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Human Tousled like kinases are targeted by an ATM- and Chk1-dependent DNA damage checkpoint

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All eukaryotes respond to DNA damage by modulation of diverse cellular processes to preserve genomic integrity and ensure survival. Here we identify mammalian Tousled like kinases (Tlks) as a novel target of the DNA damage checkpoint. During S-phase progression, when Tlks are maximally active, generation of DNA double-strand breaks (DSBs) leads to rapid and transient inhibition of Tlk activity. Experiments with chemical inhibitors, genetic models and gene targeting through RNA interference demonstrate that this response to DSBs requires ATM and Chk1 function. Chk1 phosphorylates Tlk1 on serine 695 (S695) *in vitro*, and this UCN-01- and caffeine-sensitive site is phosphorylated *in vivo* in response to DNA damage. Substitution of S695 to alanine impaired efficient downregulation of Tlk1 after DNA damage. These findings identify an unprecedented functional cooperation between ATM and Chk1 in propagation of a checkpoint response during S phase and suggest that, through transient inhibition of Tlk kinases, the ATM–Chk1–Tlk pathway may regulate processes involved in chromatin assembly.

Keywords: ATM/checkpoints/Chk1/chromatin assembly/Tlk

Introduction

Faithful replication of the genome and proper chromatin assembly are mandatory for retaining genomic integrity and fulfilling developmental programmes in higher organisms. The importance of tight control of DNA replication, allowing space and time for DNA repair, has long been appreciated, and recent research indicates that regulation of chromatin assembly plays an important role during both unperturbed DNA synthesis and DNA repair (Krude, 1999; Green and Almouzni, 2002). Recently, a new type of nuclear serine/threonine kinases, the Tousled like kinases (Tlks), which are potentially involved in regulation of chromatin assembly, has been identified in human cells (Silljé *et al*., 1999). Mutations of the prototypic member of this family, the Tousled (Tsl) kinase from the plant *Arabidopsis thaliana* (Roe *et al*., 1993), lead to a pleiotropic phenotype with abnormal flower development and a stochastic decrease in organ number, raising speculations that Tsl regulates cell division during patterning of plant organs (Roe *et al*., 1997). In mammals, Tlks are regulated in a cell cycle-dependent manner with maximal activity in S phase, and their link to chromatin assembly was established by the identification of the human chromatin assembly factors Asf1a and Asf1b (hAsf1) as Tlk substrates (Silljé *et al*., 1999; Silljé and Nigg, 2001). Apart from this link, the role of Tlks in chromatin assembly and perhaps other S-phase events remains elusive. The two known human Tlks, Tlk1 and Tlk2, are 84% similar at the amino acid sequence level, ubiquitously expressed and probably act as dimers/oligomers (Silljé *et al*., 1999). The properties of Tlk1 and Tlk2 appear similar, with both kinases super-activated during S phase and sensitive to DNA-damaging agents and inhibitors of DNA replication, such as aphidicolin, which inactivate Tlk1 and Tlk2 (Silljé *et al*., 1999). The latter phenomenon inspired the idea that Tlk activity is linked to ongoing DNA synthesis (Silljé *et al*., 1999).

There is no obvious Tlk homologue in yeast, but the evolutionarily conserved Asf1 histone chaperone was first cloned in *Saccharomyces cerevisiae* as an antisilencing factor required for timely completion of S phase (Le *et al*., 1997; Singer *et al*., 1998). Human, yeast and *Drosophila* Asf1 bind histone H3 and H4, and synergize with the CAF-1 complex in replication- and repair-coupled chromatin assembly (Tyler *et al*., 1999; Munakata *et al*., 2000; Sharp *et al*., 2001; Mello *et al*., 2002). Supporting a role for Asf1 in DNA repair, yeast *asf1* mutants are hypersensitive to inhibitors of DNA replication and agents causing single- and double-strand DNA breaks (Le *et al*., 1997; Singer *et al*., 1998; Tyler *et al*., 1999). Interestingly, yeast Asf1 is regulated by the DNA damage checkpoint. During unperturbed growth, Asf1 exists in a complex with the central DNA damage checkpoint protein kinase Rad53 (Chk2 in humans), and is released to bind acetylated histone H3 and H4 in response to DNA damage and stalled replication forks in a Mec1-dependent manner (Emili *et al*., 2001; Hu *et al*., 2001).

Given the checkpoint-regulated function of Asf1 in DNA repair, the fact that Tlks are inactivated by inhibitors of DNA replication suggests that these kinases, which are the only ones known to target hAsf1, may be regulated by the DNA damage checkpoint in mammalian cells. Central to all DNA damage-induced checkpoint responses, including DNA repair, cell cycle control and apoptosis, is a pair of large protein kinases: ATM (ataxia telangiectasia mutated) and ATR (ataxia and Rad3 related) (Abraham, 2001; Wahl and Carr, 2001). ATM is the key regulator of the immediate response to double-strand breaks (DSBs), as illustrated by the hypersensitivity to ionizing radiation

(IR), defects in IR-induced G_1 arrest, reduction in DNA synthesis and G_2 arrest in cells from patients suffering from ataxia telangiectasia (AT) (Kastan and Lim, 2000), a severe disease caused by *ATM* mutations. Cells lacking ATM respond normally, and therefore are not hypersensitive, to ultraviolet light (UV) and replication inhibitors such as hydroxyurea (HU) (Zhou and Elledge, 2000). In contrast, ectopic expression of kinase-inactive ATR sensitizes mammalian cells to all kinds of DNA damage, indicating a prominent role for ATR in responses to UV and replication inhibitors (Cliby *et al*., 1998; Wright *et al*., 1998). Moreover, the embryonic lethality of *ATR* null mice, probably caused by a mitotic catastrophe (Brown and Baltimore, 2000), suggests that ATR, like its *S.cerevisiae* homologue Mec1 (Desany *et al*., 1998), is required during normal S phase to deal with replicational stress and spontaneous DNA damage.

The principal kinases relaying the ATM/ATR-initiated checkpoint signalling appear to be preferentially Chk2 for ATM and Chk1 for ATR. ATM phosphorylates Chk2 at threonine 68 (Kastan and Lim, 2000), followed by Chk2 autophosphorylation and activation, also reflected by its reduced electrophoretic mobility. Importantly, ATM-dependent Chk2 activation is critical for IR-induced inhibition of DNA synthesis (Falck *et al*., 2001), enforcement of G_1 arrest through p53 stabilization (Hirao *et al*., 2000) and maintenance of G_2 arrest (Hirao *et al*., 2000). Chk1 is phosphorylated at S317 and S345 by ATR in response to UV and HU (Liu *et al*., 2000; Zhao and Piwnica-Worms, 2001), leading to a 3- to 5-fold increase in Chk1 activity (Zhao and Piwnica-Worms, 2001). Chk1 is required for early embryogenesis, suggesting a role analogous to ATR in surveillance of normal S phase (Liu *et al*., 2000). In response to IR, Chk1 is phosphorylated at S317 and S345, correlating with a moderate increase in kinase activity (Liu *et al*., 2000; Zhao and Piwnica-Worms, 2001; Zhao *et al*., 2002), but whether this IR-induced response depends on ATM or ATR is unclear. This observation, together with the impaired G_2 DNA damage checkpoint in Chk1-deficient embryonic stem cells and radioresistant DNA synthesis in cells treated with siRNA against Chk1 (Liu *et al*., 2000; Zhao *et al*., 2002), supports a role for Chk1 in cellular responses to DSBs.

Inspired by the link between the DNA damage checkpoint and regulation of Asf1 in yeast, and the fact that the S-phase Tlks target hAsf1 in mammalian cells, we investigated the regulation of human Tlks in response to DNA damage. We found that Tlk1 and Tlk2 are rapidly inactivated upon IR. Surprisingly, this did not correlate with inhibition of DNA synthesis, but was directly mediated by the S-phase DNA damage checkpoint. IR-induced Tlk1 inactivation required ATM and Chk1 function, and Chk1 phosphorylated Tlk1 *in vitro* and *in vivo* at a site required for the inhibition of Tlk1 in response to DNA damage. Thus, Tlk1 is a novel target of Chk1 in the intra-S-phase DNA damage checkpoint. Given that the hAsf1 are physiological substrates of Tlks (Silljé and Nigg, 2001), we speculate that the mammalian DNA damage checkpoint may, through transient inhibition of Tlks, regulate processes involved in chromatin assembly.

