, *chk1::ura4 ura4-D18 leu1-32 ade6-216/pSP1:chk1:ep*
NW1428	*chk1:L291A:ep leu1-32 ade6-216*
NW1429	*chk1:L295A:ep leu1-32 ade6-216*
NW1430	*chk1:L299A:ep leu1-32 ade6-216*
NW1431	*chk1:L303A:ep leu1-32 ade6-216*
NW1422	*chk1:L291A:ep cdc17-K42 leu1-32 ade6-216*
NW1423	*chk1:L303A:ep cdc17-K42 leu1-32 ade6-216*
NW1432	*chk1:L295A:ep cdc17-K42 leu1-32 ade6-216*
NW1433	*chk1:L299A:ep cdc17-K42 leu1-32 ade6-216*
NW1424	*chk1:L291A:ep cdc25-22 leu1-32 ade6-216*
NW1425	*chk1:L303A:ep cdc25-22 leu1-32 ade6-216*
NW1426	*chk1:L295A:ep cdc25-22 leu1-32 ade6-216*
NW1427	*chk1:L299A:ep cdc25-22 leu1-32 ade6-216*
NW1461	*h*$^+$, *chk1::ura4 ura4-D18 leu1-32 ade6-216/pSP1:chk1:L291A:ep*
NW1462	*h*$^+$, *chk1::ura4 ura4-D18 leu1-32 ade6-216/pSP1:chk1:L295A:ep*
NW1439	*h*$^+$, *chk1::ura4 ura4-D18 leu1-32 ade6-216/pSP1:chk1:L291A:NLS:ep*
NW1444	*h*$^+$, *chk1::ura4 ura4-D18 leu1-32 ade6-216/pSP1:chk1:L295A:NLS:ep*
NW1442	*h*$^+$, *chk1::ura4 ura4-D18 leu1-32 ade6-216/pSP1:chk1:4L-A:ep*
NW1454	*h*$^+$, *chk1::ura4 ura4-D18 leu1-32 ade6-216/pSP1:chk1:4L-A:NLS:ep*

medium supplemented with adenine. Standard recipes were used for YE (Moreno et al., 1991) and minimal medium was prepared using Edinburgh minimal medium (EMM) from Bio-101. Strains were grown at 30°C unless otherwise indicated.

Two-hybrid screen

Plasmids and strains for the two-hybrid analysis were generously provided by Dr Stephen J. Elledge (Baylor College of Medicine, Houston, TX). Chk1 cDNA was cloned into the pAS vector and Rad24 cDNA was cloned into the pACT vector as described (Chen et al., 1999). The *chk1L→A* mutations were cloned into pAS-Chk1 using *Spe*I and *Bam*HI sites creating pAS2-Chk1L→A constructs. The reporter strain Y190 was used in these assays (Bai and Elledge, 1997). To test for interaction between Chk1 point mutants and Rad24, pAS-Chk1 (or point mutants described above) and pACT-Rad24 were transformed into Y190 and tested for His prototrophy and β-galactosidase production. β-galactosidase production was assayed on nitrocellulose filters (Bai and Elledge, 1997).

Camptothecin treatment

Camptothecin lactone (CPT) was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Cells were grown to mid-log phase and treated with 40 μM CPT for 2-3 hours as described (Wan et al., 1999).

Lysate preparation and immunoblotting

For lysate preparation, cells were harvested by centrifugation and lysed in phosphate-buffered saline (PBS) containing complete protease inhibitor (Roche Diagnostics) using glass beads and a FastPrep (Bio101) vortexing machine. Supernatant was collected from the lysed cells by centrifugation at 3000 rpm for 5 minutes in an Eppendorf microcentrifuge. Supernatants were then subject to an additional spin at full speed for 10-20 minutes and collected again. Aliquots were separated on SDS-PAGE, transferred to nitrocellulose membrane (BA83, Schleicher and Schuell), and probed with 12CA5 antibody to detect the HA epitope at the C-terminus of Chk1. Blocking of the membranes and all antibody incubations and washes were performed in 1% non-fat dried milk and 0.05% Tween-20 in PBS. 12CA5 antibody was used at 1:500 dilution and a peroxidase-coupled secondary antibody (Boehringer Mannheim) at 1:1000. The chemiluminescence detection system Western Lightning (NEN/Perkin Elmer) was used for detection. Filters were exposed to Kodak Biomax film.

Immunoprecipitation

Lysates were prepared as described above. Prior to incubation with antibody the lysates were incubated with 15 μl protein A-Sepharose (Pharmacia) for 30 minutes. The Sepharose beads were removed by centrifugation at 1000 rpm in an Eppendorf microcentrifuge for 1 minute. The lysate (500 μl) containing 3 mg protein was then incubated with 60 μl Rad24 antibody that had been crosslinked to protein A-Sepharose. Incubation was performed at 4°C for 1 hour on a rotator. The immune complexes were then washed four times with PBS. Precipitates were resuspended in 2× Laemmli sample buffer and resolved on SDS-PAGE. Immunoblotting was performed as described above.

UV survival and checkpoint assay

UV survival was determined as described (Walworth et al., 1993). The *chk1L→A cdc25-22* strains were generated by random spore analysis. The ability of *chk1L→A* mutants to arrest cell cycle progression in the presence of DNA damage generated by UV was performed as described (Wan and Walworth, 2001).

Synthetic lethality of *chk1L→A* mutants with cdc17-K42

chk1L→A cdc17-K42 strains were generated by random spore analysis. Cells were then plated on YEA plates and incubated at 25°C, 32°C and 36°C for 3-4 days (Walworth et al., 1993).

Immunofluorescence

Cells were fixed with 37% paraformaldehyde stock used at 1:10 culture volume for 27 minutes and then centrifuged. Cells were washed twice with 1× PEM (15.1 g PIPES, 500 μl 1 M $MgCl_2$, 1 ml 0.5 M EDTA in 500 ml), then once in PEMS (10 ml 2× PEM, 4.4 g sorbitol in 20 ml). Cells (4×10^7) were digested with 1.5 mg/ml Zymolyase 100T (Seikagaku Corporation) in PEMS for 10 minutes at 37°C. Cells were washed twice with PEMS and incubated in 1% Triton X-100 (Sigma) for 1 minute. Cells were then washed once with PEMS and twice with 1× PEM. 1.5×10^7 cells were then resuspended with PEMBAL (0.1 g BSA and 0.18 g l-l-Lysine in 10 ml 1× PEM) and incubated for at least 1 hour. Cells were incubated in PEMBAL with F7 anti-HA antibody (Santa Cruz) at 1:100 overnight. Cells were washed three times with PEMBAL for 30 minutes each and incubated in PEMBAL with CY3 antibody (Jackson Labs) at 1:100 for 4 hours. Cells were then washed once with PEMBAL for 30 minutes and twice with PEM and then resuspended in 10 μl Vectashield (Vector Laboratories). Cells were viewed using a rhodamine filter on a Zeiss Axioplan 2 fluorescence microscope equipped with a Zeiss Axiocam utilizing Openlab software (Improvision). The intensity of the nuclear and cytoplasmic signals was determined by measuring pixel intensity in the nucleus and of an equivalent area in the cytoplasm. The ratio of the nuclear intensity (N) to the cytoplasmic intensity (C) was determined. Individual cells were then binned into one of three categories: N<C for cells with a ratio of $\leqslant$0.94; N>C for cells with a ratio of $\geqslant$1.15; and N=C for cells with ratios between 0.95 and 1.14. At least 300 cells were quantified for each experiment.

