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Chk1 C-terminal regulatory phosphorylation mediates checkpoint activation via derepression of Chk1 catalytic activity

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Abstract

Chk1 is phosphorylated within its C-terminal regulatory domain by the upstream ATM/ ATR kinases during checkpoint activation, however how this modulates Chk1 function is poorly understood. Here, we show that Chk1 kinase activity is rapidly stimulated in a cell cycle phase-specific manner in response to both DNA damage and replication arrest, and that the extent and duration of activation correlates closely with regulatory phosphorylation at serines (S) S317, S345, and S366. Despite their evident co-regulation, substitutions of individual Chk1 regulatory sites with alanine (A) residues have differential effects on checkpoint proficiency and kinase activation. Thus, whereas Chk1 S345 is essential for all functions tested, mutants lacking S317 or S366 retain partial proficiency for G2/ M and S/ M checkpoint arrests triggered by DNA damage or replication arrest. These phenotypes reflect defects in Chk1 kinase induction, since the mutants are either partially (317A, 366A) or completely (345A) resistant to kinase activation. Importantly, S345 phosphorylation is impaired in Chk1 S317A and S366A mutants, suggesting that modification of adjacent SQ sites promotes this key regulatory event. Finally, we provide biochemical evidence that Chk1 catalytic activity is stimulated via a de-repression mechanism.

Introduction

DNA damage or arrested replication forks activate multiple cell cycle checkpoint responses which maintain genome integrity (Bartek and Lukas, 2003). Cell cycle checkpoints are controlled by a network of signal transduction pathways. Key transducers are the phosphatidyl-inositol-3 kinase-like kinases, ATM and ATR, which target the Chk1 and Chk2 effector kinases leading to arrest in specific phases of the cell cycle and DNA repair. ATR is activated by recruitment to regions of single stranded DNA at stalled replication forks or at bulky lesions induced by UV, leading to Chk1 phosphorylation (Dart et al., 2004; Lupardus et al., 2002; Zou and Elledge, 2003). ATM, by contrast, is activated primarily by DNA double-strand breaks (DSBs) caused by irradiation or radiomimetic drugs (Lee and Paull, 2005; Smith et al., 1999; Suzuki et al., 1999) and targets both Chk1 and Chk2 (Gatei et al., 2003; Matsuoka et al., 2000). There is however cross-talk and under some circumstances ATR can be activated in response to DSBs in an ATM-dependent manner (Adams et al., 2006; Jazayeri et al., 2006; Myers and Cortez, 2006). Downstream of ATM/ ATR, evidence from studies in human, mouse, and avian cells indicates that Chk1 is the

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dominant effector of both the DNA and replication checkpoints in vertebrates, with Chk2 playing an auxiliary role (Chen and Sanchez, 2004).

ATM and ATR preferentially phosphorylate serine and threonine residues followed by glutamine (SQ/TQ motifs). Their substrates often have clusters of SQ/TQ motifs, termed SQ/TQ cluster domains (SCDs). Multisite phosphorylation of SQ/TQ motifs is required for DNA-damage responses, often by mediating protein-protein interactions (Traven and Heierhorst, 2005). Chk1 comprises an N-terminal kinase domain and a C-terminal regulatory domain. Structural studies indicate that the isolated kinase domain can exist in an open, potentially active conformation (Chen et al., 2000), however the regulatory domain is thought to act in an autoinhibitory fashion by binding and constraining kinase activity (Katsuragi and Sagata, 2004; Oe et al., 2001).

The Chk1 regulatory domain contains an SCD consisting of four SQ sites conserved from humans to xenopus (Zachos et al., 2003). All four sites can be phosphorylated in an ATR dependent manner in xenopus cell free extracts (Guo et al., 2000), however in other species only Ser317 and Ser345 have been shown to be phosphorylated in response to genotoxic stress *in vivo* (Liu et al., 2000; Zhao and Piwnica-Worms, 2001). Phosphorylation at these sites has been linked to increased Chk1 kinase activity and checkpoint activation (Feijoo et al., 2001), although whether Chk1 kinase induction is required for checkpoint responses is unclear (Zou and Elledge, 2003). In addition, Ser345 phosphorylation can mediate protein-protein interactions with 14-3-3 proteins (Jiang et al., 2003) and target Chk1 for degradation (Zhang et al., 2005), suggesting that this site plays a key regulatory role. This is supported by experiments mouse ES cells that replaced S345 in endogenous Chk1 with a non-phosphorylatable alanine residue (Niida et al., 2007). The resulting mutant cells were checkpoint defective but also died rapidly in culture. No effect of S345 mutation on Chk1 kinase activity was however detected (Niida et al., 2007).

Here we establish that checkpoint activation in response to both DNA damage and replication arrest in vertebrate cells requires induction of Chk1 catalytic activity, that this depends on phosphorylation of multiple SQ sites within the SCD by ATM/ ATR, and that both processes are regulated in a strongly cell cycle-dependent fashion. Using Chk1 null cells stably reconstituted with individual Chk1 SQ site point mutants we show that whilst Ser345 is essential for Chk1 activation and checkpoint proficiency, mutants lacking adjacent phosphorylated residues at S317 and S366 retain partial G2/ M and S/ M checkpoint proficiency. Finally, we report biochemical evidence which suggests that multi-site SQ phosphorylation may stimulate Chk1 catalytic activity via a de-repression mechanism.

Results

Chk1 Kinase Activation is Tightly linked to Phosphorylation at multiple ATM/ATR Consensus Sites within the C-terminal Regulatory Domain

The G2/ M and S/ M checkpoints triggered by DNA damage or replication arrest in DT40 cells are dependent on Chk1 (Zachos et al., 2003; Zachos et al., 2005). To determine how Chk1 activity fluctuates under these conditions, DT40 cultures were treated with either the DNA polymerase inhibitor aphidicolin or 10 Gy of ionising radiation. Chk1 was precipitated from each sample and assayed for its ability to phosphorylate a synthetic peptide substrate (Chktide) using an optimised immunoprecipitation kinase assay (see Suppl. Fig 1 for details). As shown in Fig. 1A, treatment with aphidicolin induced a marked induction of kinase activity within 15 mins of treatment. Kinase activity remained elevated for 1 hour after treatment and then fell back to near basal levels by 3 hours. Irradiation, by contrast, induced a more modest increase in kinase activity which also declined after 3 hours (Fig. 1A).

In addition to the well-characterised Ser317 and Ser345 phosphorylation sites there are additional conserved SQ sites within the Chk1 SCD at serines 357 (S357) and 366 (S366) which could serve as potential targets for ATM/ ATR. Attempts to generate phospho-specific antibodies specific for S357 were technically unsuccessful (data not shown), however we were able to confirm that S366 is subject to phosphorylation during checkpoint activation using this approach (see below and methods for details).