Results

Tlks are inactivated by IR

To investigate whether Tlk1 activity is modulated after generation of DSBs, we measured the *in vitro* kinase activity of endogenous Tlk1 immunoprecipitated from U-2-OS cells in a time course after IR. The specific activity of Tlk1 rapidly decreased 3- to 4-fold in response to IR and then recovered quickly (Figure 1A). The Tlk1 activity reached a minimum after 30 min and began to recover after 1 h, although the time to full recovery varied from 2 to 4 h. Both the kinetics and the relative decrease in Tlk1 activity were equivalent for Tlk1 autophosphorylation and for phosphorylation of its physiological substrate Asf1a (Figure 1A). Thus, the ability of Tlk1 to autophosphorylate correlates with its activity towards exogenous substrates *in vitro*. In agreement with the reported Tlk1 inactivation after aphidicolin treatment (Silljé *et al*., 1999), we found no correlation between the IR-induced inhibition of Tlk1 and any specific changes in Tlk1 mobility assayed by immunoblotting (Figure 1A, lower panel). We then compared the kinetics of Tlk1 inactivation with the activation of the checkpoint kinases Chk1 and Chk2, using the ATM-dependent retardation of electrophoretic mobility to illustrate Chk2 activation (Matsuoka *et al*., 1998) and an antibody against the phosphorylated S317 in Chk1, an ATM/ATR-dependent phosphorylation that correlates with induction of Chk1 activity (Liu *et al*., 2000; Zhao and Piwnica-Worms, 2001; Zhao *et al*., 2002). Chk2 migration was retarded throughout the time course (Figure 1A, lower panel) and Chk2 remained activated for at least 12 h after IR (10 Gy; data not shown). Phosphorylation of Chk1 at S317 occurred rapidly, but declined 3–4 h after IR (Figure 1A, lower panel). Thus, Tlk1 inhibition in response to IR correlates in time with maximal ATM/ATR-dependent phosphorylation of Chk1 and thus with its engagement in checkpoint signalling.

To compare the effect of IR on Tlk1 activity with responses to other types of DNA damage and replicative stress, we investigated the impact of UV irradiation and HU treatment. Whereas UV irradiation inhibited Tlk1 activity only transiently, similarly to IR (Figure 1B), continuous treatment with HU led to sustained inactivation of Tlk1 (Figure 1C). Notably, the sustained inhibition of Tlk1 activity in response to HU correlated with sustained phosphorylation of Chk1 at S317 (data not shown).

Our initial experiments, as well as the previous characterization of Tlk function and regulation, were carried out in tumour cell lines which could have acquired abnormal Tlk regulation. Therefore, we measured the activity of Tlk1 in asynchronously growing normal BJ fibroblasts after exposure to IR or HU. We used a Tlk1-specific antibody, an antiserum recognizing both Tlk1 and Tlk2 (TlkN), and an antiserum specific for Tlk2 to address the impact on both Tlk1 and Tlk2 activity. Tlk1 was rapidly inactivated in these cells after both IR and HU treatment (Figure 1D). Importantly, similar results were obtained with both the antiserum specific for Tlk2 and that recognizing both Tlk1 and Tlk2 (Figure 1D). Thus, both Tlk1 and Tlk2 are subject to fast inactivation in response to DSBs.

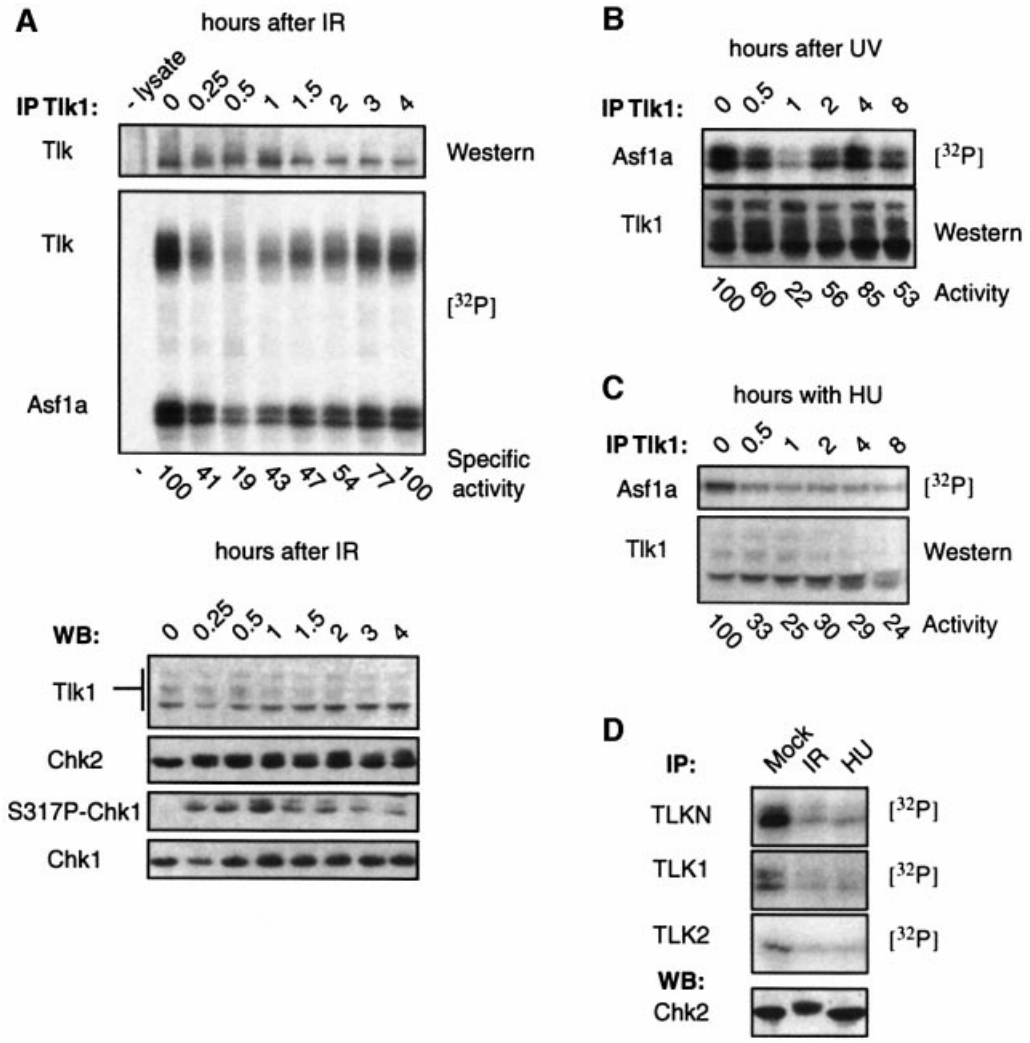


Fig. 1. Tlk1 is transiently inactivated in response to IR and UV irradiation, but continuously inhibited in the presence of HU. (**A**) Asynchronously growing U-2-OS cells were exposed to IR (10 Gy) and Tlk1 kinase activity was measured at the indicated times. The amount of Tlk protein in each kinase reaction was determined by immunoblotting (upper panel). The specific activity of Tlk1 was determined as the ratio of ^{32}P incorporated into the substrate GST–Asf1a to the amount of Tlk protein in the individual reactions. The level and electrophoretic mobility of Tlk1 throughout the time course is shown by immunoblotting on whole-cell lysates run on a high-resolution SDS–PAGE gel (lower panel). Chk2 mobility and Chk1 phosphorylation on S317 were determined by immunoblotting (lower panel). (**B**) Asynchronously growing U-2-OS cells were exposed to UV and Tlk1 activity, and total protein levels were measured at the indicated times as in (A). (**C**) Asynchronously growing U-2-OS cells were treated with HU and analysed as in (B). (**D**) Asynchronously growing BJ fibroblasts were left untreated, exposed to IR (10 Gy) or treated with HU. Kinase activities were measured using either Tlk1- or Tlk2-specific antibodies or pan-Tlk antibody (TlkN). Chk2 activation was revealed by its mobility shift seen in SDS–PAGE on total cell lysates as a control for activation of the DNA damage response.