Results

Identification of residues in Chk1 that mediate its interaction with Rad24

Chk1 is composed of two distinct halves (Fig. 1A). The N-terminal half of the protein encodes the catalytic domain, which has been crystallized for mammalian Chk1 (Chen et al., 2000). The C-terminal half encodes a putative non-catalytic regulatory domain (Chen et al., 2000; Shann and Hsu, 2001). It is within this C-terminus that we previously identified a region of Chk1 that was required for mediating the interaction between Chk1 and the 14-3-3 protein, Rad24 (Chen et al., 1999). Although that portion of Chk1 does not contain a typical phosphoserine-binding motif for 14-3-3 protein association, inspection of the amino acid composition of the region did reveal a domain enriched in hydrophobic residues. We investigated if these hydrophobic residues were important for mediating the interaction with Rad24 as early structural studies of the target protein binding pocket on 14-3-3 proteins suggested that it might accommodate an amphipathic α-helix (Liu et al., 1995). To that end, point mutations were generated to mutate some of the hydrophobic leucines (L) to alanines (A) (Fig. 1A). These mutants were cloned into the appropriate yeast two-hybrid expression vectors, transformed into the Y190 reporter strain and assayed for β-galactosidase activity. Mutation of individual leucines was sufficient to disrupt the

Chk1/Rad24 interaction, whereas mutation of a critical lysine in the N-terminus that renders Chk1 catalytically inactive had no effect on the interaction (Fig. 1B).

To test the importance of these residues for interaction with Rad24 in vivo, strains were created that carry individual L→A mutations in HA-tagged *chk1*. These alleles were integrated at the *chk1* locus under control of the *chk1* promoter as described (Walworth and Bernards, 1996). Western blot analysis confirmed expression of the mutant alleles and revealed that the mutants failed to undergo the DNA damage-induced mobility shift (Fig. 1C) typical of wild-type Chk1 (Walworth and Bernards, 1996; Wan et al., 1999). Antibodies against Rad24 were used to co-immunoprecipitate Chk1 (Chen et al., 1999). As previously demonstrated (Chen et al., 1999), Rad24 preferentially co-immunoprecipitates the phosphorylated form of Chk1. No damage-induced increase in Chk1 association with Rad24 was apparent in the L→A mutants consistent with the lack of a damage-induced mobility shift. Some unphosphorylated Chk1 is present in all samples, including controls using a non-immune serum (Fig. 1C). For this reason it was difficult to determine conclusively from these experiments whether the L→A mutants were defective in association with Rad24 in vivo. Similar difficulties were encountered when GST-Rad24 expressed in *S. pombe* was used to precipitate Chk1 (data not shown). Taking into account these and the two-hybrid experiments, we conclude that the L→A mutants are apparently defective in Rad24 binding and that phosphorylation of the mutant proteins is also compromised.

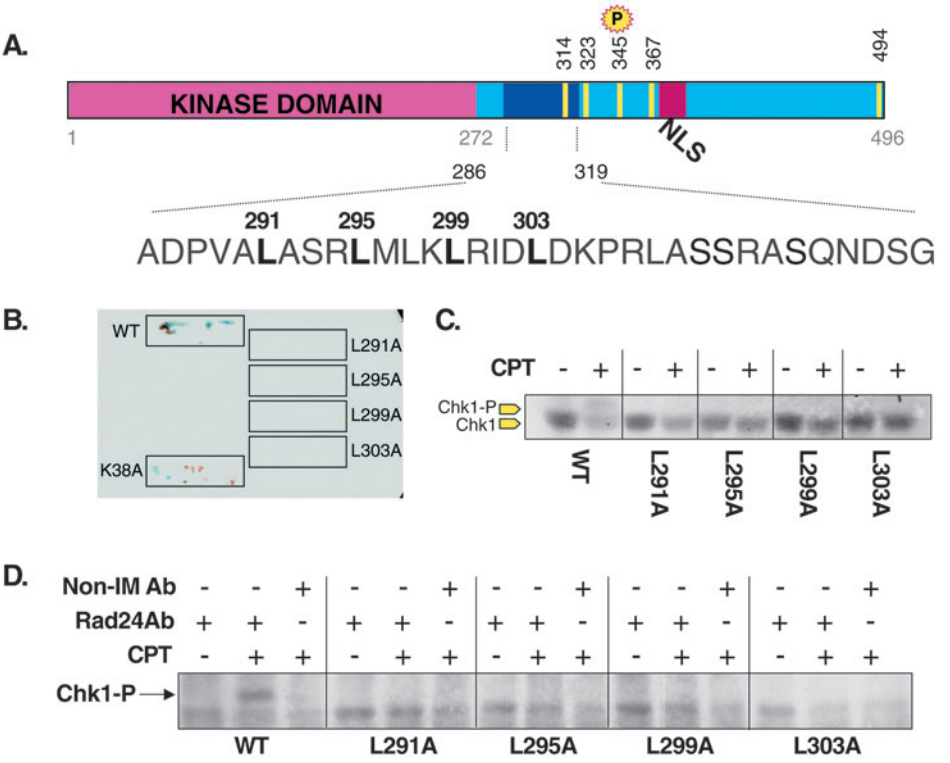

Fig. 1. Identification of residues in Chk1 required for the interaction with Rad24. (A) Domain structure of Chk1 highlighting the region required for interaction between Chk1 and Rad24 (amino acids 286-319). (B) Result of yeast two-hybrid β-galactosidase assay. Strains transformed with the pACT/Rad24 plasmid and the indicated alleles of *chk1* in the pAS2/Chk1 plasmid were exposed to X-Gal. Only the wild-type allele and the kinase domain mutant K38A show expression of the *lacZ* gene as revealed by X-gal reactivity. (C) Western blot of lysates from cells expressing the indicated alleles of Chk1 that had (+) or had not (–) been exposed to the topoisomerase-I poison camptothecin (CPT). Chk1 is detected with anti-HA antibody to detect the tag on the C-terminus of Chk1. Only the wild-type protein undergoes the mobility shift representative of Chk1 phosphorylation. (D) Immunoprecipitation of lysates shown in C to evaluate the interaction of alleles of Chk1 with Rad24. Immunoprecipitations were carried out with antibodies against Rad24 or with a non-immune antibody. The immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose and probed by immunoblot with antibody against the HA tag on the C-terminus of Chk1.

chk1L→A mutants are UV sensitive and checkpoint defective

To investigate further the effect these mutations had on Chk1 function, assays were performed to test the function of these alleles in vivo. To this end, we monitored the survival of the *chk1L→A* mutant strains after transient exposure to UV. As shown in Fig. 2A the mutants are indeed sensitive, exhibiting a level of survival equivalent to cells that lack Chk1 (*chk1::ura4*) altogether.

To confirm the results from the UV survival assay, strains were created with the *chk1* mutants in a background containing a temperature-sensitive allele of the gene encoding DNA ligase, *cdc17-K42*. At a fully restrictive temperature of 36.5°C, the unligated DNA fragments act as a DNA damage signal culminating in a checkpoint arrest and inviability (Nasmyth, 1977). At the semi-permissive temperature (32°C) *cdc17-K42* retains partial ligase activity and cells are slightly elongated. Cells can form colonies on a plate presumably by virtue of delaying mitosis for a sufficient period to allow the partially active ligase to carry out its activity. Thus, the ability of a *cdc17-K42* mutant to form colonies at 32°C requires a functional checkpoint (al-Khodairy and Carr, 1992). The combination of *cdc17-K42* with a checkpoint mutant, such as a non-functional allele of *chk1*, results in loss of viability of the double mutant strain at 32°C (al-Khodairy and Carr, 1992; Walworth et al., 1993). When the leucine mutants were crossed with *cdc17-K42* and plated at 32°C, they failed to form colonies suggesting that the leucine mutants are checkpoint defective (Fig. 2B).