Using phospho-specific antisera specific for S317, S345, and S366, we then compared the extent and duration of phosphorylation at each of these sites in relation to Chk1 kinase activity after replication arrest or irradiation-induced DNA damage. Phosphorylation of each of these sites increased within 15 mins of treatment with either aphidicolin or IR (Fig. 1B, C). In each case phosphorylation persisted at maximum levels up to one hour post treatment but then declined between 1 and 3 hours, closely paralleling kinase activity (Fig. 1A).

When the immunoprecipitated Chk1 proteins were eluted and resolved by SDS-PAGE after the kinase assay shown in panel (A) had been performed, we observed that increased kinase activity against Chktide was paralleled by a corresponding increase in auto-phosphorylation of Chk1 itself (Fig. 1D). This was most clearly seen in aphidicolin-treated cells, where it was evident that auto-phosphorylation was largely confined to a subset of Chk1 isoforms that exhibited retarded electrophoretic mobility (Fig. 1D, arrows).

Chk1 is preferentially activated in response to DNA damage and replication arrest in late S and G2 phase of the cell cycle

To determine whether basal Chk1 kinase activity varied during an unperturbed cell cycle, we used centrifugal elutriation to separate an asynchronous DT40 culture into fractions enriched in specific cell cycle phases (Fig. 2A). Fraction 1 (F1) contained predominantly G1 cells, F4 almost exclusively S phase, whilst F6 was highly enriched for cells in G2/ M. The intermediate fractions contained variable proportions of cells from sequential but progressively later phases of the cell cycle. Chk1 was immunoprecipitated from each of these fractions and its ability to phosphorylate Chktide determined (Fig. 2B). Interestingly, cell cycle-specific variations in basal Chk1 kinase activity were detectable, with higher levels of Chk1 kinase activity in the elutriated fractions enriched for S-phase cells (F3, F4 and F5).

We then investigated whether Chk1 activation in response to replication inhibition or DNA damage was similarly cell cycle dependent. Replicate cultures of DT40 cells were treated with aphidicolin or irradiated with 10Gy and after 30 mins separated by elutriation. Remarkably, aphidicolin and IR each induced a very similar pattern and level of cell cycle phase-specific increases in Chk1 catalytic activity. In each case kinase activity was highest in fractions enriched for mid to late S-phase (F3, F4, and F5) and lower but still significant in F6 where G2- and M-phase cells predominated. By contrast fraction F1, composed primarily of G1 cells with some very early S-phase cells (Fig. 2A), contained lower levels of active Chk1 kinase than the corresponding asynchronous populations, and was only weakly activated, if at all, as a result of aphidicolin or IR treatment (Fig. 2B, C, D).

To determine if cell cycle-specific variations in kinase activation were linked to SQ site phosphorylation, fractions were analysed by western blotting using anti-phospho-S317, -S345 or -S366 antibodies. In untreated cells a low basal level of Ser317 phosphorylation was noted in all fractions, however no modification of S345 or S366 was detectable (Fig. 2B). By contrast, a similar pattern of induced SQ site phosphorylation was detected in aphidicolin- and IR-treated cultures, with the highest levels of phosphorylation in fractions containing the highest percentage of S-phase cells, F3, F4 and F5 (Fig. 2C, D).

Differential requirement for phosphorylation of individual SQ residues within the Chk1 SCD for G2/M and S/ M checkpoint proficiency

To determine whether modification of specific SQ sites by ATM/ATR was required for checkpoint proficiency, we stably reconstituted Chk1-deficient DT40 cells with mutant forms of Chk1 in which S317, S345, and S366 were replaced by alanine (A) residues (Fig. 3A). S317 and S345 were also mutated to aspartic acid (D) with the objective of mimicking constitutive phosphorylation. For comparison we included KD-Chk1, plus a mutant in which S280 was replaced by alanine. S280 is an established phosphorylation site in Chk1 but it is not thought to be a target for ATM/ATR (Puc et al., 2005). Stable expression of each of the mutant Chk1 proteins was verified by western blotting (Fig. 3B).

We then determined whether the mutant Chk1 proteins could complement the G2/ M and S/ M checkpoint defects previously documented in Chk1-deficient cells (Zachos et al., 2003). To quantify G2/ M checkpoint proficiency, cells were incubated with nocodozole for 10 hours with or without prior irradiation and the percentage of phospho-Ser 10 histone H3 positive (mitotic) cells determined. In this assay G2/ M arrest in checkpoint proficient DT40 cells manifests as a 70-80% reduction in the number of cells accumulating in mitosis after irradiation compared to control (Fig. 3C, black bars). This response is completely absent in Chk1-deficient (KO) cells but is restored upon stable re-expression of wild-type Chk1 (Rev) but not KD-Chk1. Remarkably, mutant Chk1 proteins where S345 was replaced by either alanine (345A) or aspartic acid (345D) were completely unable to restore G2/ M checkpoint proficiency. In contrast, mutants where alanine or aspartic acid replaced S317 conferred an intermediate phenotype where G2/ M arrest was detectable but significantly impaired compared to controls (Fig. 3C). This was also true for Ser366A, but not for Ser280A, which alone was able to restore full checkpoint proficiency (Fig. 3C).

To determine whether the S-M checkpoint was similarly reliant on phosphorylation of specific SQ sites, cells were incubated in nocodozole for 10 hours in the presence of aphidicolin and the percentage and DNA content of mitotic cells determined. Under these conditions (Zachos et al., 2005), S/ M checkpoint failure in Chk1-deficient cells results in an overall increase in the total percentage of mitotic cells compared to control (Fig. 3C, grey bars), together with the characteristic appearance of cells which have entered mitosis with unreplicated DNA (ie with 2N DNA content; Fig. 3C, red bars).

As with the G2/M checkpoint, re-introduction of wild-type and S280A but not kinase-dead Chk1 restored full S/ M checkpoint proficiency as judged by both criteria, however the S345A/ D mutants exhibited complete checkpoint failure (Figure 3C). In comparison, the S317A/ D and S366A mutants exhibited a more complex phenotype. These cultures accumulated few, if any, mitotic cells with 2N DNA content (Fig. 3C, red bars), indicating that these mutants remain capable of restraining mitosis in cells with completely unreplicated DNA. There was however a modest increase in the overall proportion of mitotic cells compared to control (Fig. 3C, gray bars), suggesting that mitotic delay at later stages of the cells cycle is impaired. Thus, phosphorylation of S345 is essential for both the G2/ M and S/ M checkpoint responses, whereas mutants lacking S317 and S366 retain partial proficiency for both.

