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Mammalian homologues of the plant *Tousled* gene code for cell-cycle-regulated kinases with maximal activities linked to ongoing DNA replication

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The *Tousled* (*TSL*) gene of the plant *Arabidopsis thaliana* encodes a serine/threonine kinase that is essential for proper flower development. Here we report the cloning and characterization of two human putative homologues of the *Arabidopsis TSL* gene, termed *TLK1* and *TLK2* (Tousled-like kinase). At the protein level, the two human Tlks share 84% sequence similarity with each other and almost 50% with *Arabidopsis* Tsl. Furthermore, nuclear localization signals and predicted coiled-coil regions are conserved in the N-terminal domains of all three kinases. The mammalian Tlks share several functional properties with plant Tsl, including a broad expression, a propensity to dimerize and autophosphorylate, and a preference for similar substrates. Most interestingly, human Tlks are cell-cycle-regulated enzymes, displaying maximal activities during S phase. Whereas protein levels are virtually constant throughout the cell cycle, both Tlks appear to be regulated by cell-cycle-dependent phosphorylation. Drug-induced inhibition of DNA replication causes a rapid loss of Tlk activity, indicating that Tlk function is tightly linked to ongoing DNA replication. These findings provide the first biochemical clues as to the possible molecular functions of Tlks, a highly conserved family of kinases implicated in the development of multicellular organisms.

Keywords: cell cycle/development/protein kinase/S phase/*Tousled*

Introduction

Reversible protein phosphorylation is one of the major mechanisms by which cellular processes are regulated. Accordingly, many kinases and phosphatases have been highly conserved during evolution. As illustrated by the cyclin-dependent kinases, which control cell cycle progression in all eukaryotic cells (Norbury and Nurse, 1992; Nigg, 1995; Morgan, 1997), the characterization of evolutionarily conserved protein kinases may provide important insights into fundamental regulatory mechanisms. On the basis of this rationale, we have embarked on a study of putative animal homologues of the *Tousled* (*TSL*) gene of the plant *Arabidopsis thaliana*.

The plant *TSL* gene encodes a nuclear serine/threonine kinase (Roe *et al*., 1997a). Based on its primary structure, Tsl does not fall into any well-studied kinase subfamily, although it is distantly related to the STE11/MEKK and polo-like kinase families. Its molecular function is presently unknown, but its mutational phenotype emphasizes a role in plant development (Roe *et al*., 1993, 1997b). Recessive tsl mutants show a pleiotropic phenotype, the most obvious aspect of which is an abnormal flower development (Roe *et al*., 1993, 1997b). However, tsl mutants also show defects in other aspects of development, including leaf morphology and flowering time (Roe *et al.*, 1993). Most strikingly, the flowers of tsl mutants show a stochastic decrease in the number of organs, as well as various morphological alterations. Detailed examination of plant gynoecium development has shown that all tissue types are present in tsl mutants, albeit at a reduced level. This has been interpreted to indicate that *TSL* may not be an organ identity gene, but instead may be involved in pathways regulating cell divisions during the patterning of plant organs (Roe *et al*., 1997b). So far, no direct biochemical or unequivocal genetic link has been established between *TSL* and other developmental regulators, and in spite of a fascinating mutant phenotype (Roe *et al*., 1993, 1997b), the biological function of the *TSL* gene product remains mysterious.

Database searches indicate that likely homologues of the Tsl kinase are also present in animals, including the nematode *Caenorhabditis elegans* and mammals. Remarkably, however, no closely related homologue is present in the unicellular eukaryote *Saccharomyces cerevisiae*. This suggests that Tsl-like kinases are specific for multicellular organisms, and that they most likely function in some fundamental aspect of development, common to both plants and animals. Here we describe the cloning and initial characterization of two human homologues of the plant *TSL* gene, termed Tousled-like kinase 1 and 2 (*TLK1* and *TLK2*). Both human Tlks show extensive sequence similarity with *Arabidopsis* Tsl and our data suggest substantial conservation also at the functional level. In particular, both plant Tsl and human Tlks localize to the nucleus, and they display a similar substrate specificity. In addition, they both autophosphorylate and dimerize. Most interestingly, we found that the activities of human Tlks are regulated during the cell cycle, most likely by phosphorylation, and that Tlk activities are directly linked to ongoing DNA replication. These results suggest that Tlk family members most likely perform cell autonomous functions and they directly implicate these kinases in the regulation of cell-cycle-dependent events.

Results

Isolation of two distinct human TLK cDNAs

A partial human cDNA sharing extensive sequence similarity with *A.thaliana TSL* was first isolated during a PCR-based search for human protein kinases (Schultz and Nigg, 1993). This original PCR fragment, HsPK41, was used as a probe for screening several human and murine cDNA libraries, and during these screens it became apparent that mammalian genomes harbour at least two putative homologues of plant Tsl. Human cDNAs encompassing the complete coding sequences of both Tsl-like kinases were isolated and named *TLK1* and *TLK2* (DDBJ/EMBL/GenBank accession Nos AF162666 and AF162667, respectively). The predicted amino acid (a.a.) sequences of the corresponding translation products are shown in Figure 1A, along with the sequence of the *Arabidopsis* Tsl kinase. Assuming that these human sequences represent the complete proteins (see the legend to Figure 1A), it follows that the human *TLK1* gene codes for a 718 a.a. protein with a calculated molecular mass of 81.9 kDa, whereas the human *TLK2* gene encodes a protein of 749 a.a. with a calculated molecular mass of 85.3 kDa. At the protein level, Tlk1 and Tlk2 share 84% similarity with each other and almost 50% with *Arabidopsis* Tsl. Over the catalytic domains, sequence similarity between human Tlks and plant Tsl is ~70%.

A schematic comparison of the different domains present in the Tsl-like kinases is shown in Figure 1B. Like *Arabidopsis* Tsl, human Tlk1 and Tlk2 have their catalytic domains located at the C terminus, and in all three kinases the ATP-binding domain displays a motif GXGXXS instead of the canonical consensus sequence GXGXXG within subdomain I (Hanks *et al.*, 1988). None of the three kinases contains an RD motif in subdomain VI, suggesting that phosphorylation of a threonine in the activation loop may not be required for their activation (Johnson *et al.*, 1996). In the N-terminal domain, conserved features include three potential nuclear localization sequences and three putative coiled-coil regions. In contrast, the human Tlks lack a glutamine-rich stretch and a putative leucine zipper motif that have been described in *Arabidopsis* Tsl.

Several human and murine cDNAs related to Tlk1 and Tlk2 have been reported previously, albeit under different names (Nagase *et al.*, 1995; Yamakawa *et al.*, 1997; Huang *et al.*, 1998; Shalom and Don, 1999). Some of these were partial cDNAs, whereas others differ from the sequences reported here by small insertions or deletions within the N-terminal domains. This suggests that Tlk genes may undergo differential splicing. In direct support of this view, a recent report describes differentially spliced Tlk2 transcripts in mouse testis (Shalom and Don, 1999).

TLK1 and TLK2 are widely expressed in mouse tissues

Plant *TSL* has been shown to be highly expressed in developing floral meristems, but lower levels of expression could be observed in other tissues as well (Roe *et al.*, 1993). To determine *TLK* expression levels in mammalian tissues, two murine cDNA fragments of *TLK1* and *TLK2* were cloned and used for RNase protection experiments with RNAs isolated from various adult mouse organs. A cDNA coding for the ubiquitously expressed transcription factor NF-Ya was used as a control (Schmidt and Schibler, 1995). As shown in Figure 2, *TLK1* and *TLK2* transcripts could be detected in all organs analysed. Expression levels differed substantially among the various organs, but no obvious correlation could be established with mitotic activity. *TLK2* was highly expressed in testis, with lower levels seen in heart, brain, liver and other organs. In contrast, *TLK1* showed a rather uniform expression level, although *TLK1* transcripts were rather low in lung, kidney and spleen. A high expression of the *TLK* gene in human testis has been reported previously, but this former study could not distinguish between *TLK1* and *TLK2* (Nagase *et al.*, 1995). Our data indicate clearly that the high *TLK* expression in human testis can be attributed to *TLK2*, suggesting that this isoform presents the primary *TLK* gene functioning in male meiosis (see also Shalom and Don, 1999).