To confirm that the DNA damage sensitivity of these *chk1* mutants results from a defective DNA damage checkpoint, checkpoint integrity was investigated (Dunaway and Walworth, 2004). Cells were arrested in G2 then irradiated with UV and

allowed to proceed from the arrest. In this assay, a *chk1*$^{+}$ strain delayed mitotic entry in response to DNA damage indicating a functional DNA damage checkpoint response (Fig. 2C). However, the *chk1* leucine mutants, like a Chk1 deletion mutant, were unable to delay mitotic progression following exposure to a DNA damaging agent (Fig. 2C). These results indicate that the 14-3-3 binding domain mutants are checkpoint defective.

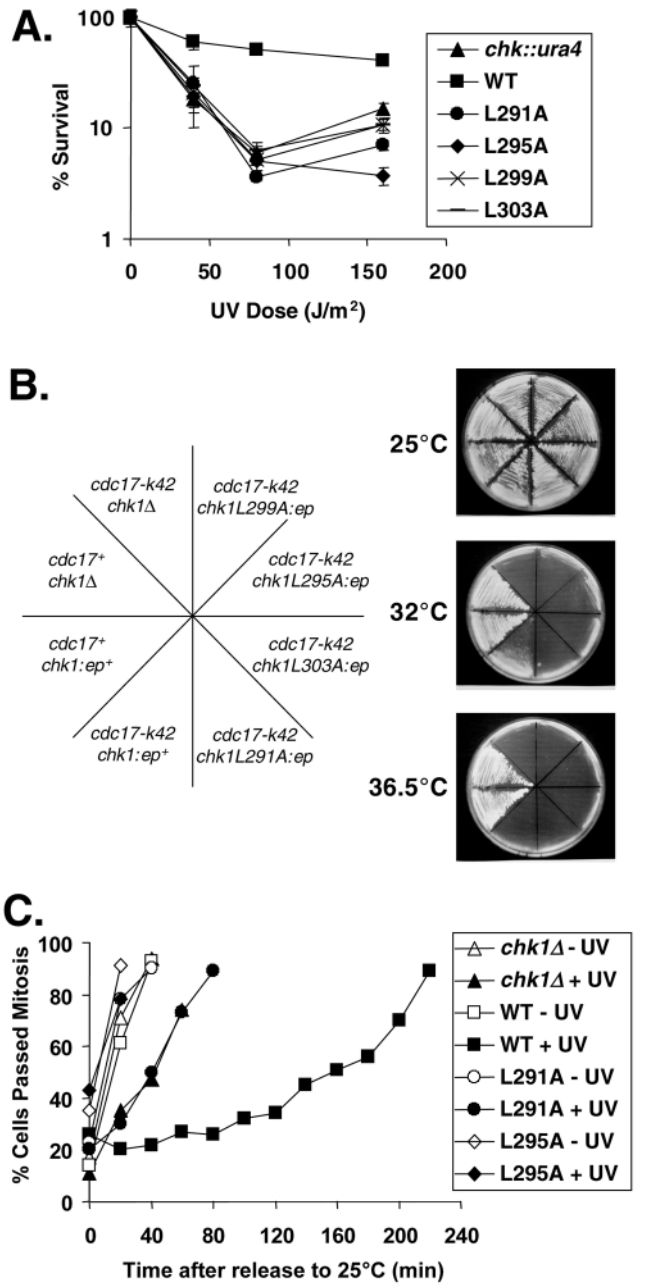

Fig. 2. *chk1L→A* mutants exhibit a loss-of-function phenotype. (A) UV survival was monitored for the indicated strains by a colony survival assay after exposure to 40, 80 or 160 J/m^2 UV. Triplicate plates were prepared and the data represent the mean survival with error bars indicating the standard deviation. (B) Cells from the indicated strains were plated on YEA plates and incubated at 25°C, 32°C and 36°C to assay for synthetic lethality in a DNA ligase-deficient background *cdc17-K42*. (C) The indicated *chk1* alleles were crossed into a *cdc25-22* background in order to allow synchronization. G2-blocked cells were exposed to UV and released at 25°C. Cells were then scored for passage through mitosis (see Materials and Methods).

Chk1 accumulates in the nucleus in response to DNA damage in a Rad24-dependent fashion

Given the observations that Chk1 interacts with 14-3-3 proteins and that 14-3-3 proteins are involved in the subcellular localization of many of their binding partners (Brunet et al., 1999; Brunet et al., 2002; Peng et al., 1997; Rittinger et al., 1999; Seimiya et al., 2000; Zha et al., 1996), we chose to investigate the localization of Chk1 before and after DNA damage. Chk1 localization was determined by immunofluorescence using antibodies to the HA epitope incorporated at the C-terminus of Chk1. As shown in Fig. 3A, localization of Chk1 is pancellular. When the cells were treated with the DNA damaging agent CPT, Chk1 accumulated in the nucleus (Fig. 3A). To quantitatively evaluate changes in localization, fluorescence intensities of the Chk1 signal were determined for a circular region corresponding to the nucleus and a comparable area in the cytoplasm as explained in the Materials and Methods. The ratio of the nuclear signal to the cytoplasmic signal was then determined and cells were assigned to categories as outlined in Fig. 3B. Results for Chk1 before and after CPT treatment are shown in Fig. 3C. The cytoplasmic protein Ded1 (Liu et al., 2002) remains cytoplasmic in the presence of CPT (Fig. 3A) indicating that relocalization to the nucleus is not a general property of proteins following CPT treatment of cells. A similar phenotype was observed when wild-type Chk1 was expressed from a plasmid rather than its endogenous locus (Fig. 3D). These observations indicate that Chk1 subcellular localization dramatically changes in response to DNA damage.

The next question addressed was whether Chk1 localization was dependent on 14-3-3 proteins. Chk1 immunofluorescence was performed in a strain devoid of Rad24. In the absence of CPT, the cells displayed a pancellular-staining pattern for Chk1 and the cells showed the short and clumpy phenotype characteristic of cells lacking Rad24 (Fig. 4) (Ford et al., 1994). When these cells were treated with CPT, there was no apparent change in the localization of Chk1. Quantitative analysis of these experiments proved too difficult owing to the altered morphology of the cells caused by deletion of Rad24. However, the observations indicate that the DNA damage-induced accumulation of Chk1 in the nucleus is dependent on Rad24, a 14-3-3 protein.