Phosphorylation of Chk1 at ATM/ATR Consensus Phosphorylation Sites is Required for Kinase Activation in Response to Stress

These observations raised the issue of whether partial or complete defects in checkpoint proficiency resulting from SQ site mutations would be reflected in altered Chk1 kinase activation in response to replication arrest or DNA damage. To address this, wild-type and S317A-, S345A- and S366A-reconstituted cell lines were treated with either aphidicolin or

irradiation and harvested over a 3 hour time course. The wild-type and mutant Chk1 proteins were then immunoprecipitated and their kinase activities determined. Values are presented as fold increase or decrease from control at each time point for each cell type. Subsequently the immunoprecipitated Chk1 proteins were eluted and analysed by western blotting to confirm equal recovery in each assayed sample (data not shown).

As shown in Fig. 4A and 1A, aphidicolin induced a substantial induction in the kinase activity of wild-type Chk1 in DT40 cells within 15 mins of treatment. In comparison, the catalytic activity of the S317A and S366A mutant proteins was delayed and induced to a much lesser degree, whilst S345A kinase activity did not increase at all. This was not due to a total lack of kinase activity in the S345A protein as all mutants exhibited basal kinase activity (Suppl. Fig. 2). Similar results were observed after irradiation, although in this case S366A Chk1 kinase activity increased only marginally at later times (3 hours; Fig 4B).

Taken together, these experiments established that Chk1 S345, presumably through its capacity to serve as a site of phosphorylation catalyzed by ATM/ ATR, was essential both for checkpoint function and kinase activation under conditions of genotoxic stress. The basis for the partial checkpoint proficiency and diminished kinase activation resulting from mutations in other SQ sites however was less clear. To explain these effects, we considered that mutation of adjacent SQ sites might affect phosphorylation of S345. To test this, wild type and mutant cell lines were treated with aphidicolin or irradiated for 15 mins then analysed by western blotting to establish the relative degree of S345 phosphorylation in each mutant protein. As expected, the S345A mutant Chk1 protein was not recognised by anti-pS345 antibodies, however it was also clear that this site was phosphorylated to much a lesser degree in the S317A and S366A mutant proteins than wild-type or kinase-dead Chk1 following both replication arrest and damage (Fig. 4C and D). We also noted that phosphorylation of S317 occurred efficiently in the S345A mutant protein but less so in S366A, whilst S366 phosphorylation was slightly reduced in both S317A and S345A mutants, at least after aphidicolin treatment when this modification was most strongly induced (Fig. 4C).

Chk1 S345 phosphorylation de-represses latent kinase activity under native conditions

S345 phosphorylation could stimulate Chk1 catalytic activity by inducing a conformational change in the kinase domain to an inherently more active configuration, or alternatively, by modulating interaction with regulatory factors. Under our standard assay conditions, where Chk1 shows S345 phosphorylation-dependent kinase activation, cells are lysed and the protein immunopurified under mild, non-denaturing conditions (Lysis buffer: LB). To gain insight into the possible presence of co-precipitating regulatory factors under these conditions, we investigated the effect of washing Chk1 immunoprecipitates with a more stringent buffer (RIPA) prior to performing the kinase assay. Immunoprecipitates prepared from control and aphidicolin-treated DT40 cells under standard conditions (LB) were divided and washed three further times with LB or RIPA buffer. A portion of each immunoprecipitate was then analysed by western blotting for Chk1 and the remainder for kinase activity.

As shown in Fig. 5A (upper panels), RIPA washing did not result in any detectable loss of Chk1 from the precipitates, however the kinase activity of Chk1 immunopurified from both control and aphidicolin-treated cells was increased (Fig. 5B). This effect was relatively greater in the untreated sample than that in the pre-activated, aphidicolin-treated sample (Fig. 5B). In addition to increased activity against Chktide, Chk1 autophosphorylation was elevated in the RIPA-washed immunoprecipitates (Fig. 5A).

We next asked whether the Chk1 S345A mutant, which is resistant to activation under native conditions (Fig. 4), could be stimulated in a similar fashion *in vitro*. To this end Chk1, Chk1 S345A, and Chk1 KD (kinase dead) proteins were immunoprecipitated under native conditions and then washed either in LB or RIPA buffers prior to measurement of kinase activity. As shown in Fig. 5C, basal kinase activity of both the wild-type and S345A mutant Chk1 proteins was greatly increased after RIPA washing, whereas K38R protein was not. Phosphorylation of S345 is therefore not obligatory for high levels of Chk1 kinase activity *per se*, even though it is essential for biological function and for increased kinase activity under native assay conditions in response to checkpoint activating signals.

One means by which RIPA buffer might stimulate Chk1 kinase activity could be by dissociating the kinase domain from the Chk1 C-terminal regulatory tail to release autoinhibition (Katsuragi and Sagata, 2004). To test this, we expressed a C-terminally truncated form of Chk1, Δ400, together with increasing amounts of the isolated C-terminal regulatory domain (Chk1 amino acids 260-475) in Cos1 cells. Lysates were precipitated under standard conditions using C-terminal antiserum, which reacts with only the Chk1 260-475 polypeptide, and analysed for co-precipitation of Chk1 Δ400. As shown in Fig. 5D, Chk1 Δ400 was efficiently co-precipitated with Chk1 260-475, indicating that these proteins form a complex *in vivo*. The Chk1 Δ400 protein was however retained within immunoprecipitates after RIPA washing (Fig. 5E), indicating that dissociation of the inhibitory C-terminal and kinase domain alone is unlikely to account for the resulting increase in kinase activity.

Discussion

Chk1 is a key effector of checkpoint responses to a wide range of genotoxic stresses in vertebrate cells, including the G2/ M and S/ M arrests which delay the onset of mitosis whilst DNA is damaged or replication incomplete (Bartek and Lukas, 2003) . In many situations these responses are triggered in a rapid and acute fashion, suggesting that Chk1 function must be subject to stringent regulation. DNA damage or DNA synthesis inhibition triggers ATM/ ATR-mediated phosphorylation of Chk1 on two SQ motifs, S317 and S345, within the C-terminal regulatory domain, however it remains unclear exactly how these modifications affect Chk1 biochemical and biological function. Measurements of Chk1 kinase activity after exposure to genotoxic stress have produced conflicting results; some studies have documented modest increases during checkpoint activation (Feijoo et al., 2001; Zhao and Piwnica-Worms, 2001), whereas others have failed to detect any change (Kaneko et al., 1999). An alternative view holds that subcellular localisation play a key role in Chk1 regulation. In its most extreme form this hypothesis suggests that Chk1 is constitutively active and that the role of regulatory ATM/ ATR phosphorylation is simply to dissociate the protein from chromatin and enable it to migrate to other subcellular locations such as the cytoplasm or centrosome (Kramer et al., 2004; Shimada et al., 2008; Smits et al., 2006).