Recombinant Tlk1 and Tlk2 are active enzymes

To ascertain that human Tlk1 and Tlk2 are functional enzymes, myc-tagged Tlk proteins were expressed in Sf9 insect cells, using recombinant baculoviruses. As negative controls, catalytically inactive myc-tagged Tlk1 and Tlk2 proteins were generated by mutating critical residues (D559A and D590A, respectively) within the corresponding catalytic domains and expressed in parallel. Overexpressed kinases were purified to near homogeneity and analysed by SDS–PAGE. The active forms of both enzymes showed a significantly retarded electrophoretic mobility when compared with their inactive counterparts (Figure 3A), suggesting that Tlk1 and Tlk2 undergo autophosphorylation (see below). Using the purified enzymes, *in vitro* kinase assays were then performed in the presence of [γ-^{32}P]ATP, using either myelin basic protein (MBP), casein or histone H1 as exogenous substrates. MBP was strongly phosphorylated by both Tlk1 and Tlk2, whereas casein was a poor substrate and histone H1 was hardly phosphorylated at all (Figure 3B). Virtually no activity was associated with the catalytically inactive mutants, attesting to the specificity of the observed reactions (Figure 3B). Furthermore, both wild-type Tlk1 and Tlk2 were labelled by ^{32}P, consistent with the view that these kinases were able to autophosphorylate (Figure 3B). This was confirmed further by treating the wild-type enzymes with alkaline phosphatase, which abolished the electrophoretic mobility difference between catalytically active and inactive Tlks (Figure 4A; data not shown). Strikingly, however, dephosphorylation of mycTlk1 did not substantially diminish kinase activity (Figure 4B). Although ^{32}P incorporation into MBP was slightly decreased, we interpret this to result from substrate competition, since phosphate incorporation into the dephosphorylated mycTlk1 was correspondingly increased. Thus, the results shown in this section indicate that recombinant Tlks are active as protein kinases, that Tlk activity can readily be assayed using MBP as a convenient substrate, and that autophosphorylation causes a retardation in the electrophoretic mobility of Tlks, but is not required for activity.

Detection of active Tlks in human cells

To study the properties of mammalian Tlk1 and Tlk2, three different antibodies were produced. A first antibody

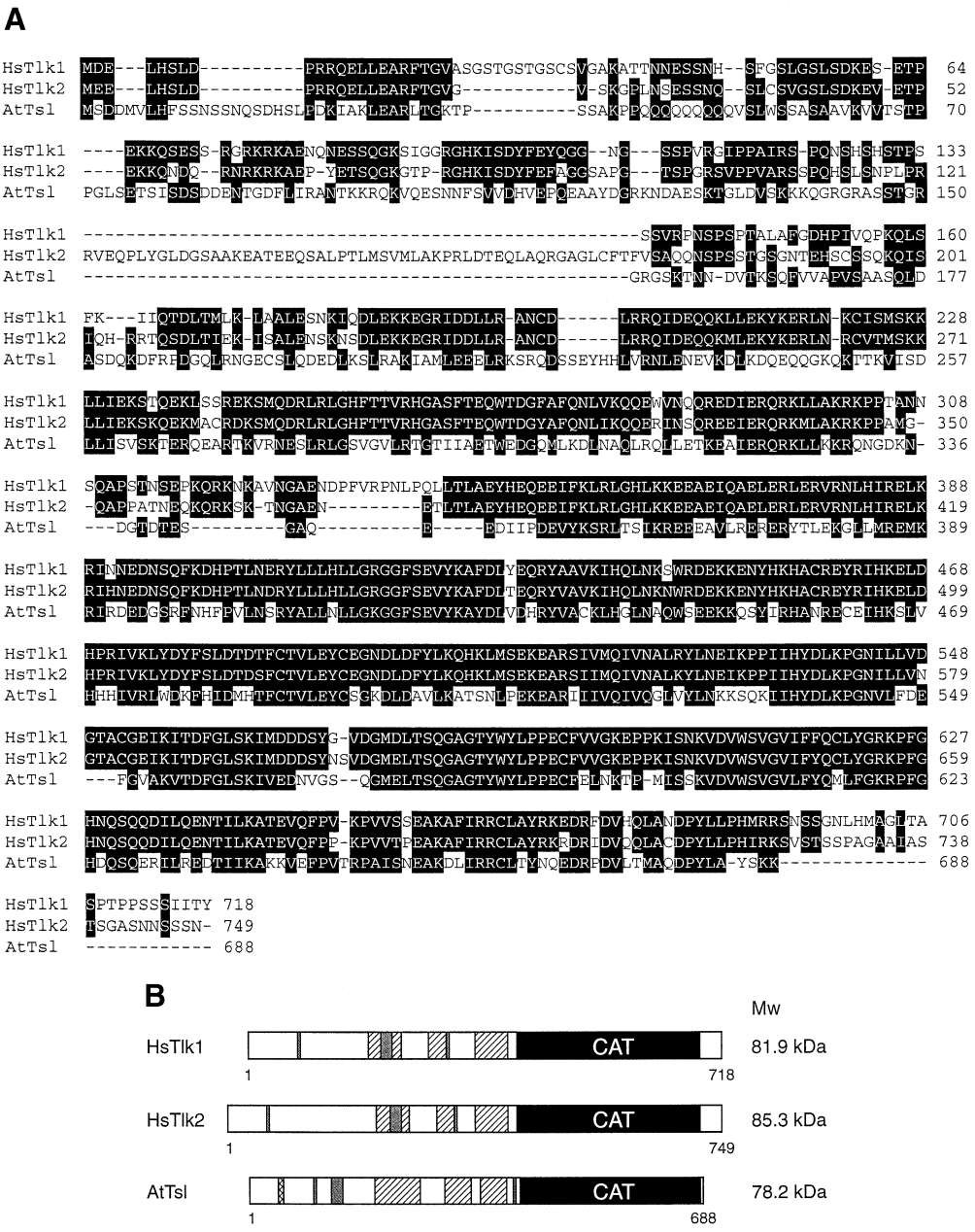

Fig. 1. Comparison of human Tlk1 (HsTlk1), human Tlk2 (HsTlk2) and *Arabidopsis* Tsl (AtTsl). (**A**) Alignment of the deduced amino acid sequences of human Tlk1, human Tlk2 and *Arabidopsis* Tsl, shown in single-letter code. Identical residues or conservative replacements are shaded black and dashes denote gaps in the alignments. The human sequences are believed to be full length for the following reasons: the *TLK1* cDNA shows an upstream stop codon, and the one in-frame methionine codon present between this stop and the proposed start codon is in a poor context for translational initiation (Kozak, 1991). In the *TLK2* cDNA, no upstream stop codon was found, but the homology with other Tlks ends abruptly upstream of the proposed start codon; although there is one methionine directly upstream of the proposed start codon, this methionine is in a poor context for translational initiation (Kozak, 1991). (**B**) Schematic representation of the Tousled-like kinases, with the different domains indicated. The catalytic domains (cat) are shown in black, the putative nuclear localization sequences in grey, the predicted coiled-coil regions by striped boxes and the glutamine-rich domain in *A.thaliana* Tsl by a zig-zagged box.

(R69; anti-TlkN) was raised against a region within the N-terminal, non-catalytic domain of Tlk1 (a.a. 52–443); this region shows a substantial degree of similarity between Tlk1 and Tlk2, and accordingly, anti-TlkN was not expected to discriminate between the two kinases. An antibody specific for Tlk1 (anti-Tlk1; R94) was made against a C-terminal peptide specific to Tlk1 (a.a. 691–705) (Zymed Laboratories), and an antibody specific for Tlk2 (R91; anti-Tlk2) was raised against a Tlk2-specific sequence within the N-terminal domain (a.a. 127–179). In all experiments shown below, affinity-purified antibodies were used. Antibody specificity was first assessed by Western blots on equal amounts of purified recombinant myc-tagged Tlk proteins (Figure 5A). Anti-TlkN recognized both Tlk1 and Tlk2, but anti-Tlk1 was clearly specific for Tlk1 and anti-Tlk2 was specific for Tlk2. Next, [^{35}S]methionine-labelled Tlk1 and Tlk2 were synthesized by *in vitro* transcription–translation (IVT) and subjected to immunoprecipitation by anti-Tlk antibodies. The precipitated proteins were then resolved by SDS–PAGE and analysed by autoradiography (Figure 5B). As expected, the anti-TlkN antibody was able to immunoprecipitate

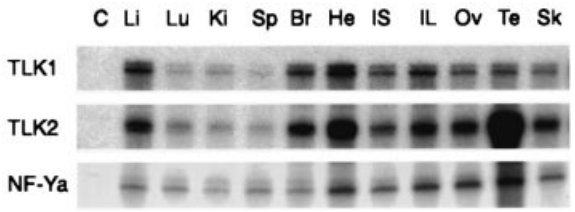

Fig. 2. RNase protection analysis of *TLK1* and *TLK2* expression in mouse tissues. RNase protection experiments were performed on RNA corresponding to 10 μg DNA equivalents of each tissue (Tanaka *et al*., 1997). Each RNA was supplemented to 100 μg with yeast RNA and hybridized with 10 fmol of murine *TLK1*, *TLK2* and *NF-Ya* probes. The control (C) contained 100 μg yeast RNA and 10 fmol of each probe. All organs were isolated from 4-month-old adult mice. Li, liver; Lu, lung; Ki, kidney; Sp, spleen; Br, brain; He, heart; IS, small intestine; IL, large intestine; Ov, ovary; Te, testis; Sk, skin.