Chk1 contains a functional NLS

These studies led to the investigation of whether or not Chk1 contains a functional nuclear localization sequence (NLS). Such sequences have been identified for many proteins and usually consist of patches of basic residues (Dingwall and Laskey, 1991; Nigg, 1997). Upon inspection of the amino acid sequence of Chk1, a potential candidate NLS was discovered in the C-terminal half of the protein (see Fig. 1A) consisting of two pairs of basic residues, a lysine and arginine at positions 377 and 378, respectively, and a pair of lysines at positions 381 and 382. When the basic residues were mutated to alanine

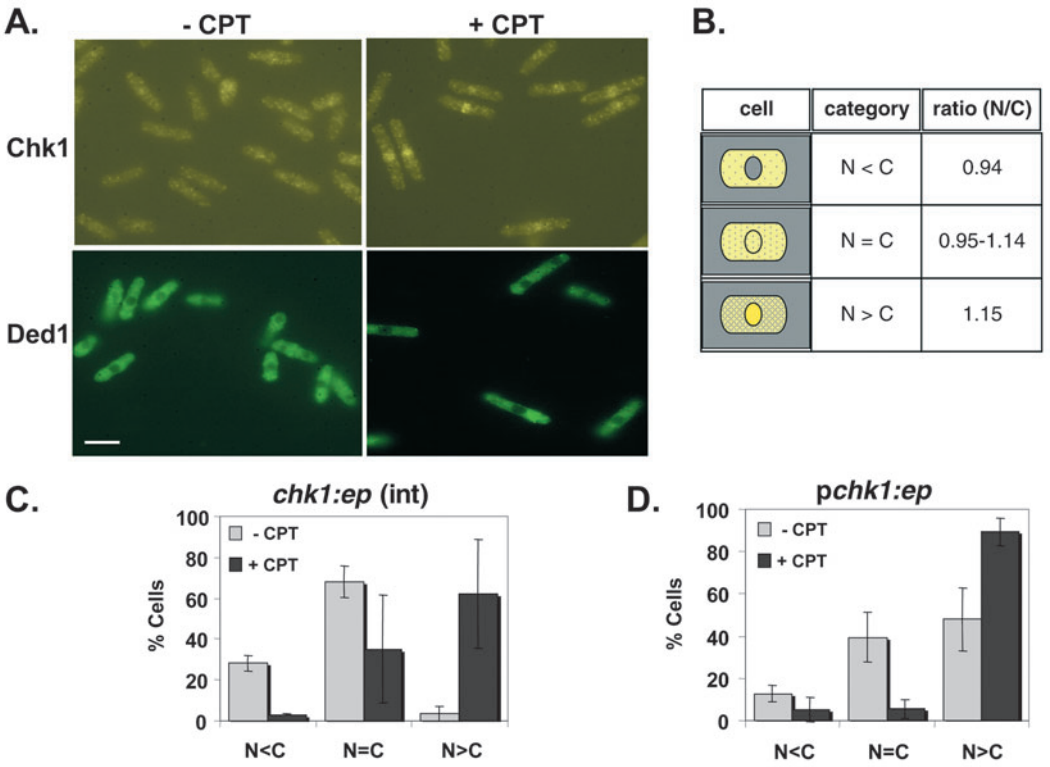

Fig. 3. Chk1 protein accumulates in the nucleus after DNA damage. (A) A strain with integrated Chk1:HA was examined by immunofluorescence with anti-HA antibody. Cultures grown to mid-log phase were treated with 40 μM CPT for 2.5 hours to generate DNA damage, then fixed and processed for immunofluorescence. Antibody to a cytoplasmic protein Ded1 is also shown. Bar, 10 μm. (B) Quantification of immunofluorescence signal was performed by measuring pixel intensity in the nuclei and in an equivalent area of the cytoplasm. Nuclear to cytoplasmic ratios of these signals were then generated and cells were sorted into the three categories shown. (C) Quantification was performed and is presented for the integrated wild-type *chk1:HA* allele (an average of two independent experiments is shown). (D) Quantification is presented on a similar immunofluorescence experiment performed with a strain deleted for endogenous *chk1* and instead expressing Chk1:HA from the pSP1 plasmid (an average of four independent experiments is shown).

(*chk1-4A*), Chk1 localization to the nucleus in response to DNA damage was disrupted as revealed by the appearance of dark circles coincident with the nucleus, suggesting a complete lack of nuclear staining (Fig. 5A). Additionally, this mutant was sensitive to UV exposure and the mutant Chk1 protein failed to be phosphorylated when the cells were treated with CPT indicative of a non-functional protein (Fig. 5B,C). An attempt to rescue these phenotypes was made by cloning the potent SV40 NLS onto the C-terminus of this mutant (*chk1-4A+NLS*). As shown in Fig. 5D, the presence of the SV40 NLS restored localization of the *chk1-4A* mutant to the nucleus as evidenced by the reappearance of staining in the nuclear region. Additionally, the appendage of the SV40 NLS largely restored the DNA damage-induced phosphorylation and rescued the DNA damage sensitivity phenotype (Fig. 5C,E). Because the nuclear stain of the 4A+NLS mutant was not robust, we examined the localization of a wild-type Chk1 protein with the NLS appended. As shown in Fig. 5F, addition of the NLS did indeed enhance nuclear localization of Chk1. Together, these results suggest that the basic residues K377, R378, K381 and K382 contribute to a functional NLS activity in Chk1 and that the NLS plays an integral role in controlling Chk1 localization allowing the protein to function properly.

chk1L→A mutants fail to accumulate in the nucleus in response to DNA damage

Given that Chk1 and 14-3-3 proteins physically interact and that the 14-3-3 protein Rad24 is necessary for the proper localization of Chk1 in response to DNA damage, we investigated the intracellular localization of the Chk1 L→A mutant protein. We performed immunofluorescence on the integrated HA-tagged mutant alleles and found that the proteins localized to the cytoplasm (data not shown). To enhance the signal for immunofluorescence, we then expressed the L→A mutants from plasmids. The mutants displayed a primarily cytoplasmic staining pattern of Chk1 in the absence of CPT treatment (data for two of the mutants is shown in Fig. 6A,B). When cells were treated with CPT for 2-3 hours, Chk1 remained cytoplasmic (Fig. 6A,B). This data indicates that these Chk1 mutants fail to accumulate in the nucleus in response to DNA damage, suggesting that the leucine residues are important for the proper localization of Chk1.

To test whether forced nuclear localization would restore function to the L→A mutants, as it did for the NLS-deficient 4A mutant, we cloned the SV40 nuclear localization sequence (NLS) onto the C-terminus of the Chk1 mutants and performed immunofluorescence on the plasmid-expressed proteins. These Chk1 proteins localized both to the cytoplasm and to the nucleus indicating that localization of the mutant proteins to the nucleus could be restored (Fig. 6C,D). However, this localization pattern remained unchanged when the cells were treated with CPT, as Chk1 L→A mutants did not accumulate in the nucleus (Fig. 6C,D).

To address whether restoration of nuclear localization rescued the DNA damage sensitivity of the leucine mutants, a UV survival assay was performed. Unlike the 4A mutant (see Fig. 5), the addition of an NLS to the C-terminus of the leucine mutants failed to restore function (Fig. 6E). Additionally, the Chk1 leucine mutants containing the SV40 NLS failed to

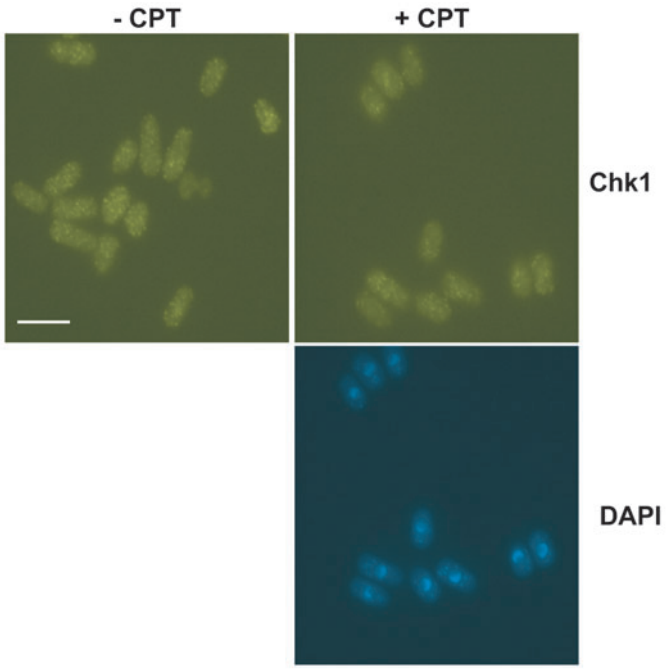

Fig. 4. Chk1 accumulation in the nucleus is Rad24 dependent. Chk1 immunofluorescence was performed in a *rad24Δ* strain after treatment for 2.5 hours with or without 40 μM CPT. Cells were also stained with DAPI. Bar, 10 μm.

undergo the phosphorylation-mediated gel mobility shift in response to DNA damaging agents (Fig. 6F). Taken together these data suggest that these leucine residues are important for the proper localization of Chk1 in response to DNA damage and that forced nuclear localization is not sufficient to correct these mutant phenotypes.