Here, we show that DNA damage and replication arrest do stimulate endogenous Chk1 kinase activity, and that activation closely parallels S317 and S345 phosphorylation. We also found that a previously unrecognised SQ phosphorylation site, S366, is modified after DNA damage and replication arrest, and that the extent and duration of its modification is similar to the better-characterised S345 and S317 sites.

Interestingly, the kinetics of Chk1 regulatory phosphorylation and kinase activation were surprisingly transient in comparison to the duration of downstream checkpoint responses. The G2/ M and S/ M checkpoint delays elicited by DNA damage or replication arrest are sustained for at least 8 hours in DT40 cells under these conditions (Zachos et al., 2003; Zachos et al., 2005), however in each case Chk1 activation and SQ site phosphorylation

peaked within 1 hour and then declined substantially by 3 hours. Presumably therefore either a brief pulse of Chk1 activity can rapidly set in motion biochemical events which lead to prolonged mitotic delay, or alternatively, once established, the checkpoints can be maintained by much lower levels of Chk1 activity.

Additional complexity was revealed when we investigated the cell cycle dependence of Chk1 activation. Even in unperturbed cell cultures it was apparent that Chk1 activity varied in a cell cycle dependent fashion, with much higher activity in fractions enriched for mid to late-S/ G2 phase cells and much lower levels in fractions containing predominantly cells in G1 and early S. Remarkably, a very similar pattern of variation was observed after either irradiation or aphidicolin treatment when the overall levels of Chk1 kinase activity were substantially amplified, and in all three situations the extent of S317, S345, and S366 regulatory phosphorylation closely paralleled kinase activation.

Irradiation will cause DNA damage in every exposed cell whereas aphidicolin should only stall replication forks in cells actively engaged in DNA synthesis, yet both induce very similar cell cycle phase-specific increases in Chk1 activity. Why should this be? We did not observe major cell cycle fluctuations in the level of Claspin, an adaptor protein required for ATR-mediated Chk1 activation (Mailand et al., 2006; Peschiaroli et al., 2006), in our elutriation experiments (data not shown). One possible explanation might be that DNA damage activates the ATR-Chk1 pathway only in S and G2 cells, as recently proposed by Jazayeri and colleagues (Jazayeri et al., 2006). Alternatively, recent evidence that Cdk activity, which increases with cell cycle progression, is required for efficient checkpoint signalling downstream of damage recognition could also be consistent with our observations (Bonilla et al., 2008). Further work will be required to clearly define the processes responsible for this pattern of cell cycle phase-specific Chk1 activation.

We also evaluated the consequences of substituting individual SQ residues with non-phosphorylatable alanine residues or, in the case of S317 and S345, with phospho-mimicking aspartic acid residues for G2/ M and S/ M checkpoint proficiency (Zachos et al., 2003). Strikingly, whereas substitution of S345 with either alanine or aspartic acid rendered Chk1 completely incapable of restoring either checkpoint response, alanine substitution mutations of S317 and S366 had more subtle and complex effects. Each of these mutants was able to partially restore the G2/ M and S/ M checkpoint arrests triggered by DNA damage and replication arrest. Notably, the S317A and S317D mutants were fully able to suppress the onset of premature mitosis in cells with unreplicated DNA (Fig. 3C). We believe that these phenotypes are most likely a consequence of altered SQ site phosphorylation, although we recognise that such substitution mutations might also affect protein structure directly.

To gain further insight into why mutations of individual SQ phosphorylation sites within the Chk1 SCD differentially affect checkpoint proficiency, we determined how the kinase activity of each mutant responded to genotoxic stress. Whereas the biologically inactive Chk1 S345A was completely refractory to induction in response to both DNA damage and replication arrest, the partially active S317A and S366A mutants remained weakly inducible. Interestingly, much lower levels of S345 phosphorylation occurred within the S317A and S366 mutant proteins, which could underlie the partial checkpoint phenotypes.

Our findings differ significantly from a previous study (Niida et al., 2007), which evaluated the consequences of substituting alanine or aspartic acid for S317 and S345 in endogenous Chk1 in mouse embryonic stem cells (ES cells). In this study both residues were found to be essential for the G2/ M and S/ M checkpoints yet no differences in the kinase activity of the mutant proteins was detected. S317 was also reported to be essential for DNA damage and

replication checkpoint proficiency in human cells, although kinase activity was not investigated (Wilsker et al., 2008). The basis for these differences are currently unclear; cell type-specific factors may be involved, particularly since Chk1 is essential in human and mouse cells but not in DT40. Further work will be required to clarify this issue.

Finally, we sought to gain insight into the mechanism through which SQ phosphorylation stimulated Chk1 kinase activity. In an attempt to distinguish between effects on protein conformation and altered association with potential regulatory factors, we investigated the effect of washing immunoprecipitates of soluble Chk1 formed under standard conditions with a more stringent buffer (RIPA) prior to performing a kinase assay. Remarkably, this simple manipulation greatly increased the specific activity of Chk1 obtained from unstimulated cells, which bore little or no SQ site phosphorylation, but had relatively less effect on the more active, SQ-phosphorylated Chk1 obtained from aphidicolin-stimulated cells. We considered the possibility that this effect might reflect abrogation of the intramolecular inhibitory interaction which occurs between the C-terminal regulatory region and the kinase domain (Chen et al., 2000; Oe et al., 2001), however this interaction was not dissociated by RIPA washing. Remarkably, Chk1 S345A also became highly active after RIPA washing even though it was wholly refractory to activation under native non-stringent conditions. We conclude that S345 phosphorylation is not essential for high levels of Chk1 kinase activity *per se*, even though it is required for biological function *in vivo* and for stimulation of kinase *in vitro* when the protein is purified under native conditions. These observations suggest that the role of S345 phosphorylation is to relieve repression of latent Chk1 catalytic activity under conditions of checkpoint activation, which might be achieved via altered configuration or association with as yet unknown trans-regulatory molecules. In future it will be important to elucidate the molecular mechanism that underlies this regulation.