Tlk1 inactivation occurs in S phase and is checkpoint dependent

Tlk1 activity is known to peak in S phase, and the Tlk-inhibitory effect of various DNA-damaging drugs is restricted to this phase of the cell cycle (Silljé *et al.*, 1999). This suggests that the regulation of Tlk1 activity by IR, which we describe above, might also be cell cycle dependent. To address this issue, we used T98G glioblastoma cells, which enter quiescence upon serum withdrawal and resume cell cycle progression upon addition of serum. As expected, Tlk1 activity increased as the cells entered S phase, which was verified by flow cytometry analysis of DNA content and immunoblotting analysis of cyclin A levels (Figure 2A). The basal activity of Tlk1 in starved and G_1 cells was not modulated, whereas the S-phase-induced activity was abolished by IR and HU treatment (Figure 2A). Interestingly, we again observed a clear correlation between Tlk1 inhibition and the ATM/ATR-dependent phosphorylation of Chk1 at S317 (Figure 2A, bottom panel). In contrast, IR caused activation of Chk2 irrespective of the cell cycle phase (Figure 2A, bottom panel). To verify that the IR-induced Tlk1 inhibition was S phase specific, we measured Tlk1 activity in U-2-OS cells synchronized by a double-thymidine (dT) block and released into S phase before exposure to IR or treatment with HU. Indeed, generation of DSBs by IR led to inhibition of Tlk1 activity in early, middle or late S-phase cells (Figure 2B), demonstrating that IR-induced inhibition of Tlk is an intra-S-phase response.

The above results are in agreement with the previously proposed hypothesis that Tlk activity is linked to ongoing DNA synthesis. To address the potential causative relationship between inhibition of DNA synthesis and

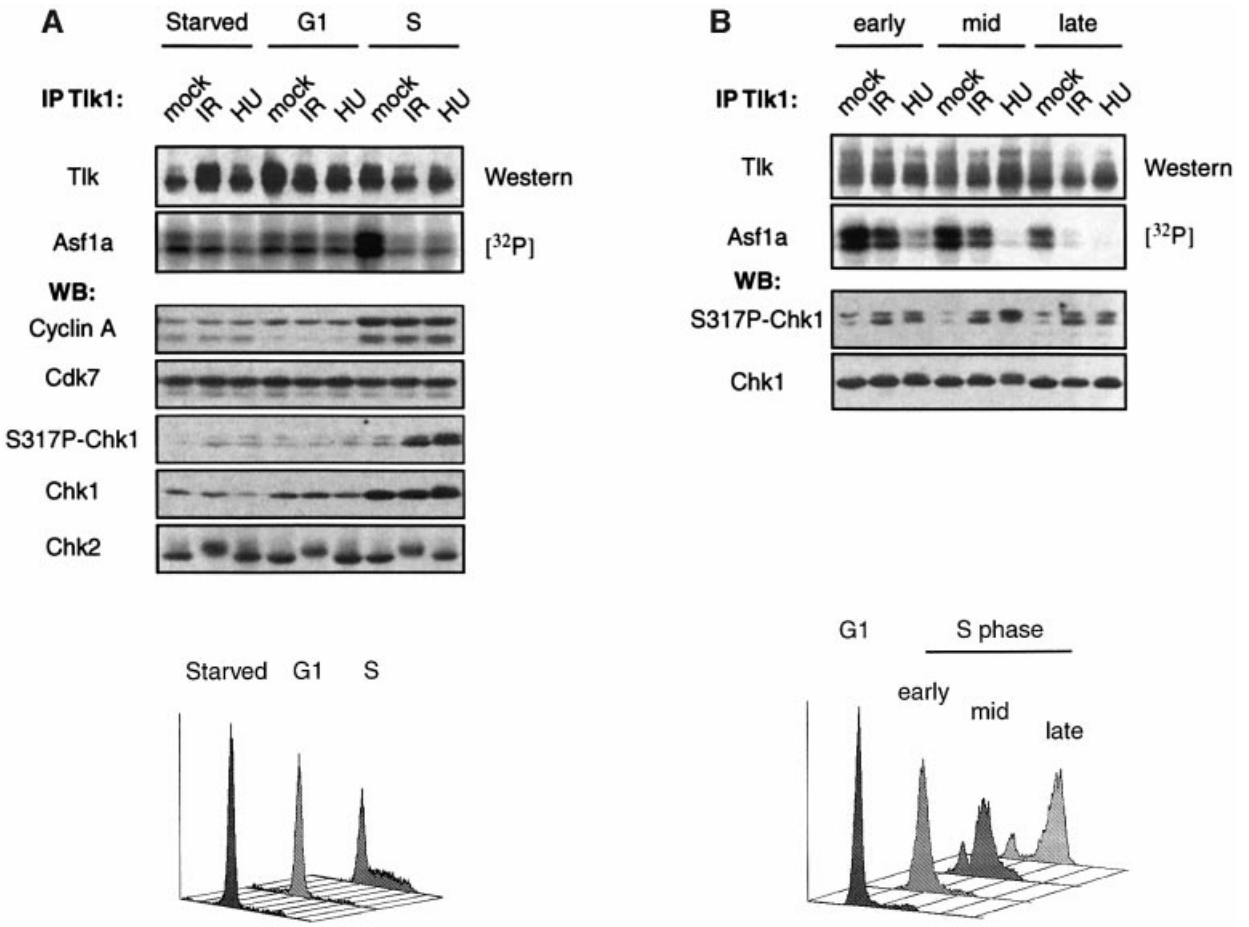


Fig. 2. S-phase-induced Tlk1 hyperactivation is inhibited by IR. (**A**) Tlk1 activity was measured in lysates of T98G cells arrested by serum deprivation (starved) or released from quiescence for 4 h (G_1) or 18 h (S). The cells were left untreated (mock), exposed to IR (10 Gy) or treated with HU, and harvested 30 min later. The amount of Tlk in each kinase reaction was determined by immunoblotting (upper panel). FACS analysis of DNA content (lower panel) and immunoblotting for the S-phase marker cyclin A indicate cell cycle position. The blot of Cdk7 indicates equal loading. Immunoblotting with antibodies recognizing total Chk1, S317-phosphorylated Chk1 and total Chk2 were performed in parallel as indicated. (**B**) Tlk1 activity was measured in lysates of U-2-OS cells released into S phase from a dT block for 3 h (early), 6 h (middle) or 9.5 h (late), and treated as in (A). Progression through S phase was determined by flow cytometry and is shown next to cells in G_1 for comparison. The amount of Tlk in each reaction and Chk1 phosphorylation at S317 were determined as in (A).

Tlk1 inactivation directly, we measured the rate of DNA synthesis in parallel with the activity of Tlk1 in a time course after IR. In response to IR, the activity of Tlk1 rapidly decreased (Figure 3A), reached a minimum after 30 min and then quickly recovered to its starting level. In comparison, the rate of DNA synthesis decreased quickly by ~2-fold, but then remained low for up to 4 h. Likewise, we found no correlation between the rate of DNA synthesis and Tlk1 activity after UV irradiation (data not shown), which evokes a longer inhibition of DNA synthesis than does IR. Thus, Tlk1 inactivation does not appear to be caused directly by lack of DNA replication after DNA damage, although the initiation of both responses could be co-ordinated. To test whether Tlk activity can be uncoupled from DNA replication, we attempted to abrogate HU-induced Tlk1 inactivation with drugs known to block the replication checkpoint yet insufficient to restore normal productive DNA synthesis. We used caffeine and UCN-01 to inhibit ATR/ATM and Chk1 function, respectively (Sarkaria *et al*., 1999; Graves *et al*., 2000). As expected, HU treatment led to a dramatic inhibition of both Tlk1 activity and DNA synthesis (Figure 3B). However, in cells pretreated with either caffeine or UCN-01, HU treatment failed to inactivate Tlk1 efficiently despite the blockade of productive DNA synthesis (Figure 3B). Even when Tlk1 had been efficiently inactivated by HU treatment for 2.5 h, addition of caffeine under continuous exposure to HU allowed it to regain activity within 30 min to a similar extent as when HU was removed (Table I). Thus, an intact replication checkpoint is required for both initiating and sustaining Tlk inactivation in response to stalled DNA replication. These results suggest the involvement of the key regulators of the replication checkpoint, ATR and Chk1, in regulating Tlk1 in the response to replication block. Based on these data and the fact that Tlk1 activity does not correlate with the rate of DNA synthesis after IR, we conclude that Tlk1 inactivation in response to DNA damage and replication blocks is checkpoint dependent.