Chk1 contains a functional NES

Given the recent functional role ascribed to 14-3-3 proteins in controlling localization of certain binding partners, we sought to determine the mechanism by which 14-3-3 protein binding governs Chk1 localization (van Hemert et al., 2001). It has recently been demonstrated that 14-3-3 proteins can affect localization of their targets by a molecular interference mechanism through which binding masks sequences such as nuclear export or import signals (Muslin and Xing, 2000). Since the 14-3-3 binding domain on Chk1 resembles a nuclear export sequence (NES) we speculated that the binding of 14-3-3 protein to Chk1 might function similarly.

To determine if the putative NES is functional, immunofluorescence was performed on cells treated with Leptomycin B (LMB), an inhibitor of Crm1-dependent nuclear export (Fornerod et al., 1997; Fukuda et al., 1997; Kudo et al., 1998; Ossareh-Nazari et al., 1997). When wild-type cells are treated with LMB, Chk1 accumulates in the nucleus indicating that Chk1 indeed contains a functional NES (Fig. 7A).

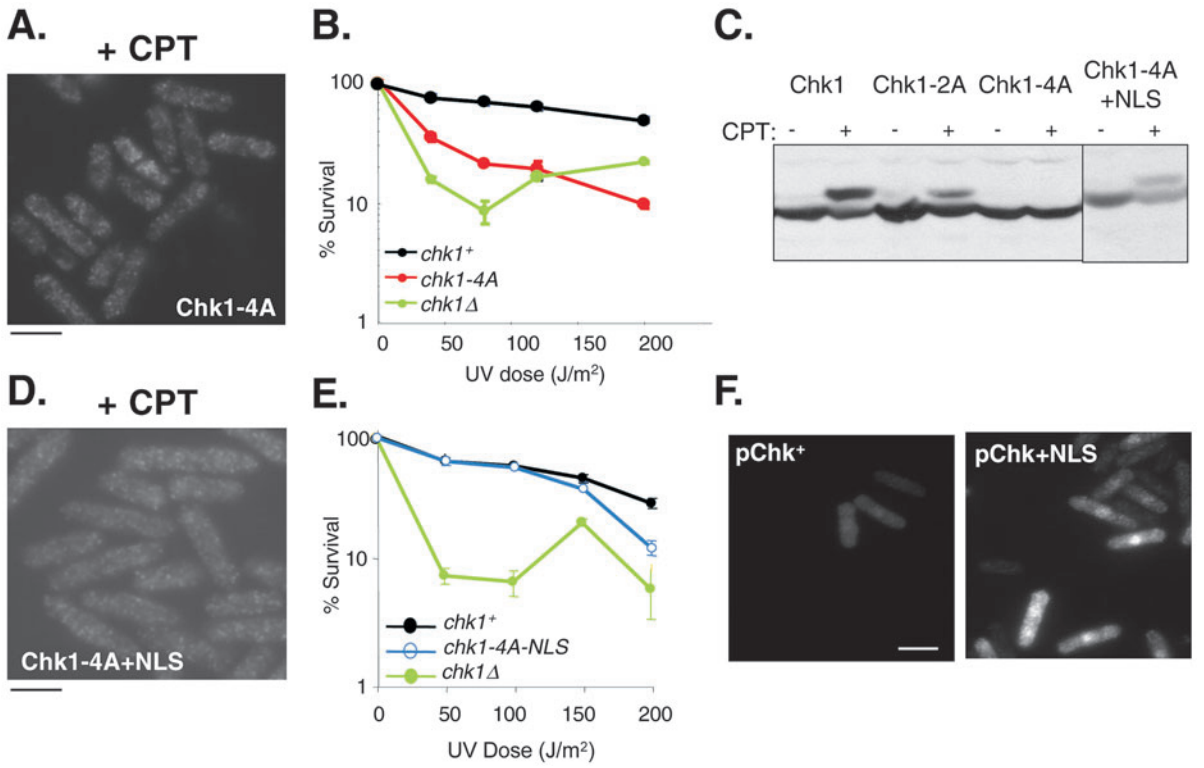

Fig. 5. Chk1 contains a functional NLS. (A) A strain expressing the *chk1-4A* mutant integrated at the *chk1* locus was treated with 40 μM CPT and processed for immunofluorescence. (B) UV survival was measured for the indicated strains after exposure to 50, 100, 150 and 200 J/m^2 UV. UV survival experiments were conducted in triplicate. (C) Western blotting was performed on protein lysates from the indicated strains that were treated with or without 40 μM CPT for 2.5 hours. Chk1 mobility shift was monitored after SDS-PAGE using anti-HA antibody. (D) A strain expressing the *chk1-4A* + SV40 NLS was treated with 40 μM CPT and processed for immunofluorescence. (E) UV survival was measured for the indicated strains as in B. (F) Addition of the SV40-NLS to wild-type Chk1 enhances nuclear localization as detected by immunofluorescence. Bars, 10 μm.