Materials and Methods

Cell Culture and Treatments

DT40 avian B-lymphoma cells and isogenic cell lines were grown in DMEM (GIBCO) containing 10% fetal bovine serum, 1% chicken serum and 10^{-5}M β-mercaptoethanol at 39.5°C. Cells were irradiated with 10 Gy γ-radiation using an Alcyon II CGR MeV cobalt source, or treated with 60μM Aphidicolin (Sigma), 1 μg/ml Nocodozole (Sigma) and 20μM Bromodeoxyuridine (BrdU; Sigma) as appropriate.

Antibodies

Monoclonal antibody against Chk1 (G-4) and polyclonal Chk1 antibody (FL-476) were purchased from Santa Cruz Biotechnology. Polyclonal anti-phospho-S345 and — phospho-S317 Chk1 were from New England Biolabs. Anti-phospho Ser10 of histone H3 was from Upstate Biotechnology. Polyclonal rabbit antiserum were raised against a synthetic peptide containing the C-terminal 15 amino acids of Avian Chk1 and a peptide containing phospho-S366 of Avian Chk1, both conjugated to keyhole limpet haemocyanin. The crude anti-phospho-S366 serum was affinity purified first on a phospho-S366 peptide column and then depleted of non-phospho-specific antibodies using a column prepared from a non-phosphorylated version of the same peptide. Western blotting experiments in which the antiserum was first pre-adsorbed with either phosphorylated or non-phosphorylated peptide demonstrated that the purified antibodies were specific for Chk1 phosphorylated on S366.

Chk1 Kinase Assay

Immunoprecipitates were washed twice in kinase buffer (KB, 50 mM Hepes, 10 mM $MgCl_2$, 0.8 mM EDTA, 0.8 mM DTT, 10 mM B-glycerophosphate). They were then incubated in

KB containing 100 μM ATP, 2.5 μCI (γ-ATP) and 100 μM of human Cdc25C-derived Checktide, KKKVSRSGLYRSPSMPENLNRPR (Cross and Smythe, 1998) for 30 mins at 30°C. Reactions were stopped by spotting onto 2cm squares of P81 paper, before dropping into 500 ml of 0.5% phosphoric acid. Papers were washed twice with 0.5% phosphoric acid, rinsed in acetone, and γ^{32}P incorporation measured by Cerenkov counting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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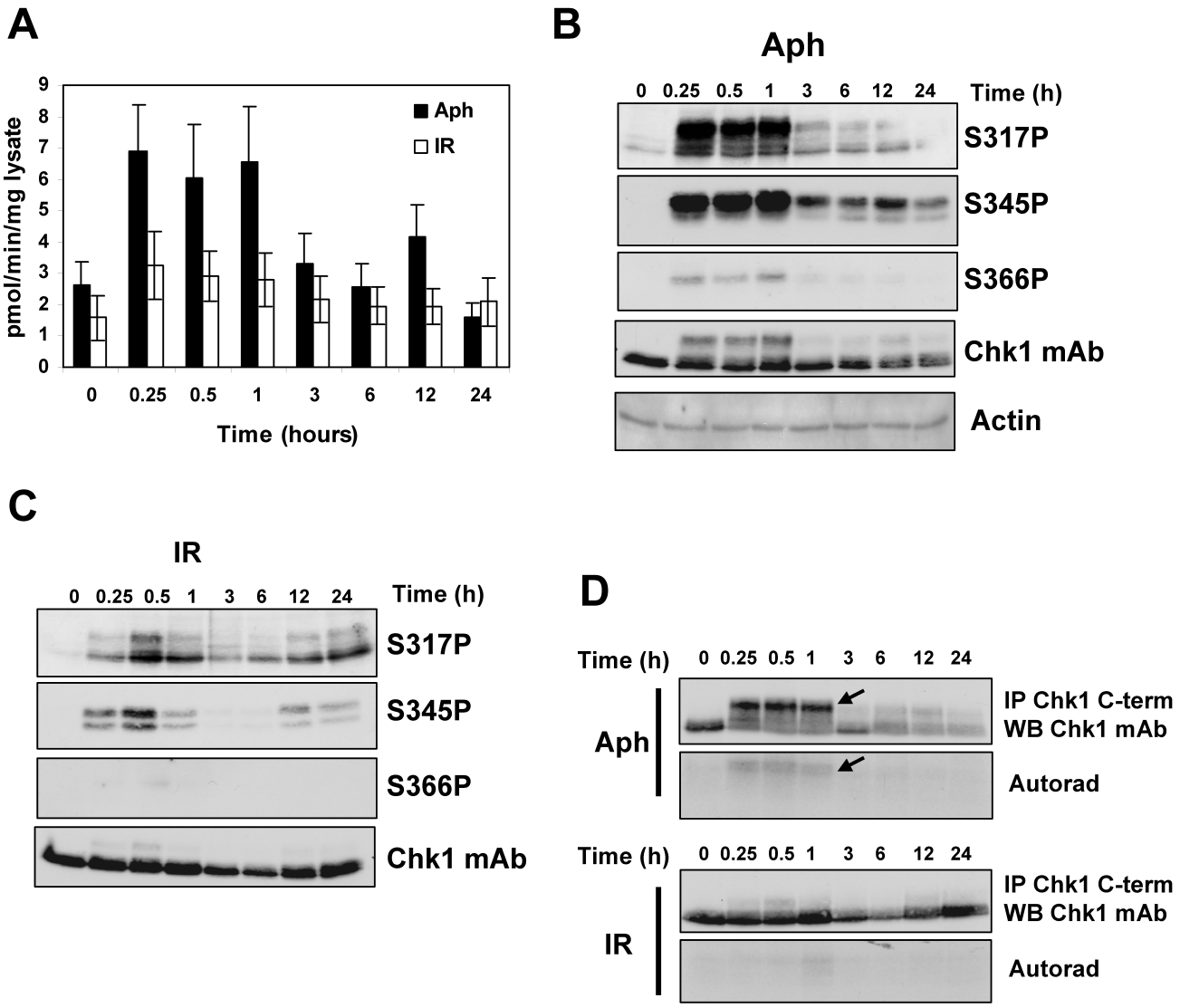

Figure 1. Activation and phosphorylation of Chk1 in response to irradiation and DNA synthesis inhibition

(A) Kinase activity of Chk1 immunoprecipitated from wild type DT40 (WT) cells treated with 60 μM aphidicolin or 10Gy IR. Shown is the mean +/− S.E.M of ≥ 4 independent experiments. Immunoprecipitation and lysis conditions are described in detail in Supplementary methods. (B, C) Western blot analysis of cell lysates from the aphidicolin or IR treated cultures using anti-S317, anti-S345 and anti-S366 phospho-specific Chk1 antibodies plus anti Chk1 MAb (G-4). (D) Western blot and autoradiograph of Chk1 immunoprecipitates shown in (A) analysed post kinase assay.