Tlk1 is regulated by an ATM-dependent pathway

To gain more insight into the upstream regulators of Tlks in the response to DSBs, we employed the chemical checkpoint inhibitors described above in cells exposed to

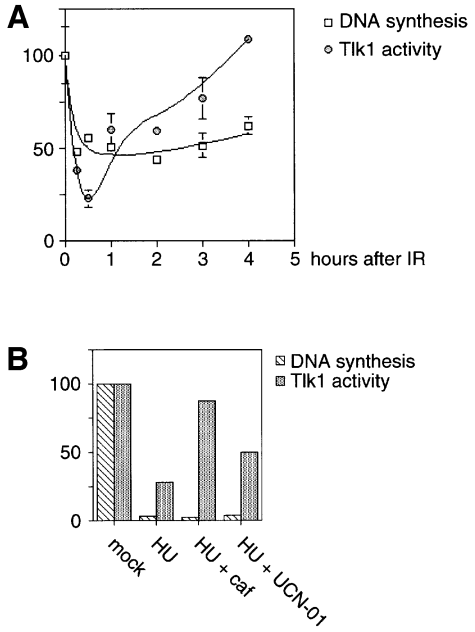


Fig. 3. Tlk1 activity is not directly dependent on ongoing DNA replication. (**A**) Graph showing relative Tlk1 activity and the rate of DNA synthesis in U-2-OS cells at the indicated times after IR (10 Gy). The relative Tlk1 activity was calculated as a mean value of two independent measurements and the deviation is illustrated by error bars. All DNA synthesis measurements were made in duplicate and similar results were obtained in three independent experiments. (**B**) Tlk1 activity and the rate of DNA synthesis were measured in U-2-OS cells left untreated or treated for 2 h with HU. Caffeine and UCN-01 were added 30 min prior to HU treatment as indicated. Tlk1 activity is given relative to the amount of Tlk in each reaction (specific activity) and DNA synthesis measurements were made in duplicate as in (A).

Table I. Sustained Tlk1 inhibition requires an intact replication checkpoint

	Tlk1 activity[a]
Mock	100
HU, 30 min	3
HU, 3 h	8
HU, 3 h. Caffeine added at 2.5 h	161
HU, 3 h. Washed off at 2.5 h	176

[a]Counts incorporated into GST–Asf1a relative to the amount of GST–Asf1a.

IR. As expected, pretreatment with caffeine prevented the ATM-dependent activation of Chk2, whereas UCN-01 did not (data not shown). Surprisingly, both inhibitors efficiently prevented inhibition of Tlk1 activity in response to IR (Figure 4A). This result was intriguing since it pointed towards ATM and/or ATR together with Chk1, rather than Chk2, as the main upstream regulators of Tlk1 in response to IR.

ATM is the major upstream kinase co-ordinating the immediate response to IR in all phases of the cell cycle (Abraham, 2001). To clarify the role of ATM in the rapid IR-induced inhibition of Tlk1 activity, we used cells from ataxia telangiectasia patients (AT cells). The IR-induced Tlk1 inactivation was severely impaired in a panel of such AT fibroblasts and lymphoblast cell lines (Figure 4B; data not shown) compared with normal BJ fibroblasts (Figure 4B) and U-2-0S cells (Figure 1A). Notably, this

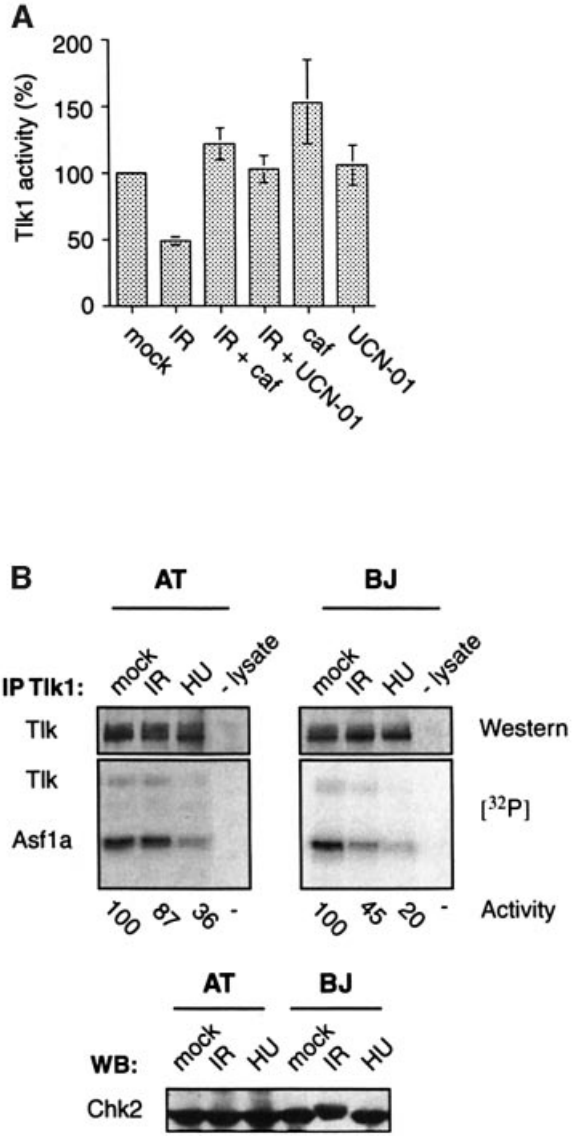


Fig. 4. Tlk1 inactivation in response to IR is ATM dependent and can be abrogated by caffeine and UCN-01. (**A**) Bar chart illustrating Tlk1 activity in untreated (mock) or IR-exposed U-2-OS cells pretreated with caffeine or UCN-01 for 30 min as indicated. Each bar represents at least three independent experiments. (**B**) AT fibroblasts (GM05849) and normal BJ fibroblasts were left untreated (mock), exposed to IR or treated with HU, and were analysed for Tlk1 activity. The amount of Tlk in each kinase reaction was revealed by immunoblotting (upper panel). The BJ fibroblasts had been released from contact inhibition to increase the fraction of cells in S phase, but similar results were obtained with asynchronously growing cells (Figure 1D). Compared with BJ fibroblasts, the response to HU in the AT cells was slightly less pronounced. Whether this reflects a minor role for ATM in HU-induced Tlk1 inhibition or simply cell type differences is unclear. The lack of ATM function in the AT fibroblasts was demonstrated by the absence of Chk2 electrophoretic mobility shift in response to IR (lower panel).

was not due to a general defect in Tlk1 regulation, since in AT cells Tlk1 activity was still downregulated to a significant extent in response to inhibitors of DNA replication, such as HU and aphidicolin, which trigger a largely ATR-dependent checkpoint (Figure 4B; data not shown). We conclude that the rapid inactivation of Tlk1 in response to DSBs depends on the DNA damage checkpoint kinase ATM.