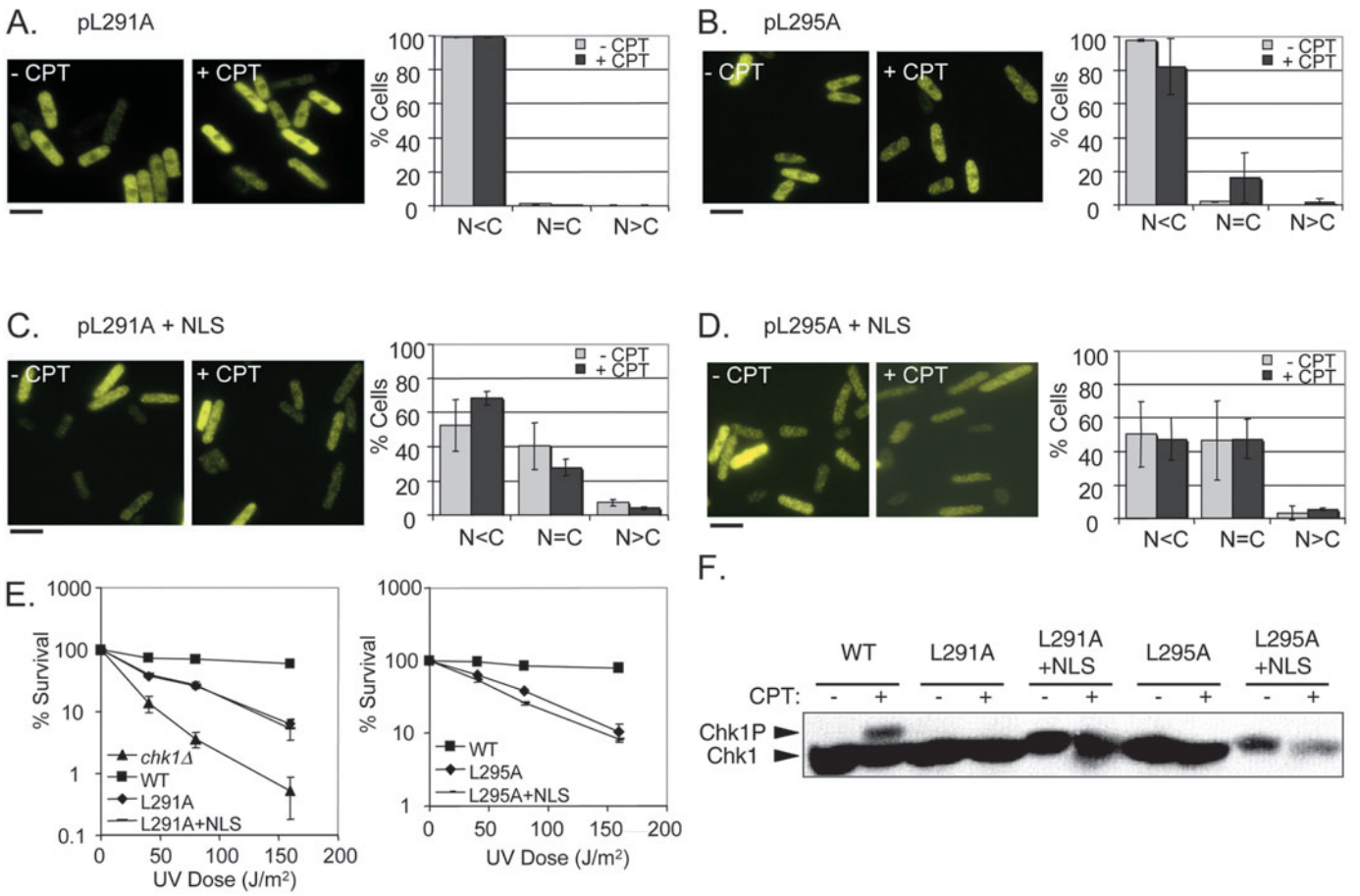

Fig. 6. *chk1L→A* mutants display the same phenotypes as a *chk1*$^{-}$ strain. (A-D) Chk1 immunofluorescence was performed on strains expressing the indicated plasmid-borne Chk1 mutants after treatment with or without 40 μM CPT for 2.5 hours. Quantification was then performed (an average of two independent experiments is shown). Bar, 10 μm. (E) UV survival was monitored for the indicated strains by colony counting after exposure to 40, 80 or 160 J/m^2 UV. (F) Western blotting was performed with 12CA5 antibody on protein lysates from the indicated strains treated with or without 40 μM CPT for 2.5 hours.

Two potential overlapping consensus NES sequences (LxxxLxI/L) are present in the C-terminus of Chk1 (Bogerd et al., 1996). Interestingly, this region contains the leucine residues important for binding to Rad24. To test whether the leucine residues act as an NES we treated cells expressing the NLS-modified L→A Chk1 proteins with LMB. Like wild type Chk1, the L→A + NLS mutants accumulated in the nucleus following treatment with LMB (Fig. 7B,C). Thus, single amino acid substitutions within the putative NES do not abolish NES activity. Therefore, we generated a mutant with each of the four leucines substituted with alanine, along with and without an NLS at the C-terminus (Chk1 4L→A and Chk1 4L→A + NLS). The NLS modified protein exhibits nuclear localization that is not enhanced upon treatment with LMB (Fig. 7D). The failure of this mutant to respond to the drug suggests that this region does indeed contain a functional NES.

Discussion

Chk1 is an essential component of the highly conserved DNA damage checkpoint pathway that contributes to the preservation of genomic integrity. As a downstream effector kinase in the pathway, Chk1 relays the checkpoint signal from detection of DNA damage to control of the cell cycle machinery. Chk1 is phosphorylated and activated in response to several types of DNA damage in a manner that requires the activity of the ATM/ATR-like kinase Rad3 (Capasso et al., 2002; Walworth and Bernards, 1996; Wan et al., 1999). Activation of this pathway in the presence of DNA damage leads to a delay of the cell cycle prior to mitosis in fission yeast. As such it has been demonstrated that Chk1 can phosphorylate key regulators of the cell cycle, the tyrosine phosphatase Cdc25 and the tyrosine kinase Wee1 (O'Connell et al., 1997; Peng et al., 1997; Sanchez et al., 1997) that together control tyrosine phosphorylation of the cyclin dependent kinase, Cdc2/cyclin B. These observations suggest a model whereby activated Chk1 indirectly modulates the activity of Cdc2/cyclin B through its regulators Cdc25 and Wee1.

Evidence in support of this model has arisen from experiments performed in several different systems. Chk1 phosphorylates human Cdc25 in vitro on Ser216 (Peng et al., 1997; Sanchez et al., 1997). This phosphorylation event creates a binding site for a family of proteins known as 14-3-3 proteins (Muslin et al., 1996). 14-3-3 proteins were first found as