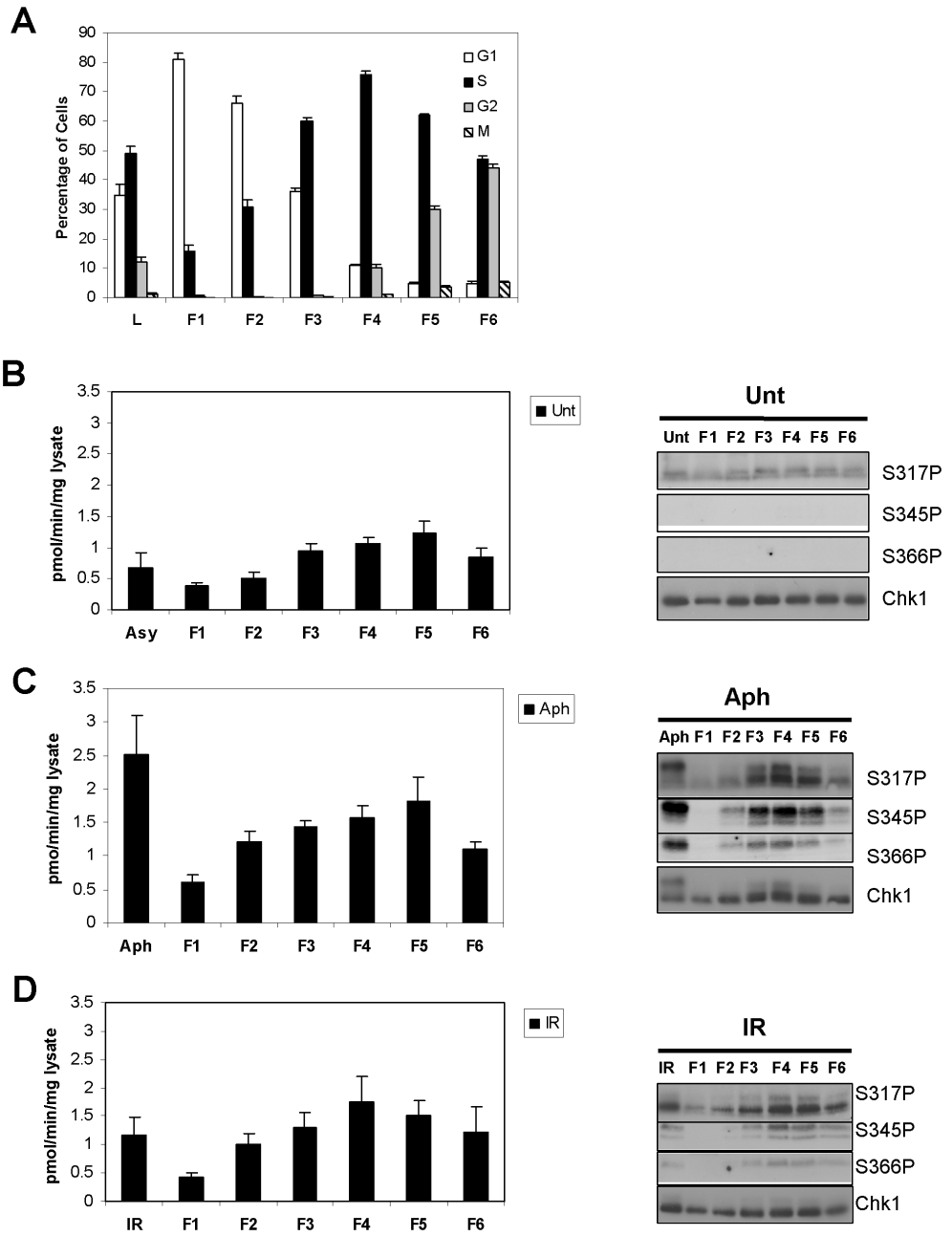

Figure 2. Cell cycle phase specificity of Chk1 activation and phosphorylation

(A) Percentage of DT40 cells in each phase of cell cycle before (L) and after fractionation of an asynchronous culture by centrifugal elutriation, as determined by flow cytometry analysis of DNA content, BrdU incorporation and pS10 histone H3 positive mitotic cells. Centrifugal elutriation was performed as described previously (Zachos et al., 2005) and in Supplementary methods. (B, C, and D) Kinase activity of Chk1 immunoprecipitated from untreated (B), aphidicolin treated (C), or irradiated (D) cells before and after fractionation. Values represent mean +/− S.E.M of four independent experiments Extracts assayed for Chk1 kinase activity (left panels) were also analysed by western blotting (right panels) using anti-S317, anti-S345 and anti-S366 phospho-specific antibodies and anti Chk1 MAb (G-4).