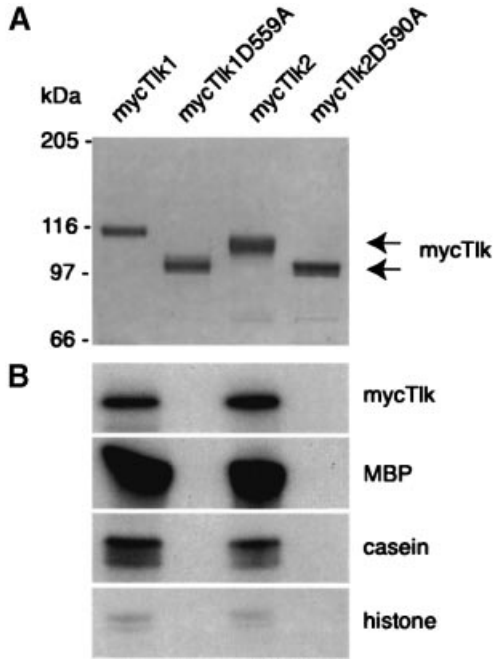

Fig. 3. Substrate specificity of purified recombinant mycTlk1 and mycTlk2. Sf9 cells were infected with mycTlk1, mycTlk1D559A, mycTlk2 or mycTlk2D590A recombinant baculoviruses and whole-cell extracts were made 36 h post-infection. Recombinant proteins were subsequently purified with monoclonal 9E10 myc-specific antibody coupled to protein G–Sepharose beads. (**A**) Equal amounts of the purified kinases were separated by SDS–PAGE and subsequently stained with Coomassie Blue. Note that wild-type kinases show a retarded electrophoretic mobility as compared with the catalytically inactive kinases. (**B**) Kinase assays with similar amounts of purified kinases were performed with MBP, casein or histone H1 as substrates (0.2 mg/ml) in the presence of [γ-^{32}P]ATP. Phosphorylation of the substrates was visualized by autoradiography after SDS–PAGE. Autophosphorylation is shown in the panel denoted mycTlk.

both Tlk1 and Tlk2, whereas anti-Tlk1 and anti-Tlk2 specifically precipitated Tlk1 and Tlk2, respectively. The *in vitro* translated Tlk1, and to a minor extent also Tlk2, migrated as multiple bands (Figure 5B). This most likely reflects autophosphorylation, as similar experiments with the catalytically inactive kinases yielded only one discrete band in each IVT (data not shown).

To examine the expression of endogenous Tlks in mammalian cells, Western blots were performed on HeLa cell extracts (Figure 6A). All three antibodies recognized one major protein migrating with an apparent molecular

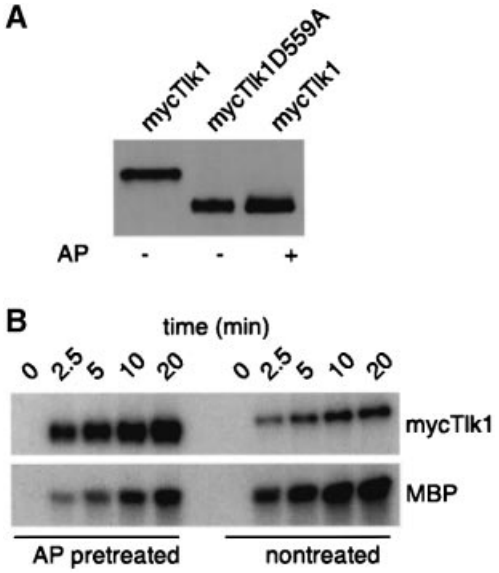

Fig. 4. Analysis of recombinant mycTlk1 autophosphorylation. (**A**) Recombinant mycTlk1 was isolated from Sf9 cells as described in the legend to Figure 3 and treated with alkaline phosphatase (AP). It was then separated by SDS–PAGE along with untreated mycTlk1 and mycTlk1D559A. Proteins were subsequently detected by Western blot analysis, using myc antibody 9E10. (**B**) Kinase assays were performed with alkaline phosphatase-treated and untreated mycTlk1, using MBP as a substrate in the presence of [γ-^{32}P]ATP. Reactions were stopped at the times indicated and, following SDS–PAGE, ^{32}P incorporation into mycTlk1 itself and into MBP was determined by autoradiography.

mass of ~85 kDa, suggesting that both Tlk1 and Tlk2 are expressed in HeLa cells, but that the two proteins co-migrate in the SDS–PAGE system used here. When Tlk immunoprecipitates from HeLa cell extracts were analysed by Western blotting, all three antibodies were found to immunoprecipitate an 85 kDa band, whereas the corresponding pre-immune IgGs did not precipitate any proteins (Figure 6B). As expected, anti-TlkN immunoprecipitates contained both Tlk1 and Tlk2. Somewhat surprisingly, however, anti-Tlk1 and anti-Tlk2 immunoprecipitates contained not only the corresponding cognate antigens, but also the second isoforms, albeit in lower amounts (Figure 6B). Considering that the anti-Tlk1 and anti-Tlk2 antibodies showed strict specificity for their respective antigens when used for immunoprecipitation of IVT products (Figure 5B), the most likely interpretation of this result is that Tlk1 and Tlk2 interact with each other *in vivo*. In support of this view, plant Tsl has previously been shown to dimerize (Roe *et al.*, 1997a).

When kinase assays were performed with MBP as a substrate, high levels of activity were associated with all Tlk immunoprecipitates, but not with control immunoprecipitates (Figure 6C). This demonstrates that all three antibodies can precipitate endogenous Tlks in active forms; however, because some Tlk1 co-precipitates with Tlk2 and vice versa, presumably due to heterodimerization (Figure 6B), we are presently unable to measure Tlk1 or Tlk2 activity exclusively.

Both Tlk1 and Tlk2 localize to the nucleus

The subcellular localization of Tlks was examined in exponentially growing HeLa cells, using anti-Tlk1 and anti-Tlk2 antibodies for indirect immunofluorescence

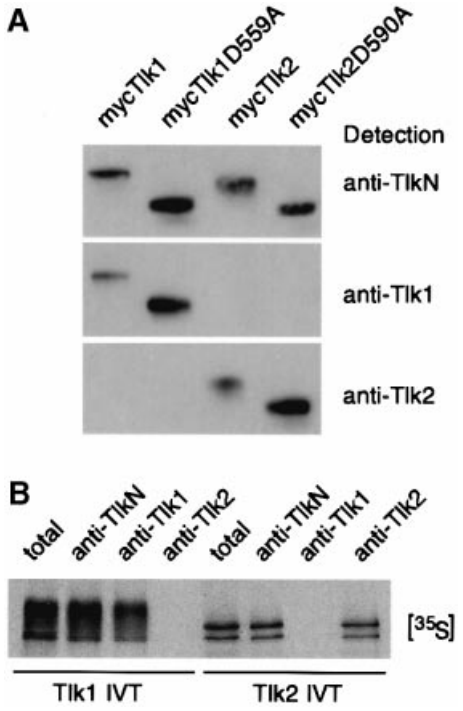

Fig. 5. Specificity of anti-Tlk1, anti-Tlk2 and anti-TlkN antibodies. (**A**) Nitrocellulose membranes containing equal amounts of baculovirus-expressed purified mycTlk1, mycTlk1D559A, mycTlk2 and mycTlk2D590A were probed with the indicated affinity-purified Tlk antibodies. Note that in all cases the phosphorylated wild-type kinases are less well detected than the catalytically inactive kinases. This is due to phosphorylation resulting in epitope masking (data not shown). (**B**) [^{35}S]methionine-labelled *in vitro* transcribed–translated (IVT) Tlk1 and Tlk2 were subjected to immunoprecipitation by the three Tlk antibodies. Corresponding amounts of the IVT lysates (total) and immunoprecipitates were subsequently separated by SDS–PAGE and the labelled Tlk products visualized by autoradiography. Owing to autophosphorylation, the Tlk1 and Tlk2 IVT products give rise to multiple bands.