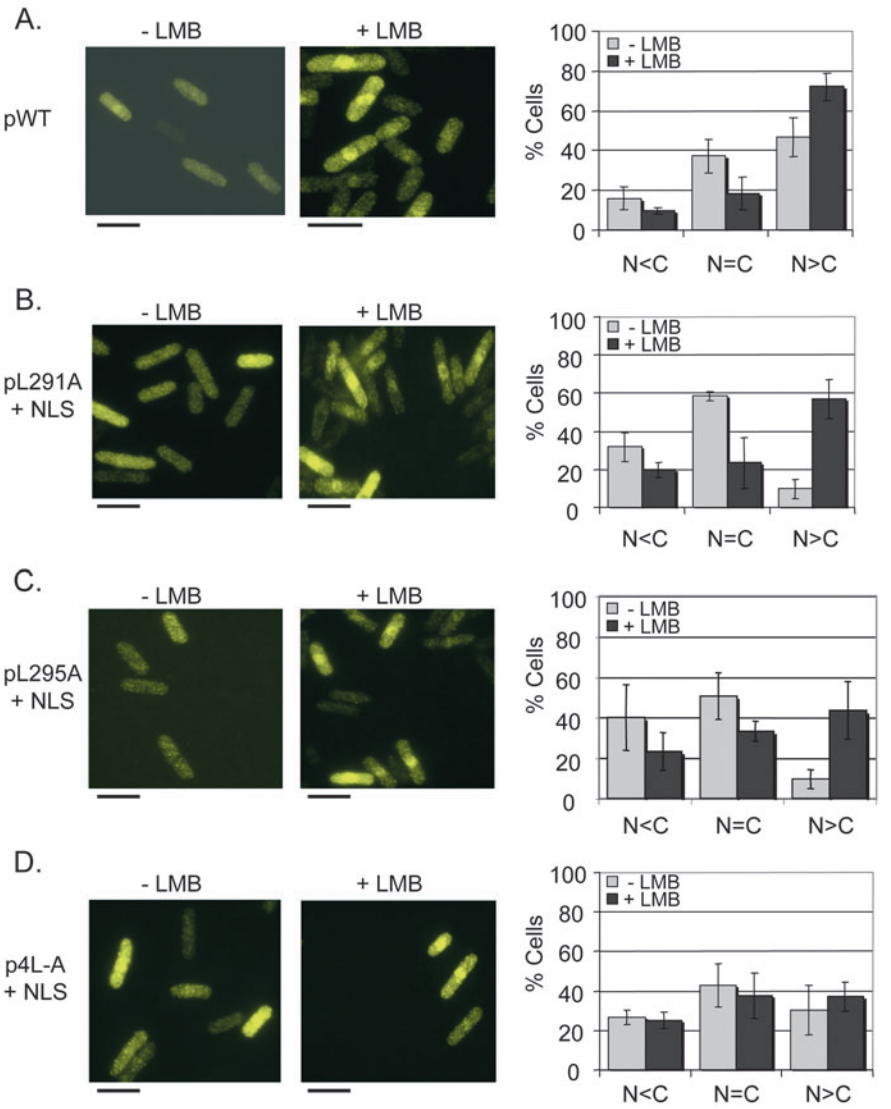

Fig. 7. Chk1 contains a functional NES. (A-D) Chk1 immunofluorescence was performed on strains harboring the indicated plasmid-borne Chk1 alleles after treatment with or without 100 ng/ml LMB for 1-2 hours. Quantification was then performed (an average of four independent experiments is shown for the wild-type, three independent experiments for each of the mutants). Bar, 10 μm.

binding partners for a number of enzymes (Aitken, 1996) and were later demonstrated to have the capacity to bind to phosphorylated amino acid residues (normally serines) in a particular sequence context (Muslin et al., 1996; Yaffe et al., 1997). Several models have been proposed for the role that 14-3-3 protein binding plays in the regulation of Cdc25. Studies of human, frog and fission yeast Cdc25 have suggested that 14-3-3 protein binding regulates the intracellular localization of Cdc25 within the cell (Dalal et al., 1999; Kumagai and Dunphy, 1999; Lopez-Girona et al., 1999; Yang et al., 1999; Zeng and Piwnica-Worms, 1999). In fission yeast, binding of 14-3-3 protein to Cdc25 leads to its cytoplasmic sequestration thereby separating it from its substrate, Cdc2/cyclin B, delaying activation of Cdc2/cyclin B and consequently, the onset of mitosis (Peng et al., 1997; Zeng and Piwnica-Worms, 1999). Although human Cdc25C is also sequestered in the cytoplasm as a result of 14-3-3 association, it is not compartmentalized away from its target because Cdc2 is also cytoplasmic in such cells (O'Connell et al., 2000). However, studies of mammalian and fission yeast Cdc25 utilizing in vitro assays have also suggested that 14-3-3 binding inactivates the phosphatase activity of Cdc25 (Blasina et al., 1999; Furnari et al., 1999). These observations are consistent with 14-3-3 proteins playing a prominent role in the execution of a checkpoint arrest, a supposition supported by the fact that cells lacking *rad24* are sensitive to DNA damaging agents and display a checkpoint defect (Ford et al., 1994).

If Chk1-dependent phosphorylation of Cdc25 and consequent association of 14-3-3 protein plays a critical role in the fission yeast checkpoint response, then it probably is the result of effects on a small pool of Cdc25. There is no detectable difference in the total amount of Cdc25 associated with 14-3-3 protein in damaged or undamaged cells (Chen et al., 1999). Furthermore, the association of Cdc25 with 14-3-3 protein is unaffected by the absence of Chk1 (Chen et al., 1999). Thus, a kinase other than Chk1 must also be capable of phosphorylating Cdc25 in such a way as to promote association with 14-3-3 proteins in the absence of DNA damage. In addition, it is probably a small, albeit critical pool of 14-3-3-associated Cdc25 that must be removed from the nucleus in order to efficiently delay the cell cycle in response to DNA damage.

The fission yeast 14-3-3 proteins Rad24 and Rad25, also associate with Chk1 itself, raising the possibility that an important additional role for these proteins in the DNA damage response is mediated through their interaction with Chk1. Indeed, treatment of cells with agents that damage DNA causes a tenfold increase in the level of Chk1 that is found in association with Rad24 (Chen et al., 1999). As demonstrated in this work, mutation of amino acids in Chk1 that disrupt the interaction between Chk1 and Rad24 confer similar phenotypes to strains devoid of Chk1 (Fig. 2), suggesting that Rad24 plays a critical role in mediating the checkpoint function of Chk1. It is important to note that the residues important for 14-3-3 binding to Chk1 do not constitute a phosphopeptide-binding motif typical of those thought to be critical for phosphoserine-mediated 14-3-3-target protein interactions (Yaffe et al., 1997). Nonetheless, other proteins have been shown to bind to 14-3-3 proteins through non-traditional motifs as well (Masters et al., 1999; Mils et al., 2000).

Results presented here demonstrate that fission yeast Chk1 accumulates in the nucleus in response to DNA damage (Fig.

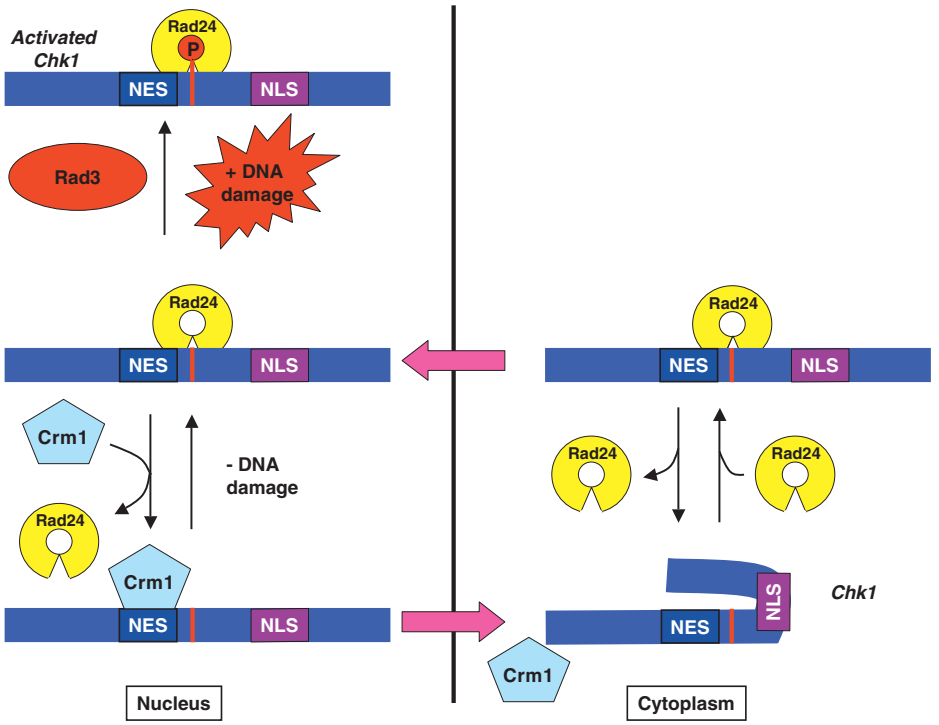

Fig. 8. Model for the role of localization in regulation of Chk1 in fission yeast. When Chk1 binds Rad24 the 14-3-3 protein may act to open up the conformation of Chk1 to expose an NLS, while at the same time blocking access of Crm1 to the NES. This would allow Chk1 to enter the nucleus. Subsequent dissociation of 14-3-3 would expose the NES permitting Crm1 to bind and mediate export. When cells incur DNA damage, Chk1 becomes phosphorylated and its interaction with 14-3-3 protein is stimulated approximately tenfold (Chen et al., 1999). Nuclear accumulation of Chk1 would thus result from the stabilized interaction of Chk1 with Rad24 and the consequent reduction in its association with Crm1.