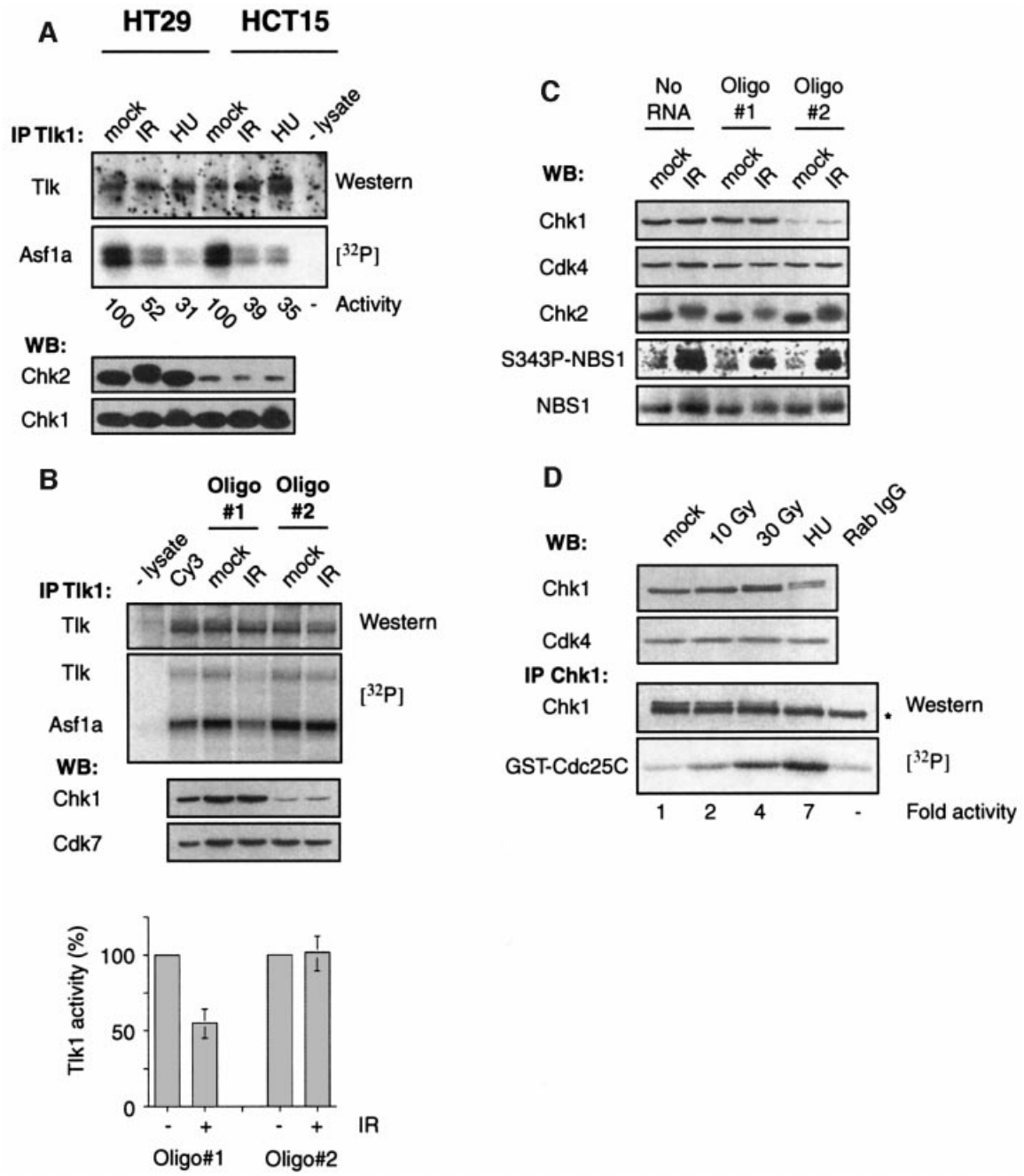


Fig. 5. IR-induced Tlk1 inactivation requires Chk1, but not Chk2. (**A**) Control HT29 and Chk2-deficient HCT15 cells were treated as in Figure 4B and analysed for Tlk1 activity. Equal input of Tlk1 in each kinase reaction was verified by immunoblotting (upper panel). The level and electrophoretic mobility of Chk2 and Chk1 were analysed by immunoblotting on total cell lysates (lower panel). (**B**) HeLa cells were transfected with two siRNA oligos specific for Chk1 or a Cy3-labelled oligo. Oligo 1 had no effect on Chk1 level and was used as a control for oligo 2 that silenced Chk1 expression. The Cy3 oligo served to estimate transfection efficiency by immunofluorescence. The efficient downregulation of Chk1 by oligo 2 was demonstrated by immunoblotting using Cdk7 as a loading control (middle panel). Forty-eight hours after transfection, the cells were either exposed to IR or left untreated (mock) and analysed for Tlk1 activity (upper panel). The input of Tlk1 in each kinase reaction was revealed by immunoblotting. The bar chart represents four independent experiments carried out as described above and normalized to untreated values. (**C**) The integrity of S-phase checkpoint responses was verified by immunoblotting, revealing an IR-induced Chk2 mobility shift and phosphorylation of NBS1 at S343 in cells transfected with oligo 1, oligo 2 or mock. (**D**) Dose-dependent activation of Chk1 in response to DSBs was measured in HeLa cells released into S phase for 2.5 h from a dT block. Cells were exposed to IR (10 or 30 Gy) or left untreated and harvested 35 min later. As a control for Chk1 activation, HeLa cells were treated for 18 h with HU (3 mM). The total level of Chk1 was determined by western blotting. The band representing cross-reacting rabbit IgG is indicated by an asterisk.

IR-induced Tlk1 inactivation requires Chk1

Current evidence points to Chk2 as the primary effector kinase downstream of ATM in checkpoint signalling during most of the cell cycle (Matsuoka *et al*., 1998; Hirao *et al*., 2000; Falck *et al*., 2001). To test the requirement for Chk2 in IR-induced Tlk inactivation directly, we used the colon carcinoma cell line HCT15, which expresses low levels of mutant Chk2 that fail to be activated in response to IR (Bell *et al*., 1999; Falck *et al*., 2001) (Figure 5A, lower panel). We found that, despite the lack of functional

Chk2, HCT15 cells rapidly inactivated Tlk1 after IR to a similar extent as the control cell line HT29 (Figure 5A, upper panel). This result and the data obtained with UCN-01 argue against Chk2 as the principal relaying kinase responsible for IR-induced Tlk1 inactivation.

Hence we set out to clarify the role of the other central effector kinase, Chk1, in IR-induced Tlk1 inactivation. Owing to the absence of a good genetic system lacking Chk1 and the fact that UCN-01 is not strictly specific for Chk1, we applied RNA interference (siRNA) to abolish Chk1 function in HeLa cells. These cells show normal regulation of Tlk1 in response to IR and replication inhibitors despite the lack of functional p53 (Figure 5B) (Silljé *et al*., 1999), indicating that IR-induced Tlk1 inactivation is p53 independent. We used two oligonucleotides corresponding to different sequences in the Chk1 mRNA. Oligo 1 failed to affect Chk1 expression and was used as a control, whereas oligo 2 led to efficient downregulation of Chk1 protein (Figure 5B, middle panel). In two independent experiments, the levels of Chk1 protein remaining after transfection with oligo 2 were 8 and 18%, respectively (Figure 5B; data not shown). The ATM-dependent activation of Chk2 and phosphorylation of NBS1 at S343, critical for other S-phase checkpoint responses, were unaffected by Chk1 siRNA treatment (Figure 5C). Yet, Tlk1 inactivation in response to IR was abrogated when Chk1 expression was silenced by oligo 2 (Figure 5B, top panel and bar chart), demonstrating a requirement for Chk1 in the checkpoint-mediated inhibition of Tlk1. Similar results were obtained in U-2-OS cells (data not shown).

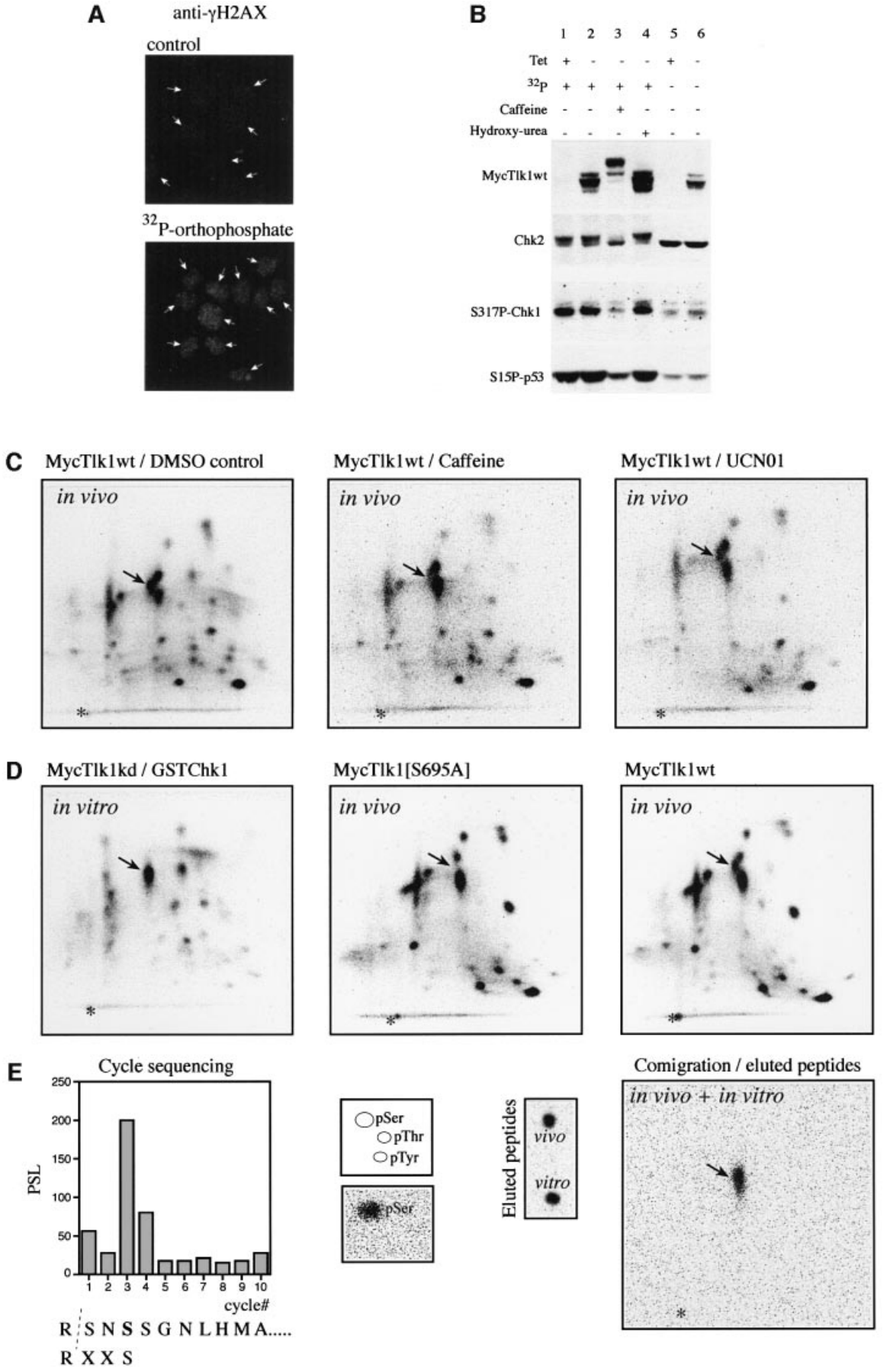
To verify that Chk1 is activated in response to DSBs in S-phase cells when Tlk1 is subject to IR-induced inactivation, we exposed cells synchronized in mid S phase to IR and measured Chk1 activity. Shortly after IR, Chk1 was activated in a dose-dependent manner (Figure 5D), correlating with the rapid S-phase-specific inhibition of Tlk1 (Figure 2B).