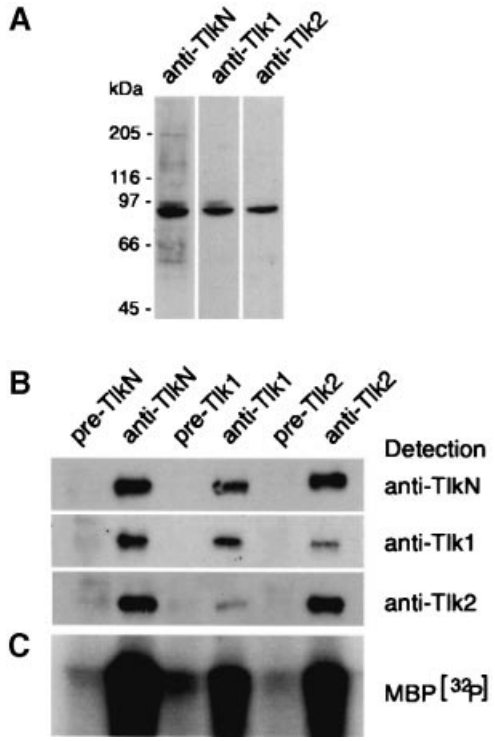

Fig. 6. Detection of endogenous Tlk1 and Tlk2 in cell extracts from exponentially growing HeLa cells. (**A**) Cell extracts were separated on a 7.5% polyacrylamide gel and probed by Western blotting with anti-Tlk1, anti-Tlk2 and anti-TlkN. All antibodies recognized a single band of ~85 kDa. (**B**) Immunoprecipitations with the three Tlk antibodies and corresponding pre-immune IgGs were carried out on equal amounts of protein extracts. Western blots of these immunoprecipitates were performed with the three Tlk antibodies. (**C**) Equal amounts of the above immunoprecipitates were used in *in vitro* kinase assays with [γ-^{32}P]ATP and MBP as an exogenous substrate. Reaction products were separated by SDS–PAGE and visualized by autoradiography.

microscopy. In interphase cells, both antibodies produced a strong and specific staining throughout the nucleoplasm, except for nucleoli (Figure 7A), and similar results were obtained with the anti-TlkN antibody (data not shown). No such staining was observed when using the corresponding pre-immune IgGs (Figure 7A; data not shown), and the nuclear staining produced by anti-Tlk1 could be abolished by competition with the peptide that had been used for immunization (Figure 7A). During mitosis, both Tlks were diffusely distributed, showing no preferential association with condensed chromosomes (Figure 7B). All results were independent of whether paraformaldehyde–Triton X-100 or methanol fixation procedures were used (data not shown). To corroborate these findings, both wild-type and catalytically inactive mycTlk1 and mycTlk2 were ectopically expressed in U2OS cells, using transient transfection. In all cases, anti-myc antibodies revealed a nucleoplasmic localization of the expressed Tlk proteins (Figure 7C; data not shown), indicating that kinase activity is not required for nuclear localization. Finally, overexpression of the N-terminal domain of Tlk1 alone was sufficient to confer nuclear localization, indicating that at least one of the three putative nuclear localization signals present in this domain is functional (data not shown).

Tlk activities are cell cycle regulated

The precise function of *Arabidopsis* Tsl remains unknown, but it has been suggested that the developmental defects observed in tsl mutant plants might arise from impaired cell proliferation (Roe *et al.*, 1997b). Prompted by this suggestion, we have examined the expression levels and kinase activities of human Tlk1 and Tlk2 during cell cycle progression. HeLa cells were synchronized using different drug arrest–release protocols, and the relative levels of Tlk1 and Tlk2 were determined by Western blotting on cell extracts equalized for protein content. In parallel, kinase activities were measured in immunoprecipitates prepared using either anti-Tlk1 or anti-Tlk2 antibodies and MBP as a substrate. In all immunoprecipitation experiments, recovery of Tlk protein was controlled by Western blot analysis.

In a first experiment, cells were pre-synchronized by a thymidine arrest–release protocol (see Materials and methods) and arrested in M phase by a 4 h treatment with nocodazole. Mitotic cells were then collected by shake-off and released into fresh medium. At the times indicated (Figure 8), aliquots of cells were analysed for Tlk abundance and activity (Figure 8B and C), and flow cytometry was used to confirm synchronous progression through the cell cycle (Figure 8A). As determined by Western blot analysis, Tlk1 and Tlk2 protein levels remained fairly constant throughout the cell cycle, but both proteins

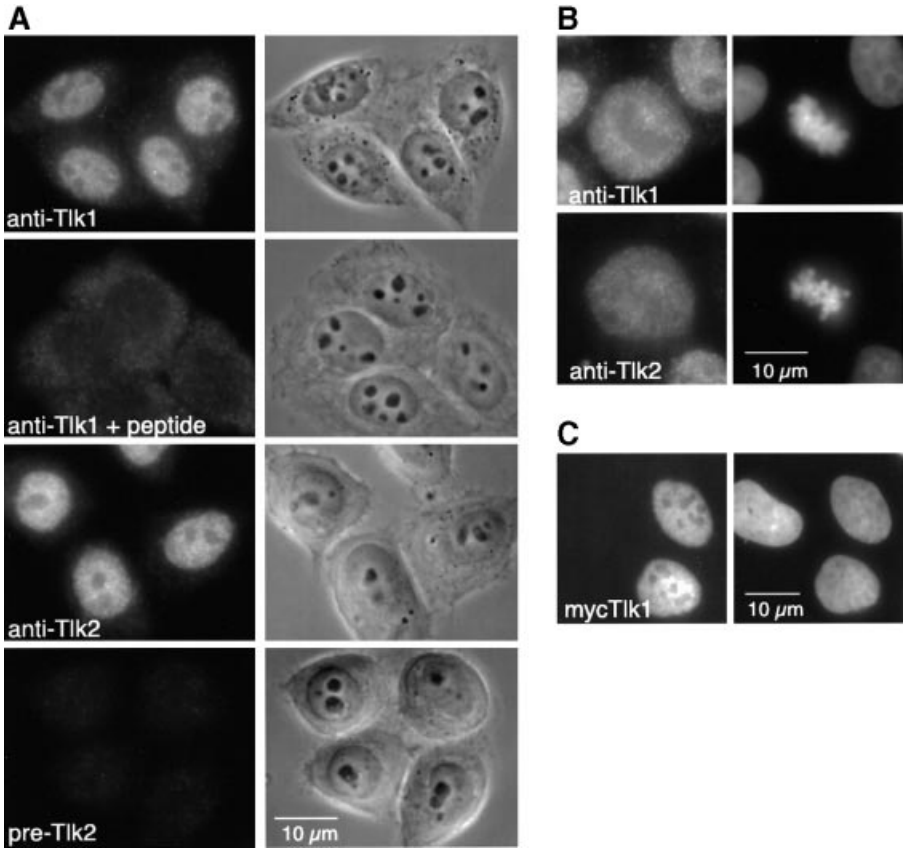

Fig. 7. Localization of endogenous Tlk1 and Tlk2 in HeLa cells and overexpressed mycTlk1 in U2OS cells. Exponentially growing HeLa and U2OS cells were cultured on glass coverslips. They were fixed with paraformaldehyde–sucrose solution followed by permeabilization with 0.5% Triton X-100. (**A**) Nuclear localization of endogenous Tlk1 and Tlk2 in interphasic HeLa cells, visualized with anti-Tlk1 and anti-Tlk2 antibodies, respectively (left). As a control, anti-Tlk1 was competed with the Tlk1 peptide used for immunization, and for anti-Tlk2 the corresponding pre-immune IgGs were used. Rhodamine-conjugated goat anti-rabbit was used as secondary antibody. Corresponding phase-contrast photographs are shown to the right. (**B**) Cellular localization of Tlk1 and Tlk2 in mitotic HeLa cells, using anti-Tlk1 and anti-Tlk2 antibodies, respectively (left). Corresponding DNA staining with Hoechst 33258 is shown to the right. (**C**) mycTlk1 was transiently overexpressed in U2OS cells for 24 h and then visualized with myc 9E10 antibody followed by rhodamine-conjugated anti-mouse IgG secondary antibody (left). DNA staining with Hoechst 33258 is shown to the right.

displayed striking changes in electrophoretic mobility (Figure 8B and C). In particular, both Tlk1 and Tlk2 showed a retarded electrophoretic mobility in M-phase extracts. A very similar retardation could be seen in cells collected by shake-off from exponentially growing populations, indicating that it did not result from drug treatment (data not shown). Most interestingly, the activities of both Tlk1 and Tlk2 increased substantially as cells entered S phase (t = 10 h) and decreased again upon exit from S phase (t = 20 h). As determined by ^{32}P incorporation into Tlk proteins *in vitro*, the apparent Tlk autophosphorylation changed in parallel with MBP phosphorylation (data not shown).