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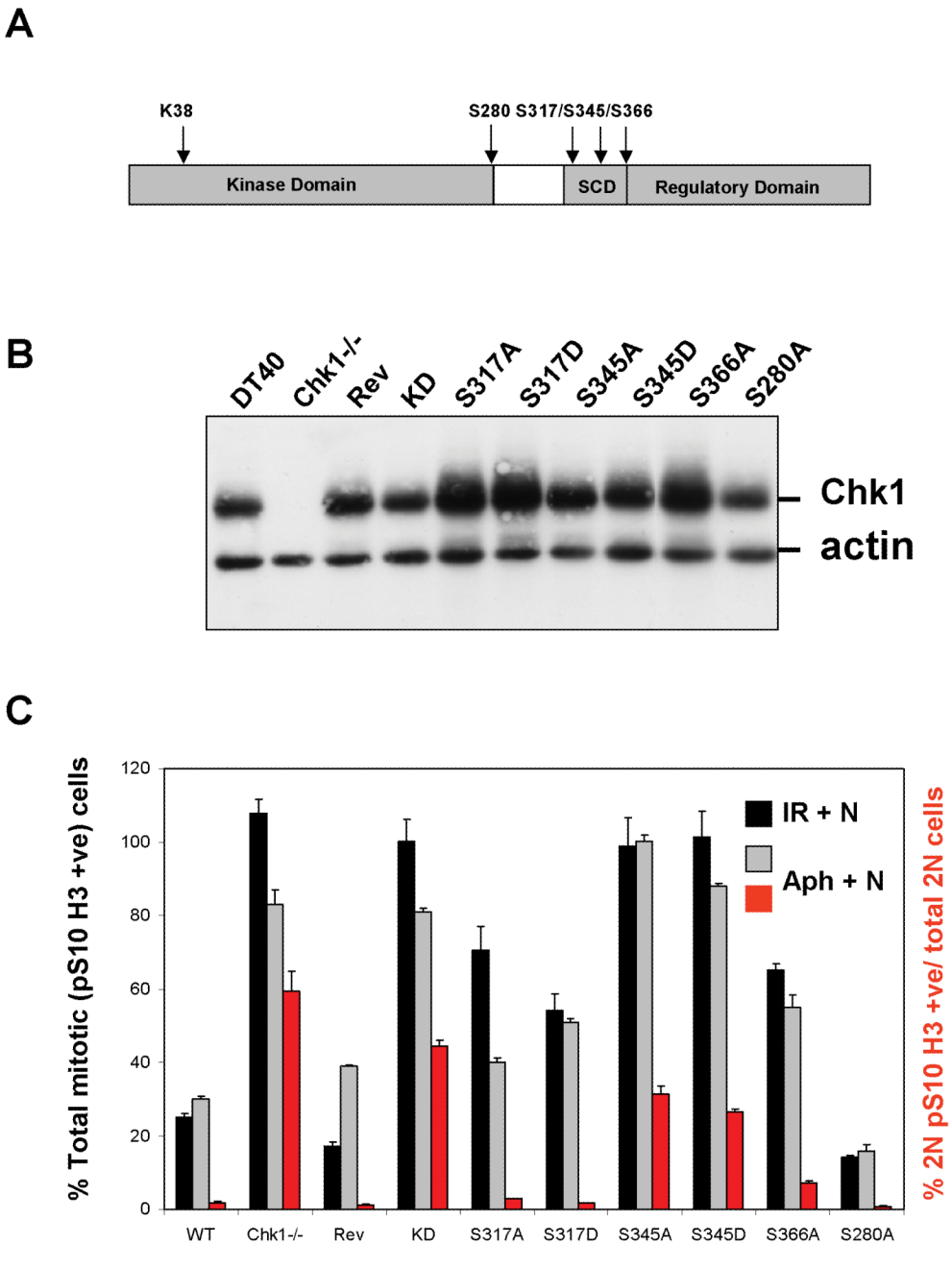

Figure 3. Determination of G2/M and S-M checkpoint proficiency of Chk1 SQ site mutants

(A) Chk1 null cells were stably reconstituted with either WT Chk1 or mutants lacking individual SQ phosphorylation sites as shown in the schematic. (B) Lysates from each cell line were analysed by western blotting using antibodies specific for Chk1 and actin as a loading control. (C) G2/M checkpoint proficiency was determined by quantifying the accumulation of pS10 histone H3 positive mitotic cells in cultures treated with either nocodozole alone or IR (10 Gy) plus nocodozole for 10 hours. Black bars represent the number of mitotic cells accumulated in the irradiated culture expressed as a percentage of the nocodazole control. S-M checkpoint proficiency was assessed by treating each cell line with aphidicolin plus nocodozole for 10 hours and quantifying both the total percentage of

mitotic cells (grey bars) and the percentage of pS10 histone H3 positive cells with 2N DNA content (premature mitosis: red bars). Flow cytometry was performed as described previously (Zachos et al., 2005) and in Supplementary methods.

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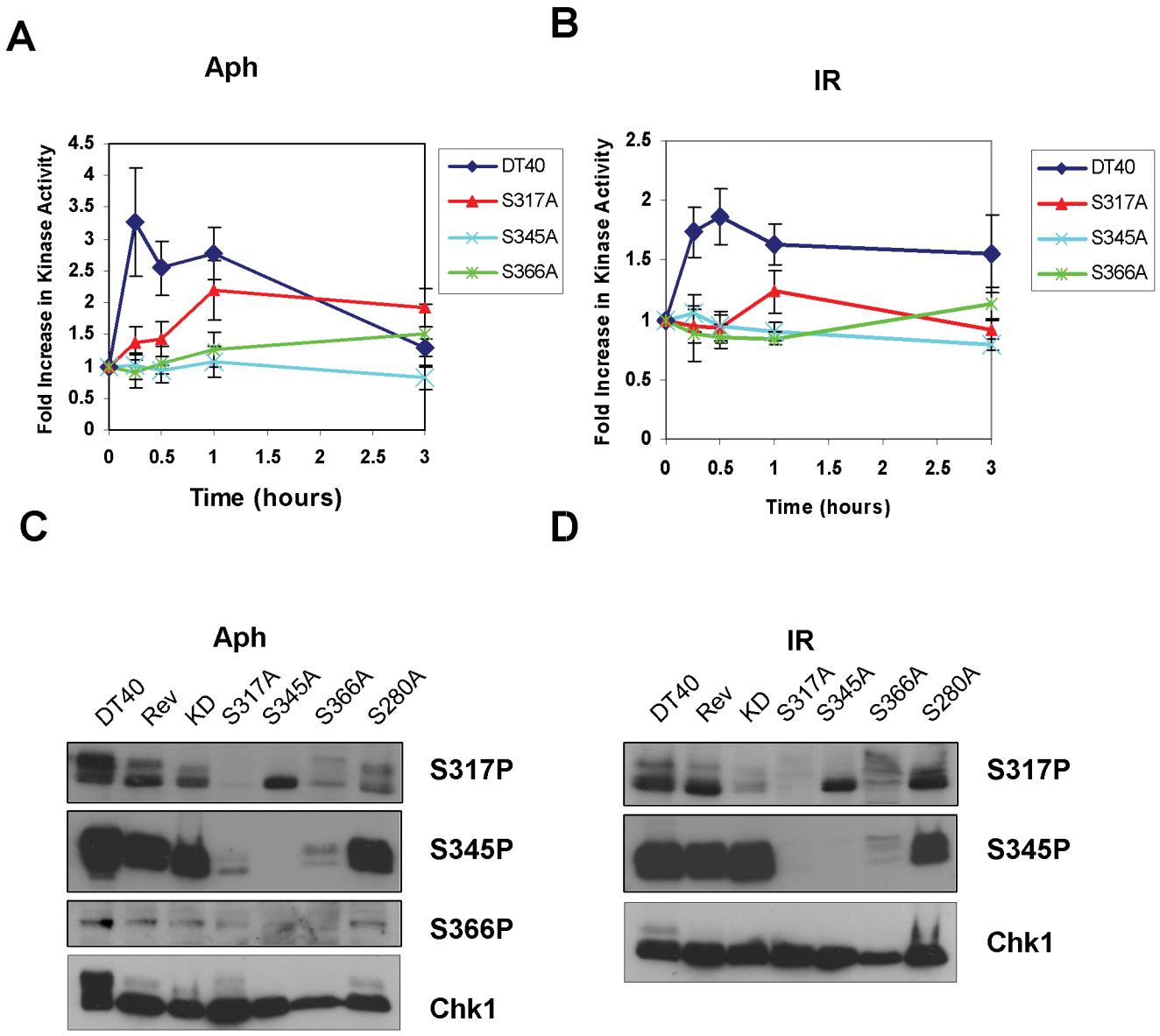