Chk1 phosphorylates Tlk1 in vitro and in vivo at S695

Based on the findings described above, we investigated whether Tlk1 might be a direct target of Chk1. We found that Chk1 phosphorylated Tlk1 *in vitro* and pre-phosphorylation of Tlk1 by Chk1 reduced the activity of Tlk1 towards its physiological substrate Asf1a by ~20% (see Supplementary figure 1 available at *The EMBO Journal* Online). To provide *in vivo* evidence for a direct link between Chk1 and Tlk1, we performed phosphopeptide mapping of wild-type MycTlk1 conditionally expressed in S-phase cells. The amount of [^{32}P]orthophosphate used for labelling generated DSBs and activated the DNA damage checkpoint, as revealed by staining for phosphorylated histone 2AX (γH2AX) (Figure 6A) and immunoblotting for Chk2, Chk1 phospho-S317 and p53 phospho-S15 (Figure 6B). To reveal checkpoint-mediated phosphorylations of MycTlk1, we compared the phosphorylation pattern in mock-treated cells (checkpoint on) with that obtained from cells where Chk1 or ATM/ATR had been inhibited by short-term treatment with UCN-01 or caffeine, respectively. From these *in vivo* maps, it was clear that the intensity of one abundant phosphopeptide was significantly reduced by both caffeine and UCN-01 (Figure 6C). Importantly, this phosphorylation site corresponded to the predominant site in Tlk1 phosphorylated by Chk1 *in vitro* (Figure 6D, left panel), as demonstrated by co-migration of the eluted phosphopeptides (Figure 6E, right panel). Edman degradation revealed that the phosphopeptide corresponds to the C-terminal part of Tlk1 phosphorylated on S695 (Figure 6E, left panel), which indeed resembles a consensus phosphorylation site of Chk1 (O'Neill *et al*., 2002). Accordingly, the map of mutant MycTlk1 with S695 changed to an alanine (S695A) differed from that of wild-type Tlk1 by specifically lacking only the S695-containing phosphopeptide (Figure 6D, middle and right panels).

To test the functional implications of the Chk1-dependent phosphorylation of Tlk1 at S695, we first attempted to recapitulate the DNA-damage-induced Tlk1 modification by expressing in human cells two MycTlk1 mutants where S695 was replaced by glutamic (E) or aspartic (D) acid residues, thought to mimic phosphorylated serine. Indeed, the activity of either mutant was <50% of wild-type (wt) MycTlk1 (Figure 7A), correlating with the degree of Tlk1 inactivation after IR-induced phosphorylation. Next, we assayed the impact of IR on the activity of a Tlk1 mutant where S695 was substituted by non-phosphorylatable alanine. We generated cell lines conditionally expressing MycTlk1 wt and S695A, respectively, to allow low and similar expression of both ectopic Tlk1 alleles that would not saturate their processing by physiological checkpoints. In response to IR, the activity of the S695A mutant was significantly less downregulated than that of Tlk1 wt (Figure 7B, middle panel and bar chart). Taken together, these data strongly suggest that the phosphorylation of Tlk1 by Chk1 regulates Tlk1 activity in response to intra-S-phase DNA damage.

Fig. 6. Chk1 phosphorylates Tlk1 at S695. (**A**) U-2-OS cells incubated in the presence or absence of [^{32}P]orthophosphate for 3 h were stained for γH2AX to reveal DSBs. (**B**) U-2-OS cells conditionally expressing MycTlk1 synchronized by a dT block were induced (– tet) or left repressed (+ tet) for 3 h before they were released into S phase in the presence or absence of [^{32}P]orthophosphate for 3 h. Where indicated, caffeine and HU were added for the last 30 min. Cell lysates were assayed by immunoblotting using the indicated antibodies. (**C**) Cells were synchronized, induced and labelled with ^{32}P as in (B), and treated with DMSO (mock), caffeine or UCN-01 for the last 30 min. Samples were immunoprecipitated using anti-Myc antibodies and processed for phosphopeptide mapping. (**D**) Phosphopeptide map of recombinant kinase-inactive (kd) MycTlk1 phosphorylated by Chk1 *in vitro* (left panel), and *in vivo* phosphopeptide maps of MycTlk1 S695A (middle panel) and MycTlk1 wt (right panel). U-2-OS-TA cells were transiently transfected with inducible vectors (pBI, tet off) before they were synchronized, induced and labelled with ^{32}P as in (B). (**E**) The phosphopeptides indicated by arrows were eluted from the maps of MycTlk1 wt (C, left panel) and MycTlk1 kd (D, left panel), and similar amounts of phosphopeptide from *in vivo* and *in vitro* labelled MycTlk1 were applied for co-migration on TLC (right panel). The *in vivo* labelled phosphopeptide was used for phosphoamino acid analysis (middle panel) and sequenced by Edman degradation (left panel). The individual cycles were analysed for ^{32}P-labelled amino acids, and the corresponding sequence of human Tlk1 with S695 highlighted in position 3 after the tryptic cleavage site is shown together with a Chk1 consensus site below.


A
anti-γH2AX
control
32P-orthophosphate
B
1 2 3 4 5 6
Tet + - - - + -
32P + + + + - -
Caffeine - - + - - -
Hydroxy-urea - - - + - -
MycTlk1wt
Chk2
S317P-Chk1
S15P-p53
C
MycTlk1wt / DMSO control
MycTlk1wt / Caffeine
MycTlk1wt / UCN01
in vivo
in vivo
in vivo
D
MycTlk1kd / GSTChk1
MycTlk1[S695A]
MycTlk1wt
in vitro
in vivo
in vivo
E
Cycle sequencing
PSL
250
200
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100
50
0
1 2 3 4 5 6 7 8 9 10
cycle#
R S N S S G N L H M A.....
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pSer
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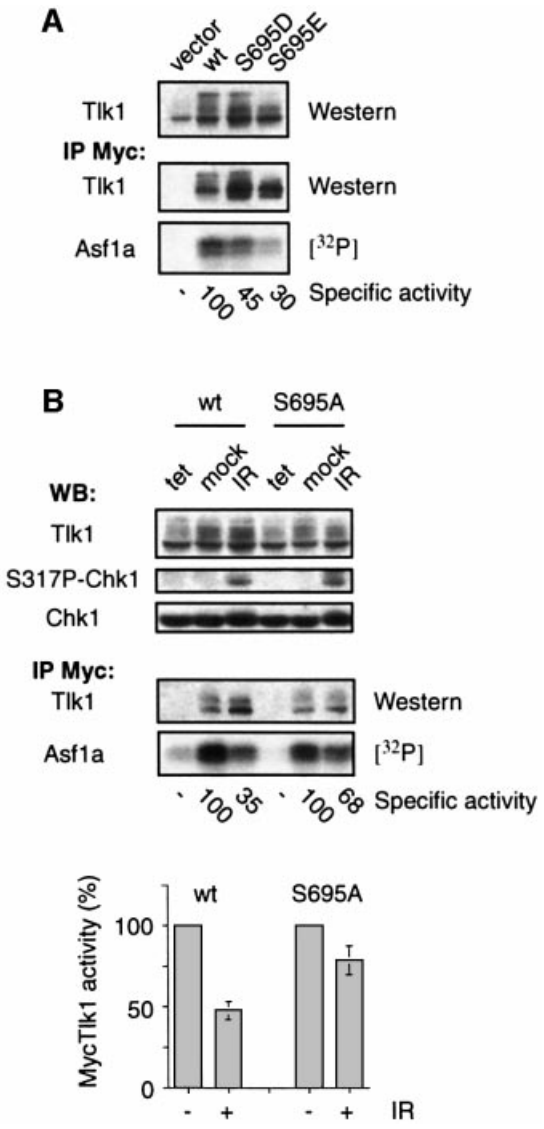