To extend these findings, a second type of drug arrest–release experiment was performed. This time, cells were blocked at the G_1–S transition, using a double block–release protocol based on the consecutive use of thymidine and aphidicolin (see Materials and methods). Using this procedure, cells progressed through S phase with a very high degree of synchrony, as indicated by flow cytometric analysis (Figure 9A). Western blot analyses suggested that the levels of Tlk1 and Tlk2 might be somewhat lower during S phase than during other phases of the cell cycle (Figure 9B and C). However, as described further below, we believe that this reflects a detection problem, related to hyperphosphorylation of Tlks during S phase (see also Figures 10 and 5A). In aphidicolin-arrested cells, the electrophoretic mobilities of both Tlks were maximal and, concomitantly, the activities were very low (Figure 9B and C). Immediately after release from the aphidicolin block, Tlk activities increased strongly and remained high until cells entered G_2 (t = 8 h), when they decreased again (Figure 9B and C). We conclude from these results that Tlk1 and/or Tlk2 are most active during the S phase of the cell cycle.

Figures 8 and 9 illustrate that endogenous Tlks undergo striking mobility shifts during cell cycle progression. On the basis of the results obtained with recombinant proteins (Figures 3A and 4A), it appeared likely that at least some of these mobility shifts were due to phosphorylation. To test this notion directly, Tlk proteins were immunoprecipitated from asynchronously growing HeLa cells, as well as from cells at the G_1–S boundary (i.e. blocked in aphidicolin) and from S phase cells (i.e. 4 h after release from the aphidicolin block). One half of each immunoprecipitate was then treated with alkaline phosphatase, and the electrophoretic mobility of Tlks was assessed by Western blotting (Figure 10). Phosphatase treatment clearly converted slow-moving forms of Tlks to fast-moving forms, indicating that the cell-cycle-dependent changes in

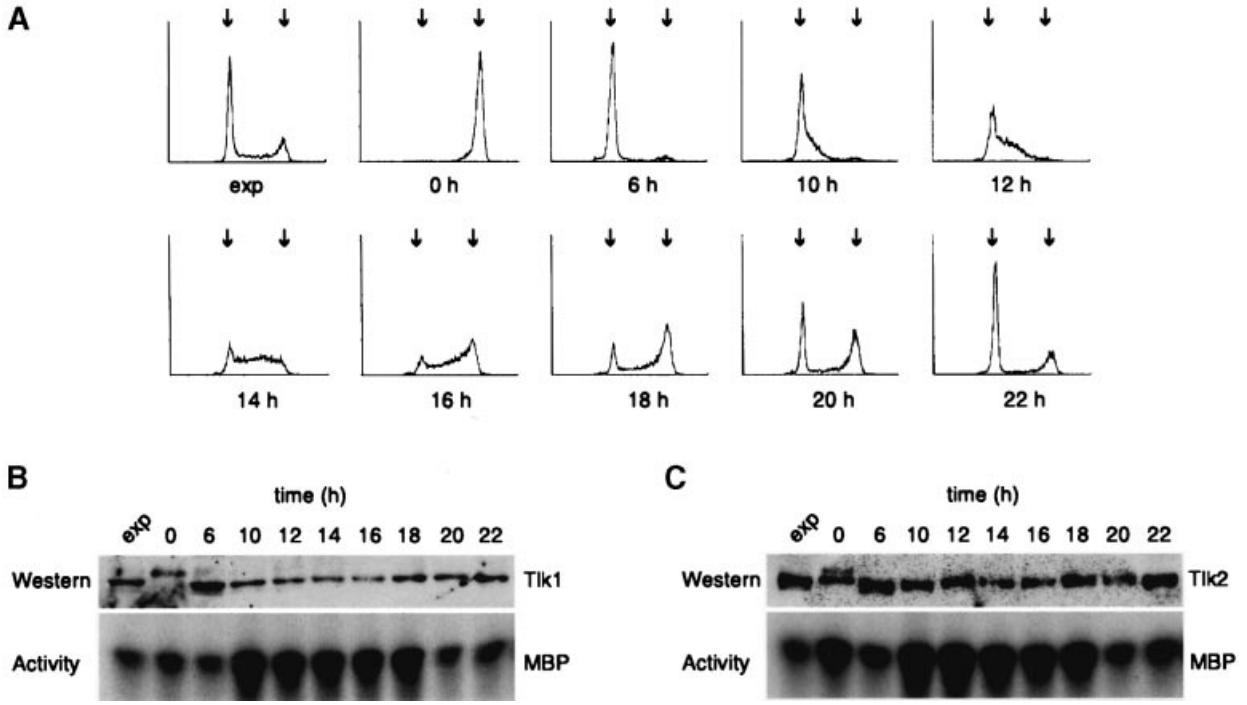

Fig. 8. Cell-cycle-dependent activation of Tlk1 and Tlk2 after release from a nocodazole block. Mitotic HeLa cells were obtained by pre-synchronization for 14 h with 2 mM thymidine followed by a release for 10 h and a subsequent block with 500 ng/ml nocodazole for 4 h. Mitotic cells were shaken off, washed with fresh medium and at $t = 0$ replated in fresh medium. At the indicated time points, samples were taken for FACS, Western blot analysis and kinase activity measurements. For comparison, exponentially (exp) growing cells were used. (**A**) FACS results of ethanol-fixed, propidium iodide-stained cells are shown as histograms and the positions of the G_1 phase peak and the G_2/M phase peak are marked by arrows. (**B**) For Western blot analysis, equal amounts of protein extracts were probed with anti-Tlk1 antibody (upper panel). For kinase activity measurents, anti-Tlk1 immunoprecipitates from equal amounts of protein extracts were used. Kinase assays were performed with 0.2 mg/ml exogenous MBP and reaction products were separated by SDS–PAGE. The amount of ^{32}P incorporated into MBP was visualized by autoradiography (lower panel). (**C**) Similar experiment to that described in (B), but performed with anti-Tlk2 antibody. Recovery of Tlk protein in the immunoprecipitates was controlled by Western blot analysis using anti-TlkN (not shown).

Tlk mobility were mostly, and perhaps entirely, due to phosphorylation. We caution, however, that there is no simple relationship between Tlk mobility, Tlk phosphorylation state and Tlk activity. In particular, Tlks display a strongly retarded mobility during M phase, although their activities are not particularly high during this stage of the cell cycle (see Figure 8B and C). It appears most likely, therefore, that Tlks are phosphorylated on multiple sites, depending on cell cycle stage, and that the functional consequences of Tlk phosphorylation differ depending on the particular sites.

Tlk activities are directly linked to ongoing DNA replication

As shown in Figure 9, Tlk activity was almost undetectable in aphidicolin-arrested cells. A priori, this observation could reflect a transient decrease in Tlk activity during a short period centred on the G_1–S transition. However, considering that no transient reduction in Tlk activity had been detected in nocodazole-release experiments (Figure 8), we reasoned that the lack of Tlk activity in aphidicolin-arrested cells might result from a drug-induced block to DNA replication. To explore this possibility further, aphidicolin was added to cells when these were already in mid-S phase, with maximal Tlk activity (4 h after release from an aphidicolin block). At short intervals thereafter, samples were collected for Western blot analyses and Tlk activity measurements. As shown in Figure 11B, Tlk1 activity fell substantially within 5–15 min after the addition of aphidicolin to mid-S phase cells, and virtually identical results were also obtained with Tlk2 (data not shown). In control cells, Tlk activity decreased only much later as cells approached the end of S phase (Figure 11B).

As cells traverse S phase, a population of Tlk1 with retarded electrophoretic mobility can readily be seen (Figure 11A). Interestingly, this slowly migrating form of Tlk1 persisted for at least 30 min after aphidicolin treatment, although Tlk1 was almost completely inactivated within 15 min. This indicates that Tlk inactivation, by an unknown mechanism, precedes the removal of phosphate groups from Tlks and it confirms that there is no simple relationship between Tlk1 activity and its phosphorylation state. Treatment of mid-S-phase cells with hydroxyurea yielded qualitatively similar results, but no Tlk inhibition could be observed when replication inhibitors were added for up to 2 h to G_1-phase cells or directly to recombinant Tlks (data not shown). Furthermore, no inhibition of Tlks was observed after inhibition of RNA polymerase II transcription by the addition of α-amanitin or 5,6-dichloro-benzimidazole riboside (DRB) to S-phase cells (data not shown). While these results do not rule out a role for Tlks in transcriptional regulation, they clearly indicate that Tlk activity is tightly coupled to ongoing DNA replication, and that the inhibition of DNA replication results in the rapid inactivation of these kinases.