Figure 4. Phosphorylation of S345 is essential for activation of Chk1 and dependent on modification of additional adjacent SQ sites

(A, B) Kinase activity of Chk1 immunoprecipitated from DT40 and SQ site mutant-expressing DT40 cells over a three hour time course following treatment with either 60 μM aphidicolin (A) or 10Gy IR (B). Shown is the mean +/− S.E.M of four independent experiments. (C, D) Western blot analysis of lysates from selected cell lines shown in (A) treated for 15 mins with aphidicolin or 10Gy IR using either anti-S317, anti-S345, anti-S366 (panel C only) phospho-specific Chk1 antibodies, or Chk1 MAb (G4).

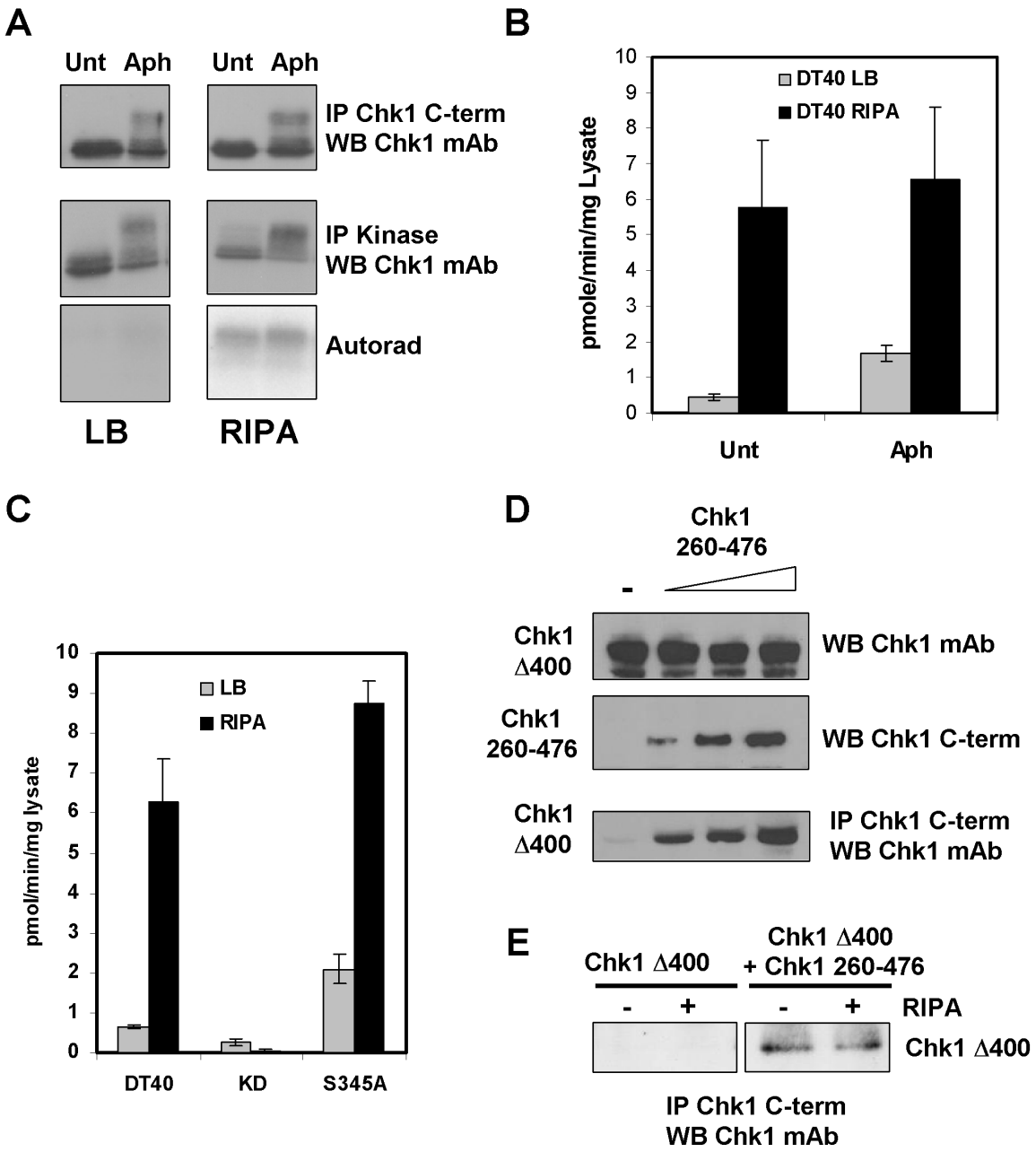

Figure 5. Chk1 SQ site phosphorylation relieves repression of latent kinase activity

(A) Chk1 was immunoprecipitated from DT40 cells treated with and without aphidicolin for 30 mins and the precipitates washed with either lysis buffer (LB) or RIPA buffer before being analysed by western blotting (upper panel) or subjected to *in vitro* kinase assay followed by western blotting (middle panel) and autoradiography (lower panel). (B) Kinase activity of replicate immunoprecipitates as shown in (A) determined using Chktide substrate, results are mean +/− S.E.M of three independent experiments. (C) Kinase activity against Chktide substrate of Chk1 immunoprecipitated from DT40, KD- and S345A-expressing cell lines, where precipitates had been washed with either LB or RIPA as in (A). Results are mean +/− S.E.M of three independent experiments. (D) Cos1 cells were co-

transfected with Chk1 Δ400 mutant minus and plus increasing amounts of a plasmid encoding the Chk1 C-terminal regulatory domain (amino acids 260-476) for 24 hours. Extracts were analysed by western blotting using anti-Chk1 MAb (upper panel) or anti C-terminal Chk1 (middle panel). Chk1 260-476 was immunoprecipitated from the lysates using C-terminal Chk1 Ab and co-precipitation of Chk1 Δ400 verified by western blotting with Chk1 MAb (lower panel). (E) Cos1 cells were transfected with plasmids encoding Chk1 Δ400 minus and plus Chk1 260-476. Chk1 260-476 was immunoprecipitated using C-terminal antiserum and the precipitates washed with either LB or RIPA as in (A) before being analysed for Chk1 Δ400 by western blotting using Chk1 MAb.

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