3) as was recently suggested for mammalian Chk1 (Jiang et al., 2003). In fission yeast, accumulation is dependent on Rad24, and if the interaction between Chk1 and Rad24 is disrupted by point mutations of Chk1, then the localization of Chk1 to the nucleus is also impaired (Fig. 5; Fig. 6A,B). This implies that Rad24 plays an active role in the subcellular localization of Chk1. Our results suggest that binding of 14-3-3 protein may be important both to permit localization of Chk1 to the nucleus and to block its export out of the nucleus in response to DNA damage.

We identified putative nuclear localization signal (NLS) and nuclear export signal (NES) sequences in the C-terminal half of Chk1. Mutation of the basic residues encoding the NLS (*chk1*-4A) was sufficient to disrupt the ability of Chk1 to localize to the nucleus (Fig. 5A). This localization defect could be overcome by forcing the *chk1-4A* mutant into the nucleus by addition of an SV40 NLS (Fig. 5D). Additionally, the phenotypes associated with this defective *chk1* allele could be restored (Fig. 5C,E). Interestingly, the *chk1-4A* mutant containing the SV40 NLS, although it localized to the nucleus and rescued the *chk1* defects, did not appear to accumulate in the nucleus, suggesting that nuclear accumulation, although a prominent feature of the damage response may not be an essential requirement for Chk1 checkpoint function. Although mammalian Chk1 has also been shown to localize to the nucleus, the exact location of the NLS has not been determined (Jiang et al., 2003). There are basic residues in the sequence of mammalian Chk1 in the region that we have identified as being important for localization of fission yeast Chk1. However, there is not a precise alignment of the critical residues when Chk1 from the two species are compared. Studies on *Xenopus* Chk1 are consistent with an NLS also being located in the C-terminal domain of the protein, although again those amino acids do not precisely align with basic residues in the fission yeast sequence (Nakajo et al., 1999; Oe et al., 2001). Given the flexibility of sequences that can act as NLS sequences, it may not be surprising that although the importance of nuclear localization is conserved for each species, the precise location of the residues that mediate that function may not be conserved.

Results presented in this study demonstrate that fission yeast Chk1 is also subject to nuclear export and that 14-3-3 protein binding may be important for that aspect of Chk1 behavior. Nuclear accumulation of Chk1 could be induced by treating cells with Leptomycin B (LMB), an inhibitor of the nuclear export factor Crm1 (Kudo et al., 1999). Chk1 contains two overlapping sequences that resemble an NES, which coincidentally are the same residues required for the Chk1/Rad24 interaction. Curiously, mutation of these leucine residues prevents Chk1 from localizing to the nucleus, indicating that the Rad24 interaction may be required for import. To test whether these leucines also encode an NES we forced nuclear localization by addition of the potent SV40 NLS onto the C-terminus of these mutants. Nuclear localization was restored, which allowed us to evaluate the contribution of the leucine residues to nuclear export. When treated with LMB, cells expressing these versions of Chk1 still displayed an enhanced nuclear signal suggesting that single mutations of the leucine residues leaves the NES intact. On the other hand, simultaneous mutation of all four leucines to alanines caused enhanced nuclear localization in the absence of LMB and subsequent treatment with LMB did not further enhance the nuclear signal. Taken together this strongly suggests that these residues, in addition to being important for Rad24 binding, also encode a functional NES.

These observations imply that Chk1 localization to the nucleus in response to DNA damage may be regulated by a 14-3-3 dependent molecular interference mechanism as has been proposed for other 14-3-3 targets, such as the catalytic subunit of human telomerase (Muslin and Xing, 2000; Seimiya et al.,

2000). We suggest that when Chk1 binds Rad24 the 14-3-3 protein may act to open up the conformation of Chk1 to expose an NLS, while at the same time blocking access of Crm1 to the NES (Fig. 8). This would allow Chk1 to enter the nucleus. Subsequent dissociation of 14-3-3 would expose the NES permitting Crm1 to bind and mediate export (Fig. 8). When cells incur DNA damage, Chk1 becomes phosphorylated and its interaction with 14-3-3 protein is stimulated approximately tenfold (Chen et al., 1999). Nuclear accumulation of Chk1 would thus result from the stabilized interaction of Chk1 with Rad24 and the consequent reduction in its association with Crm1 (Fig. 8).

Mammalian Chk1 also accumulates in the nucleus following DNA damage and has been shown to bind to 14-3-3 proteins (Jiang et al., 2003). However, the sites proposed to act as the NES and 14-3-3 protein binding site in human Chk1 are different from the site we have identified as being important for both in fission yeast Chk1. Indeed, the residues determined to be important for 14-3-3 binding and NES function for fission yeast Chk1 are not directly conserved in Chk1 from multicellular eukaryotes. Furthermore, the proposed 14-3-3 binding site on human Chk1 includes the serine residue that is phosphorylated in response to DNA damage, Ser-345, which does lie within a consensus phosphopeptide motif suitable for 14-3-3 binding. However, although fission yeast Chk1 is phosphorylated on its own Ser345, this residue does not lie within a canonical 14-3-3 binding site. It is nonetheless important for 14-3-3 binding because phosphorylated Chk1 seems to be the preferential target for 14-3-3 binding (Chen et al., 1999) and mutation of Serine345 to alanine, which abolishes phosphorylation, also eliminates the damage-dependent increase in Chk1 association with Rad24 (Capasso et al., 2002). Fission yeast Ser345 lies more than 40 residues away from the leucine residues that affect basal 14-3-3 binding. We interpret our results to suggest that 14-3-3 binding to unphosphorylated Chk1 is important for revealing the NLS and allowing Chk1 access to the nucleus, and that subsequent phosphorylation of Ser345 stabilizes the interaction of 14-3-3 protein with Chk1. Whether or not the phosphorylated Ser345 directly contacts 14-3-3 protein, albeit through a non-standard phosphopeptide binding interaction, remains to be determined.

We note that the residues we have identified as being important both for nuclear export and for 14-3-3 protein binding are conserved in budding yeast Chk1 even though they are not conserved in mammalian, frog or fly Chk1. Thus it is possible that there is a fungal-specific role for this domain. One fundamental difference between ascomycetes such as fission and budding yeast, and eukaryotic cells present in multicellular organisms is that the nuclear envelope of ascomycetes remains intact during mitosis. Thus, perhaps there is an additional requirement for nuclear export of fission and budding yeast Chk1 that is regulated by 14-3-3 association, but is not conserved in multicellular eukaryotes.

Our studies suggest that binding of Chk1 to 14-3-3 proteins may play multiple roles in Chk1 regulation. Forcing the 14-3-3 binding site mutants into the nucleus through addition of the SV40 NLS was not sufficient to restore Chk1 function. Indeed, despite their nuclear localization, these alleles of Chk1 were not phosphorylated in response to DNA damage, a necessary step for activation of the wild-type protein (Capasso et al., 2002). Thus, Rad24 association may act to promote phosphorylation of Chk1 when DNA damage is present, perhaps aiding in the targeting of Chk1 to its upstream kinase, Rad3. As has been proposed for other signaling pathways on which 14-3-3 proteins function, Rad24 may also act to direct Chk1 to its checkpoint targets, such as Cdc25 or Wee1.

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