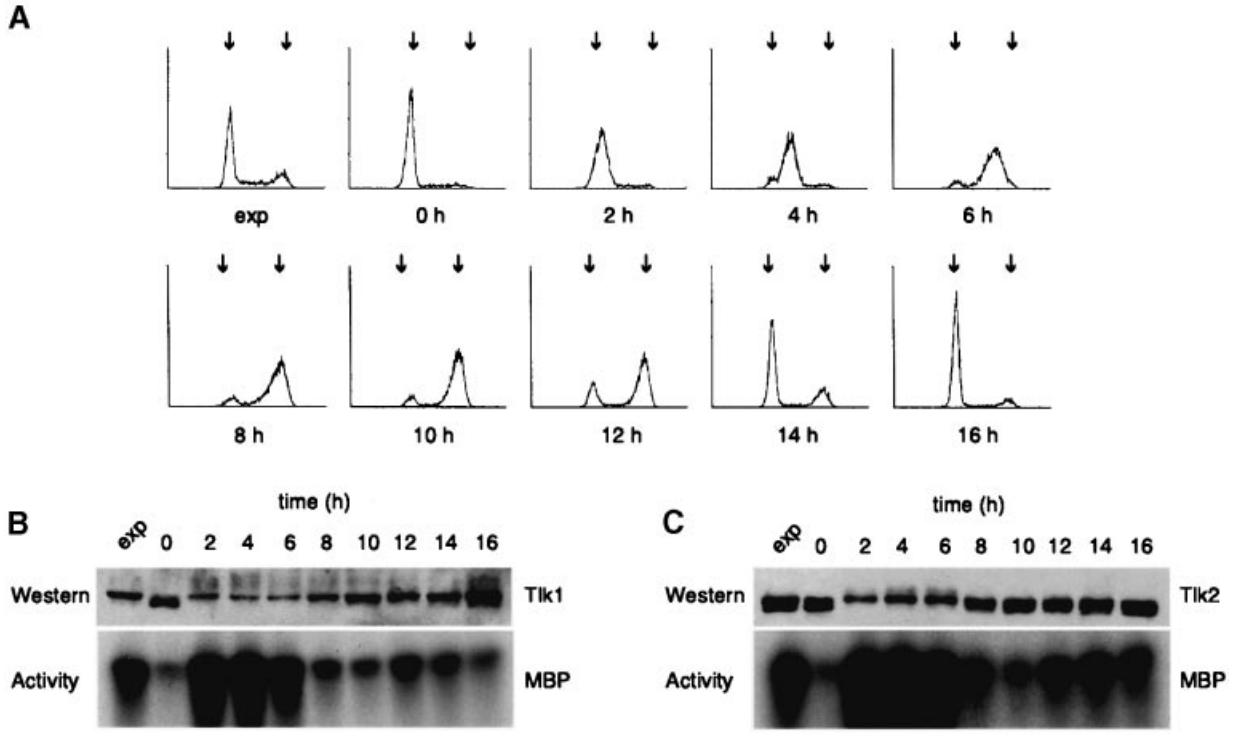

Fig. 9. Cell-cycle-dependent activation of Tlk1 and Tlk2 after release from an aphidicolin block. To obtain HeLa cells in early S phase, cells were cultured for 14 h in the presence of 2 mM thymidine, then released for 12 h in fresh medium, and finaly arrested for 14 h in the presence of 1.6 μg/ml aphidicolin. At $t = 0$, cells were released from this block and at the indicated time points samples were taken for FACS, Western blot analysis and kinase activity measurements. For comparison, exponentially (exp) growing cells were used. (**A**) FACS results of ethanol-fixed, propidium iodide-stained cells are shown as histograms and the positions of the G_1 phase peak and the G_2/M phase peak are marked by arrows. (**B**) For Western blot analysis, equal amounts of protein extracts were probed with anti-Tlk1 antibody (upper panel). For kinase activity measurements, anti-Tlk1 immunoprecipitates from equal amounts of protein extracts were used. Kinase assays were performed with 0.2 mg/ml exogenous MBP and reaction products were separated by SDS–PAGE. The amount of ^{32}P incorporated into MBP was visualized by autoradiography (lower panel). (**C**) Similar experiment to that described in (B), but performed with anti-Tlk2 antibody. Recovery of Tlk protein in the immunoprecipitates was controlled by Western blot analysis using anti-TlkN (not shown).

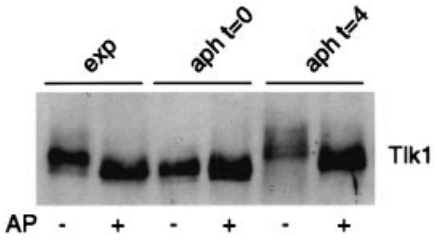

Fig. 10. *In vitro* dephosphorylation of cellular Tlk1. HeLa cell extracts were prepared from exponentially growing cells (exp), from aphidicolin-blocked cells (aph t=0) and from cells 4 h after release from an aphidicolin block (aph t=4). Tlk1 and Tlk2 were immunoprecipitated from equal amounts of extracts using anti-TlkN. Half of each immunoprecipitate was treated with alkaline phosphatase (AP) and the other half remained untreated. Equal amounts of non-treated and AP-treated sample were separated by SDS–PAGE, followed by immunoblotting with anti-Tlk1. Similar results were obtained when blots were probed with anti-Tlk2 (not shown).

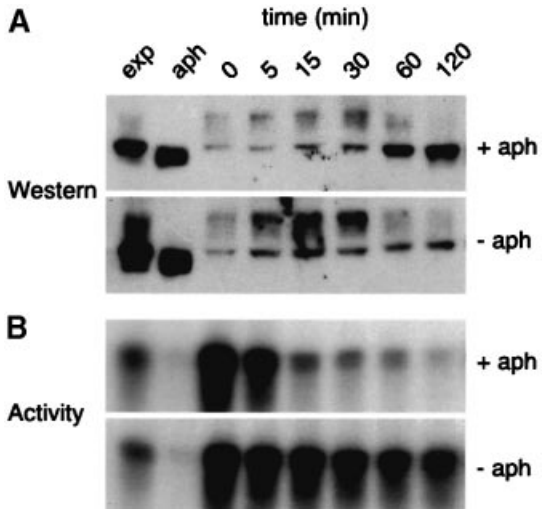

Fig. 11. Tlk activity is linked to ongoing DNA replication. Aphidicolin was added ($t = 0$) to mid-S-phase HeLa cells (4 h after release from an aphidicolin block) and samples were subsequently taken at the indicated time points (+aph). As a control, samples were also collected from cells to which dimethylsulfoxide (solvent for aphidicolin) had been added (–aph). Exponentially growing (exp) and aphidicolin-arrested (aph) cells were analysed for comparison. (**A**) For Western blot analysis, equal amounts of protein extracts were probed with anti-Tlk1 antibody. (**B**) Kinase activities of anti-Tlk1 immunoprecipitates were determined using MBP as an exogenous substrate. Similar results were obtained with anti-Tlk2 antibody (data not shown).

Sensitivity of Tlk activity to DNA-damaging agents

In a final series of experiments, we examined the effect of various DNA-damaging drugs on Tlk activity. Specifically, we tested agents expected to produce DNA cross-links (mitomycin C and cisplatin), DNA strand breaks (bleomycin) or base modifications (methyl methanesulfonate), as well as topoisomerase I and II inhibitors (camptothecin and etoposide, respectively). We found that all these agents inhibited Tlk activity when added to mid-S-phase cells (Figure 12, upper panel), albeit to different

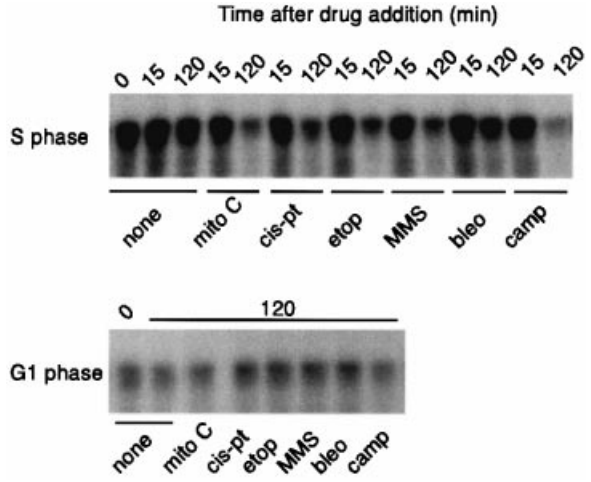

Fig. 12. Effect of DNA-damaging agents on Tlk activity. Mitomycin C (mito C), cisplatin (cis-pt), etoposide (etop), methyl methanesulfonate (MMS), bleomycin (bleo) and camptothecin (camp) were added to HeLa cells synchronized either in S phase (4 h after release from an aphidicolin block; upper panel) or G_1 phase (6 h after release from a nocodazole block; lower panel). At the times indicated, Tlks were immunoprecipitated with anti-Tlk1 antibodies and kinase activities were assayed using MBP as a substrate, followed by autoradiography. For the control, samples were taken from cells to which no drugs had been added.

extents. Interestingly, however, the same drugs did not detectably affect the low levels of Tlk activity in G_1-phase cells (Figure 12, lower panel). This argues that Tlk activity is not regulated by DNA-damage checkpoint mechanisms operating throughout the cell cycle. Although we cannot rigorously exclude the possibility that Tlks respond to an S-phase-specific checkpoint pathway, the most likely interpretation of our findings is that Tlk activity is inhibited as a consequence of DNA replication forks stalling on damaged DNA.