Fig. 7. The phosphorylation status of S695 is critical for Tlk1 activity. (**A**) T98G cells were electroporated with constructs expressing Myc-tagged Tlk1 wt, S695D or S695E. Two days later, the cells were lysed and the activity of the MycTlk1 mutants against GST–Asf1a was measured. To calculate the specific activity of each mutant, the amounts of immunoprecipitated Tlk1 were determined by immunoblotting and quantified with a CCD camera. Similar results were obtained in five independent experiments. (**B**) U-2-OS cell clones inducible for MycTlk1 wt or S695A were induced briefly (2.75 and 2.25 h, respectively) to obtain a similar low expression level before the cells were exposed to IR (10 Gy) or left untreated. The cells were harvested 30 min later and the kinase activity of MycTlk1 was measured. The specific activity was determined from the amount of MycTlk1 in each reaction and is given relative to mock values (middle panel). Direct immunoblotting for Tlk1, phospho-S317 Chk1 and Chk1 confirmed similar low expression of MycTlk1 proteins and checkpoint activation, respectively. The bar chart represents seven independent experiments normalized to untreated values; the error bars show the SEM. Only experiments where the expression level of MycTlk1 S695A was similar to or less than MycTlk1 wt were included to ensure that the level of ectopic Tlk1 did not exceed the capacity of the checkpoint.

Discussion

The human Tlks phosphorylate hAsf1 (Silljé and Nigg, 2001), which has recently been shown to synergize with CAF1 in repair-coupled chromatin assembly (Mello *et al*., 2002). Despite this intriguing functional link, very little is known about the regulation of Tlks. The present study addressed the regulation of Tlks in response to DNA damage and inhibition of DNA replication, leading to the following novel findings: (i) Generation of DSBs or exposure to inhibitors of DNA replication leads to inhibition of Tlk1 and Tlk2 activity in primary human fibroblasts as well as in tumour cell lines. (ii) Inactivation of Tlk in response to DNA damage and inhibitors of DNA replication is dependent on intact checkpoints rather than inhibition of DNA synthesis *per se*. (iii) Inhibition of Tlk activity in response to IR requires ATM and Chk1 function. (iv) Chk1 can directly phosphorylate Tlk1 *in vitro* at S695, a UCN-01-sensitive site phosphorylated *in vivo* during activation of the DNA damage checkpoint. (v) IR-induced phosphorylation at S695 is required for the efficient inhibition of Tlk1 activity in response to DSBs.

We find that DSBs caused by IR lead to fast but transient downregulation of Tlk activity that recovers substantially before the resumption of DNA synthesis. Through a combination of the DNA replication inhibitor HU and chemical inhibitors of ATM/ATR or Chk1 kinases, we show that Tlk activity can be uncoupled from ongoing DNA synthesis. Thus, our data demonstrate that the downregulation of Tlk activity observed after the generation of DSBs or treatment with inhibitors of DNA replication is a direct consequence of the activated DNA damage and replication checkpoint, respectively.

Our experiments were aimed at elucidating the upstream signalling pathway(s) responsible for silencing the Tlk activity when cells experience DNA damage. Downregulation of Tlk1 activity in response to IR was impaired in a number of AT cell lines, demonstrating that IR-induced Tlk1 inactivation depends on ATM. Moreover, both IR- and HU-induced Tlk1 inactivation were abrogated by caffeine, an inhibitor of ATM and ATR kinases. In AT cells, the downregulation of Tlk1 activity in response to replication inhibitors was less impaired than the response to IR, implying that ATR is the most likely upstream regulator of Tlk in response to replicative stress.

Currently, the prevailing evidence indicates that Chk2 is the primary kinase acting downstream of ATM in the cellular responses to DSBs (see Introduction). Our present data on checkpoint-mediated inactivation of Tlk challenge this view for the following reasons. First, although the inhibition of Tlk1 activity in response to IR is an ATM-dependent process, the response is preserved in colon carcinoma cells expressing a mutant inactive form of Chk2. Secondly, downregulation of Chk1 by RNA interference impaired the IR-induced inactivation of Tlk1 while leaving Chk2 activation intact. Thirdly, the Chk1 inhibitor UCN-01 abrogated the IR-induced Tlk1 inactivation, although efficient activation of Chk2 was preserved. Furthermore, the kinetics and cell cycle dependence of the IR-induced Tlk1 inhibition correlate with Chk1 phosphorylation on S317 (this study), a site that is phosphorylated in an ATM-dependent manner in response to IR (Sörensen *et al*., 2003). Finally, we observed that IR causes a dose-dependent activation of Chk1 in S-phase cells, when Tlk1 is targeted by the DNA damage checkpoint. Together, these data demonstrate that Chk1 is required for the ATM-dependent downregulation of Tlk1 activity in response to DSBs, and provide evidence

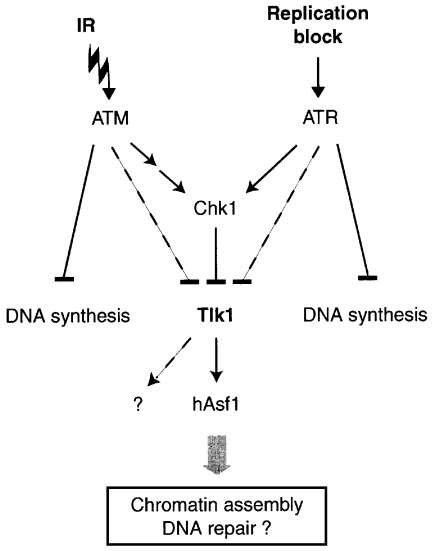

Fig. 8. Model for checkpoint regulation of Tlk1. ATM-dependent activation of Chk1 in response to DSBs leads to inhibition of Tlk1 activity through phosphorylation of Tlk1 at S695 by Chk1. This direct targeting of Tlk1 by Chk1 may work together with other ATM-dependent events to achieve efficient Tlk1 inhibition (see Discussion). A similar mode of regulation may occur in response to replication blocks and replicative stress, with ATR as the upstream kinase. The downregulation of Tlk activity would cause a delay of Tlk-dependent processes in S phase, such as the phosphorylation of hAsf1, which may be critical for efficient repair of DNA lesions.

for a functional co-operation between ATM and Chk1 in the propagation of the DNA damage-induced signalling in S phase.

Our phosphopeptide mapping data reveal that the dominant site in Tlk1 phosphorylated by Chk1 *in vitro* corresponds to a UCN-01- and caffeine-sensitive site phosphorylated during activation of the DNA damage checkpoint *in vivo*, demonstrating that Tlk1 is a novel substrate of Chk1. We used Tlk1 phosphorylation mutants to show that phosphorylation at S695 is required for the efficient inhibition of Tlk1 in response to DNA damage. Based on these novel findings we suggest that, in response to DSBs, ATM acts through activation of Chk1, rather than via Chk2, to transiently downregulate Tlk activity (Figure 8). Likewise, when cells face a replicational block due to either environmental stress or stochastic errors that occur during normal cell duplication, a signalling pathway involving ATR leads to the activation of Chk1 and downmodulation of Tlk activity. This would allow Tlk-dependent processes in S phase, such as the phosphorylation of hAsf1 (Silljé and Nigg, 2001), to be transiently reduced as part of the DNA damage response, thereby facilitating chromatin restructuring required for DNA repair. We propose that the phosphorylation of Tlk1 at S695 by Chk1 is important for Tlk1 inhibition in response to IR. However, since the MycTlk1 S695A mutant retains a weak response to IR, other checkpoint-induced modulations of Tlk1 probably act together with the phosphorylation of S695 to attain maximal inhibition of Tlk1 activity. As Tlk1 contains several S/TQ sequences, which are consensus sites for the ATM/ATR kinases, it is possible that these checkpoint kinases cause the down-regulation of Tlk1 activity both by activating Chk1 and by phosphorylating Tlk1 directly. Such a mode of regulation would be analogous to the dual role of ATM in the regulation of p53 or BRCA1, which are phosphorylated both directly by ATM and in an ATM-dependent manner by Chk2 (Abraham, 2001; Wahl and Carr, 2001).