Discussion

Mutations in the serine/threonine protein kinase Tousled (Tsl) of *A.thaliana* produce a highly intriguing phenotype, characterized by a random loss of floral organs and other develomental anomalies (Roe *et al.*, 1993). It has been suggested that Tsl may form part of a signal transduction pathway controlling cell proliferation during plant development (Roe *et al.*, 1997b), but the precise molecular functions of this kinase remain obscure. Interestingly, the *TSL* gene is not confined to plants, as apparent homologues of this kinase can be found in databases from several animal species. We emphasize, however, that no obvious Tsl homologue exists in the budding yeast *S.cerevisiae*.

We have now cloned and characterized two putative human homologues of plant *TSL*, termed *TLK1* and *TLK2*. The two human Tlk proteins share not only a high degree of sequence similarity with plant Tsl, but also several functional properties, including nuclear localization, ubiquitous expression, a preference for MBP as an exogenous substrate, and the apparent abilities to undergo dimerization and autophosphorylation (Roe *et al.*, 1997a; this study). It seems plausible, therefore, that mammalian Tlks and plant Tsl play similar if not identical functions in the animal and plant kingdoms, respectively. However, considering that mammals do not produce flowers, it seems legitimate to postulate that Tlks must control a fundamental process that has arisen during the evolution of multicellular organisms, and has subsequently been highly conserved amongst both plants and animals.

We have discovered that the activities of mammalian Tlks are cell cycle regulated. This conclusion is based on direct measurements of Tlk activity, as well as on the observation of striking changes in the electrophoretic mobilities of these kinases. Although some of the mobility changes could be attributed to autophosphorylation, their complexity suggests that Tlks are subject to post-translational modifications, most likely phosphorylation, at multiple stages of the cell cycle. Most importantly, we found that Tlk activity is maximal during S phase, indicating that one major function of these kinases relates to S-phase progression. In line with this view, inhibition of DNA replication caused a rapid inactivation of Tlks, demonstrating that Tlk activity is tightly linked to ongoing DNA replication. However, as lower levels of Tlk activity could also be detected at other cell cycle stages, the role of Tlks need not necessarily be confined to S phase.

Our results strongly suggest that Tlks perform cell autonomous functions, and they provide the first clues as to the biochemical process(es) that might be regulated by this family of kinases. Foremost amongst these processes are DNA replication, DNA structure checkpoints, S-phase-specific transcription and/or chromatin remodelling. Considering that the mechanisms underlying DNA replication are highly conserved in all eukaryotes (Waga and Stillman, 1998), and yet no obvious Tlk homologue exists in *S.cerevisiae*, a role for Tlks in the core DNA synthetic machinery appears unlikely. To examine a possible function of Tlks in DNA replication directly, we have monitored the kinetics of bromodeoxyuridine incorporation in cells overexpressing either wild-type or catalytically inactive Tlks. We found that abnormal Tlk protein levels interfere significantly with cell cycle progression, but since no correlation could be established between the levels of Tlk activity and the kinetics of S-phase progression, these preliminary data are difficult to interpret (H.H.W.Silljé and E.A.Nigg, unpublished results).

DNA-damage checkpoints have also been highly conserved from yeast to man (Elledge, 1996). However, as exemplified by p53, additional layers of regulation have clearly been added during the evolution of higher eukaryotes. Thus, a role for Tlks in DNA structure checkpoints should not be excluded a priori. We found that several DNA-damaging agents rapidly inactivated Tlks when added to S-phase cultures, but the same agents did not affect Tlk activity when added to G_1-phase cells. Since extensive DNA damage is expected to cause replication forks to stall, the most straightforward interpretation of these data is that the DNA-damaging agents inhibited Tlk activity by blocking DNA replication.

We are led to propose, therefore, that Tlks play a role in transcription and/or chromatin remodelling. While we cannot rule out a role in S-phase-specific transcription, we presently favour the hypothesis that Tlks function in the regulation of chromatin structure. First, chromatin assembly is tightly linked to DNA replication (Krude, 1999), the time period of high Tlk activity. Secondly, chromatin structure plays an important role in the establish-

ment and maintenance of gene expression patterns during development (Hagstrom and Schedl, 1997). Thus, a role for Tlks in chromatin remodelling would be consistent with both the widespread expression of Tlks among plant and animal tissues, and the pleiotropic phenotype of tsl mutations in *Arabidopsis*. Basic chromatin remodelling processes are of course already present in yeast (e.g. silencing), but the complexity of chromatin-based epigenetic mechanisms is expected to have increased considerably during the evolution of multicellular organisms (Varga-Weisz and Becker, 1998).

To the best of our knowledge, Tlks are the first kinases whose activities are directly dependent on ongoing DNA replication. Thus, it will be interesting to investigate how their activities are regulated, and how this regulatory pathway relates to the core cell cycle machinery. Genetic studies in *Arabidopsis* have revealed many genes involved in the regulation of plant development, but so far they have failed to reveal direct interactions between Tsl and other plant genes. Thus, this kinase may well represent the first component of a novel developmental mechanism or signal transduction pathway (Roe *et al.*, 1997b). To explore the molecular function of animal Tlks further, we have recently begun to characterize Tlk homologues in both genetically tractable organisms (*C.elegans* and mice) and in *Xenopus*. We hope that the combined application of genetic and biochemical approaches may soon lead to a better understanding of this novel, highly conserved family of protein kinases.

Materials and methods

Cloning and sequencing of human TLK1 and TLK2 cDNAs

A 381 bp fragment (named HsPK41) showing homology with *Arabidopsis Tousled* (Schultz and Nigg, 1993) was used to screen human λgt11 and λZAPII placental cDNA libraries (Stratagene) as described previously (Tassan *et al*., 1994). A total of seven partial *TLK1* cDNAs were obtained and two of these (showing 501 bp overlap) were fused to create a full-length *TLK1* cDNA. When database searches revealed an EST (IMAGE cloneID 197429) closely related to, but distinct from human *TLK1*, this cDNA was used to screen a λZAPII placental and a λgt10 nasopharyngeal carcinoma cDNA library (Hitt *et al*., 1989). From six positive clones, two (showing 1844 bp overlap) were fused to obtain a full-length *TLK2* cDNA. Full-length human *TLK1* and *TLK2* were subcloned into pBluescipt SK(–) (Stratagene) and sequenced in both orientations (Chen and Seeburg, 1985) using the Sequenase 2.0 kit (United States Biochemicals). Sequence analysis was performed using the University of Wisconsin GCG package.

Plasmid constructions and site-directed mutagenesis

To prepare catalytically inactive Tlk1 and Tlk2 kinases (Tlk1D559A and Tlk2D590A), codon 559 (GAT, aspartic acid) of Tlk1 was mutated into GCT (alanine), and a corresponding D to A mutation was made in codon 590 of Tlk2. Mutagenesis was carried out with the Transformer site-directed mutagenesis kit (Clontech Laboratories) using the oligonucleotides 5′-GAAATCAAAATCGCTTTTGGTCTGTCCAAG-3′ and 5′-GAGATAAAAATTACAGCTTTTGGTCTTTCGAAG-3′ for *TLK1* and *TLK2*, respectively, and a vector-specific primer for selection. Mutations were confirmed by sequencing. Myc-tagged *TLK1* and *TLK2* plasmids were prepared as in-frame fusions in pBluescript-myc (Schmidt-Zachmann and Nigg, 1993). For *TLK2*, the myc tag was fused directly to the methionine start codon, using PCR, but for *TLK1* the myc tag was fused to the first methionine codon present in the cDNA, which resulted in the presence of an additional 144 bp (48 a.a.) upstream of the *TLK1* initiator methionine. For expression in mammalian cells, myc *TLK1* and *TLK2* were excised from pBluescript-myc and subcloned into pRcCMV (Invitrogen Corp.). To create baculoviruses encoding both active and inactive versions of myc-tagged *TLK1* and *TLK2*, the corresponding cDNAs were inserted into pVL1392 (Pharmingen Corp.). All PCR fragments used during cloning were checked by sequencing.

RNA purification and RNase protection assays

To detect mouse *TLK1* and *TLK2* transcripts, fragments of mouse homologues of human *TLK1* and *TLK2* were isolated. A mouse brain cDNA library in λZAP II (Stratagene) was screened with a ^{32}P-labelled human *TLK1* cDNA fragment encoding the catalytic domain. This screen yielded five positive clones, of which one (TH-1) had homology with human *TLK1* and another (TH-4) with human *TLK2*. RNA purification from adult mouse organs and RNase protection assays with antisense RNA probes were performed as previously described (Tanaka *et al.*, 1997).

Antibody production

To produce a Tlk1-specific antibody, a 15 a.a. peptide (residues 691–705) was synthesized, coupled to keyhole limpet haemocyanin and injected into rabbits (Zymed Labs, Inc., South San Francisco, CA). Antipeptide antibodies were purified from the immune sera by affinity chromatography, using the peptide coupled to an insoluble support (Zymed). To generate a Tlk2-specific antibody, an N-terminal fragment (residues 127–179) was fused to a polyhistidine–dihydrofolate reductase (DHFR) tag, using the bacterial expression vector pQE40 (Qiagen). This fusion protein was overexpressed in *Escherichia coli* and purified under denaturing conditions as described by the manufacturer (Qiagen). Following further purification on a preparative 12% SDS–polyacrylamide gel (Fry *et al.*, 1998), 250 μg of the protein were injected several times subcutaneously into New Zealand white rabbits (Elevage Scientifique des Dombes, Chatillon sur Chalarone, France). Anti-Tlk2 antibody was purified by applying the serum onto a Ni^{2+} resin column with the bound Tlk2 fusion protein. After washing, the antibody was eluted in 4 M $MgCl_2$ as described by Gu *et al.* (1994) and dialysed extensively. To remove antibodies directed against the DHFR moiety of the fusion protein, the purified antibody preparation was passed over a His_6–DHFR Ni^{2+} resin column. For production of an antibody recognizing both Tlk1 and Tlk2, a structurally conserved fragment (Tlk1 residues 52–443) was fused to a polyhistidine tag in the pQE9 vector. Overexpression, purification and injection of this protein were similar to the procedures described above for anti-Tlk2. For affinity purification of anti-TlkN, 1 mg of the Tlk1 fragment was covalently coupled to 400 mg CNBr-activated Sepharose 4B (Pharmacia Biotech, Piscataway, NJ) as recommended by the manufacturer. Purification was performed as described by Harlow and Lane (1988).

Expression and purification of recombinant myc-tagged Tlks

Recombinant Tlk baculoviruses were generated by co-transfection of the different pVL vectors with Baculogold DNA (Pharmingen Corp.). All procedures relating to Sf9 insect cell growth, transfection, infections and viral amplifications were performed as described by the manufacturer (Pharmingen Corp., 1998). Sf9 cells were infected with recombinant baculovirus in 100 mm dishes and cell lysates were prepared 36 h after infection. Cells were washed once with ice-cold phosphate-buffered saline (PBS), 1 mM phenylmethylsulfonyl fluoride (PMSF) and resuspended in ice-cold NP-40 buffer (50 mM Tris–HCl pH 8.0, 1% NP-40, 150 mM NaCl) containing 30 μg/ml RNase A, 30 μg/ml DNase, phosphatase inhibitors (20 mM NaF, 20 mM β-glycerophosphate, 0.3 mM sodium vanadate) and protease inhibitors (1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin). After 30 min on ice, cells were disrupted by 10 passages through a 27 G needle, and lysates were spun in a microcentrifuge at full speed for 5 min at 4°C. Recombinant myc-tagged Tlks were purified from the lysates by incubation for 1 h at 4°C on a rotating wheel with the monoclonal 9E10 myc antibody coupled to Sepharose–protein G beads. Immune complexes were pelleted and washed three times with NP-40 buffer.

Cell culture and synchronization

Human cells were grown at 37°C in a 7% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with heat-inactivated fetal calf serum (FCS) and penicillin–streptomycin (100 IU/ml and 100 μg/ml, respectively). HeLa epithelial cells were grown in 5% FCS and U2OS osteosarcoma cells in 10% FCS. Sf9 insect cells were grown in TC100 medium (Gibco-BRL) supplemented with 10% heat-inactivated FCS and penicillin–streptomycin at 27°C.

To arrest exponentially growing HeLa cells at prometaphase, they were pre-synchronized in G_1/S by treatment with 2 mM thymidine (Sigma) for 14 h. Subsequently, they were washed three times in PBS and incubated for 10 h in fresh medium before 500 ng/ml nocodazole (Sigma) was added and culturing was continued for an additional 4 h. Mitotic cells were then collected by mechanical shake-off, washed twice

in PBS and replated in normal growth medium. Samples were taken either from arrested cells or after release for various time intervals.

Cells were synchronized in early S phase by a double thymidine–aphidicolin block, essentially as described by Heintz *et al*. (1983). In brief, cells were blocked for 14 h with 2 mM thymidine, washed three times with PBS, released for 12 h into fresh medium and incubated for 14 h with 1.6 μg/ml aphidicolin. After three washes with PBS, cells were released into fresh medium. Samples were taken at various time intervals. Flow cytometric analyses were performed using a FACScan (Becton-Dickinson), as described by Fry *et al*. (1995).

Immunoblotting and immunoprecipitations

HeLa cells were washed once in ice-cold PBS, 1 mM PMSF and then resuspended in ice-cold RIPA buffer (50 mM Tris–HCl pH 8.0, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl) containing RNase A, DNase, phosphatase inhibitors and protease inhibitors (see above). After 20 min on ice, cells were collected by scraping and centrifuged for 5 min at 4°C. Protein concentrations were determined using the Dc protein assay (Bio-Rad). Immunoblotting on equal amounts of protein extracts was performed essentially as described by Fry *et al*. (1998). Tlk1- and Tlk2-specific antibodies were used at a concentration of 1.5 μg/ml and anti-TlkN at 0.2 μg/ml. Detection was performed by enhanced chemiluminescence.

For immunoprecipitations, equal amounts of RIPA cell extract were pre-cleared with Affi-Prep protein A beads (Bio-Rad) for 15–30 min at 4°C. They were then incubated for 90 min at 4°C with 5 μg/ml of purified anti-TlkN, anti-Tlk1, anti-Tlk2 or the corresponding pre-immune IgGs before Affi-Prep proteinA beads were added for 60 min. All incubations were performed on a rotating wheel. Immune complexes were spun down and washed three times with RIPA buffer before they were used for immunoblotting, kinase assays or dephosphorylation assays.

In vitro kinase assays and phosphatase treatments

For *in vitro* kinase assays, Tlk immune complexes were washed three times with Tlk kinase buffer (50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.5 mM dithiothreitol and 5 mM NaF). Kinase reactions were carried out for 30 min at 30°C in Tlk kinase buffer supplemented with 10 μM ATP, 2 μCi of [γ-^{32}P]ATP (Amersham Corp.) and 2 mg/ml of MBP as a substrate. Reactions were stopped by addition of gel sample buffer and heating to 95°C. Reaction products were visualized by SDS–PAGE and autoradiography.

For dephosphorylation experiments, Tlk immune complexes were washed three times with phosphatase buffer (50 mM Tris–HCl pH 9.0, 10 mM $MgCl_2$). Dephosphorylation was performed in phosphatase buffer at 37°C for 2 h with 5 U alkaline phosphatase (Boehringer Mannheim) in 25 μl final volume. Subsequently, immune complexes were washed twice with NP-40 or RIPA buffer, resupended in gel sample buffer, heated for 5 min at 95°C and analysed by Western blotting.

Immunofluorescence microscopy

Growth of cells on coverslips and immunostaining procedures, using either paraformaldehyde or methanol fixations, were performed essentially as described by Fry *et al*. (1998). Primary antibodies were 9E10 anti-myc monoclonal antibody (undiluted tissue culture supernatant), purified anti-TlkN IgG (0.5 μg/ml), purified anti-Tlk1 IgG (2.0 μg/ml) and purified anti-Tlk2 IgG (1.0 μg/ml). All corresponding pre-immune IgGs were used at equivalent concentrations. For peptide competition with anti-Tlk1, the antibody was pre-incubated for 45 min at room temperature with a 10-fold molar excess of the immunogenic peptide. Primary antibodies were detected with Texas Red-conjugated secondary antibodies and DNA was stained with Hoechst 33258 (0.2 μg/ml). Immunofluorescence microscopy was performed using a Zeiss Axioplan II microscope and 63× oil immersion objectives. Photographs were taken using a Quantix 1400 CCD camera (Photometrics, Inc.) and IP-Lab software, and images processed with Adobe Photoshop (Adobe Systems, Mountain View, CA).

Miscellaneous techniques

In vitro transcription and translation were performed with the TnT-coupled reticulocyte lysate system (Promega), according to the manufacturer's instructions. Transient transfections of U2OS cells were performed using the calcium phosphate method as described by Seelos (1997). DNA-damaging drugs were added to synchronized cells at the following concentrations: mitomycin C at 1 μg/ml, cisplatin at 10 μM, etoposide at 25 μM, methyl methanesulfonate to 0.01%, bleomycin at 0.06 U/ml and camptothecin at 1 μM.

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