High-order chromatin structure represents a barrier to the recognition and repair of damaged DNA (Green and Almouzni, 2002). Recent studies show that the modification of chromatin structure through ATM/ATR-dependent phosphorylation of histone H2AX is an early event in response to DNA damage critical for efficient DNA repair (Bassing *et al*., 2002; Celeste *et al*., 2002). Furthermore, in yeast, the checkpoint kinase Rad53 has been proposed to regulate repair-associated chromatin assembly through its interaction with the chromatin assembly factor Asf1 (Emili *et al*., 2001; Hu *et al*., 2001). As outlined in the model (Figure 8), we speculate that the inhibition of Tlk activity in response to DNA damage may link the DNA damage signalling to the regulation of chromatin assembly in mammalian cells. Clearly, the future challenge is to resolve the functional consequences of Tlk-mediated phosphorylation of hAsf1 on its biological function.

Materials and methods

Cell culture, synchronization, irradiation and drug treatment

U-2-OS, T98G, SV40-transformed AT (GM05849, from Coriell cell repositories), BJ, HCT15, HT29 and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics. T98G cells were synchronized in G_0 by serum withdrawal for 48 h and released by serum re-addition. HeLa, U-2-OS cells and derived clones were synchronized at the G_1–S transition by a dT block using 2 mM thymidine (Sigma) and 24 μM 2-deoxycytidine (Sigma). Cell cycle position was determined by fluorescence-activated cell sorter (FACS) analysis of DNA (Lukas *et al*., 1999). U-2-OS clones conditionally expressing MycTlk1 (wild type or S695A) from the tetracycline-repressible promoter in the pBI vector were generated as described previously (Lukas *et al*., 1999). IR was delivered by an X-ray generator (RT100; Phillips Medico; dose rate 0.92 Gy/min). Cell extracts were prepared 30 min after irradiation unless indicated otherwise. Cells were exposed to UV-C (254 nm) at a dose of 20 J/m^2. HU (2 mM) was added to the medium 45 min before cell lysates were prepared, unless indicated otherwise. Caffeine (Sigma) and UCN-01 (a gift from R.J. Schultz, National Cancer Institute) were used at final concentrations of 10 mM and 300 nM, respectively.

Plasmids and mutagenesis

MycTlk1 S695A, S695D and S695E mutants were generated from pRcCMV-Myc-Tlk1 (Silljé *et al*., 1999) using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) with the following primers: 5′-GAGAAGATCAAAT**G**CTTCAGGAAACCTACAC-3′ (A); 5′-CAC-ATGAGAAGATCAAAT**GAT**TCAGGAAAC-3′ (D); and 5′-CACAT-GAGAAGATCAAAT**GAG**TCAGGAAAC-3′ (E).

Immunochemical techniques

Cells were washed twice in ice-cold phosphate-buffered saline and lysed in ice-cold RIPA buffer as described previously (Silljé *et al*., 1999), except that 0.5 μM okadaic acid was included. Cell extracts were passed five times through a 23 G syringe and left for 20 min on ice before centrifugation for 15 min (20 000 *g* at 4°C). Immunoblotting was performed by standard techniques. Rabbit antibodies against Tlk1, Tlk2 and pan-Tlk (TlkN), and mouse antibodies against Chk2 (DCS-270, DCS-273) and Cdk7 (MO-1) have been described previously (Tassan *et al*., 1994; Silljé *et al*., 1999; Lukas *et al*., 2001). The mouse monoclonal antibody against Chk1 (Neomarkers) and the rabbit polyclonal antibodies against cyclin A (H-432; Santa Cruz), NBS1 (Novus Biologicals), γH2AX (Upstate Biotechnology), phospho-S317 Chk1 (Cell Signaling) and phospho-S343 NBS1 (Cell Signaling) were purchased.

In vitro kinase assays

Tlk1 immune complexes isolated from 200–500 μg protein extracts by immunoprecipitation were washed twice in kinase assay buffer (KAB) (50 mM HEPES pH 7.2, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 2.5 mM EGTA, 1 mM DTT, 1 mM NaF, 0.2 mM sodium *o*-vanadate, 2.5 μg/ml leupeptin, 2 μg/ml aprotinin) and incubated for 15–30 min at 30°C in KAB supplemented with 10 μM ATP, 10 μCi [γ-^{32}P]ATP (>5000 Ci/mmol) (Amersham Corp.) and 5 μg GST–Asf1a (Silljé and Nigg, 2001). Reactions were stopped by addition of Laemmli sample buffer (LSB). The mixture was boiled for 3 min and analysed on 10% SDS–PAGE gels, which were either stained with Coomassie Blue (CB) and dried, or transferred to a nitrocellulose membrane and stained with Ponceau Red (PR) before exposure on a PhosphorImager screen and quantification on an FLA3000 reader (Fujifilm, Tokyo). The amount of GST–Asf1a was determined from CB or PR staining with a Fuji LAS1000 CCD camera (Fujifilm, Tokyo). The specific activity of Tlk1 was measured as the ratio of ^{32}P incorporation in the GST–Asf1a to the amount of immunoprecipitated Tlk determined by immunoblotting and chemiluminescence (ECL; Pierce) using the CCD camera. The activity of myc-tagged constructs was measured using the monoclonal 9E10 antibody and 1.5 mg of protein lysate. Chk1 kinase activity was determined essentially as described previously (Zhao and Piwnica-Worms, 2001) using 300 μg of total cell lysate and 1.4 μg of polyclonal Chk1-specific antibody kindly provided by H.Piwnica-Worms.

In vitro phosphorylation of MycTlk1kd via GSTChk1

Recombinant MycTlk1kd (D559A) was prepared from lysates of Sf9 insect cells infected with recombinant baculovirus (Silljé *et al*., 1999). GST–Chk1 (Upstate Biotechnology) was mixed with immobilized MycTlk1kd in KAB containing 15 μM ATP and 50 μCi of [γ-^{32}P]ATP. After incubation at 30°C for 30 min, the reaction was stopped by addition of LSB including 5 mM EDTA and the proteins were resolved on a 7.5% SDS–PAGE gel. After transfer to a nitrocellulose membrane and exposure on film, the band corresponding to MycTlk1kd was processed for phosphopeptide mapping.

Phosphopeptide analysis

In vivo [^{32}P]orthophosphate labelling of cells (2 mCi/ml), two-dimensional phosphopeptide mapping, phosphoamino acid analysis and automatic Edman degradation were performed as described previously (Hansen *et al*., 2001).

siRNA technique

HeLa cells were plated at 30–40% confluence and transfected using Oligofectamine (Life Technologies) by RNA oligos against Chk1 (oligo 1, oligo 2) or an oligo labelled with the fluorochrome Cy3 to allow estimation of transfection efficiency by fluorescence microscopy. The sense sequences of the oligos used were GAAGCAGUCGCAGUGAA-GAdTdT (oligo 1), UCGUGAGCGUUUGUUGAACdTdT (oligo 2) and GCACAUCAGCCAGAACAAGdTdT (Cy3; specific for HSP70). The oligos (Dharmacon Research) were prepared for transfection as described in the manual for the siACE-RNAi kit. The transfection was carried out as recommended by the manufacturer in media with 10% fetal bovine serum and lysates were prepared 48 h later.

DNA synthesis measurements

Cells were labelled with [2-^{14}C]thymidine (10 nCi/ml, 60.0 mCi/mmol) (Amersham Corp.) for 24 h before washing and replating. One day later, the cells were pulsed with [methyl-^{3}H]thymidine (2.5 μCi/ml, 2.0 Ci/mmol) (Amersham Corp.) for 15 or 20 min, harvested by trypsinization and fixed in 70% methanol overnight at 4°C. Before the incorporation of radioactive thymidine was measured using a scintillation counter, the cells were washed twice in cold PBS and resuspended in 5 ml of LSC-cocktail (ULTIMA GOLD; Packard Bioscience Bv, The Netherlands). The rate of DNA synthesis was calculated as the ratio of ^{3}H c.p.m. to ^{14}C c.p.m. after correcting the ^{3}H c.p.m. for 'bleed-through' from the ^{14}C-channel.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

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