Tousled-like kinase in a microbial eukaryote regulates spindle assembly and S-phase progression by interacting with Aurora kinase and chromatin assembly factors

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Summary

The Tousled-like kinases are an evolutionarily conserved family of proteins implicated in DNA repair, DNA replication and mitosis in metazoans and plants. Their absence from the yeasts and other eukaryotic 'microbes' suggests a specific role for them in the development of multicellular organisms. In this study, two closely related Tousled-like kinase homologs, TLK1 and TLK2, were identified in *Trypanosoma brucei*, a unicellular protozoan parasite. Only TLK1 plays an essential role in cell growth, and a deficiency in TLK1 led to an enrichment of S-phase cells, defective spindle formation and aberrant chromosome segregation. Although both TLK proteins localize to the nucleus, only TLK1 also concentrates in the spindle poles during mitosis. Both TLK proteins are phosphorylated by the Aurora kinase (AUK1), and both can autophosphorylate and phosphorylate histone H3 and the chromatin assembly factors Asf1A and Asf1B in vitro, but only TLK1 is autophosphorylated and capable of oligomerizing and interacting with AUK1, Asf1A and Asf1B in vivo. These discrepancies between the two TLK proteins can be attributed to minor differences between their N- and C-terminal sequences. In summary, TLK1 cooperates with Aurora kinase to regulate spindle assembly and chromosome segregation, and it performs a role in DNA replication probably by regulating histone modification in trypanosomes.

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Key words: Anti-silencing function protein, Tousled-like kinase, *Trypanosoma brucei*, Mitosis, Spindle

Introduction

The Tousled-like kinases (TLKs) are a family of highly conserved serine/threonine protein kinases in both plants and animals. Tousled kinase is essential for proper flower and leaf development in *Arabidopsis* (Roe et al., 1993), whereas its homologs regulate DNA repair, DNA replication and mitosis in human and *Drosophila* (Silljé et al., 1999; Groth et al., 2003; Carrera et al., 2003). Mammalian TLK activity peaks during S phase, when it phosphorylates the anti-silencing function protein 1 (Asf1), a histone H3/H4 chaperone and a chromatin assembly factor involved in DNA repair and chromatin assembly (Silljé et al., 1999; Silljé and Nigg, 2001). In *Caenorhabditis elegans*, the Tousled-like kinase TLK-1 acts as a substrate activator of the Aurora B kinase AIR-2, a chromosomal passenger controlling chromosome segregation and cytokinesis (Han et al., 2005). TLK-1 is phosphorylated by AIR-2 and the phosphorylated TLK-1 in turn increases the kinase activity of AIR-2 towards histone H3.

No TLK homolog is present in the unicellular eukaryote *Saccharomyces cerevisiae* (Silljé et al., 1999) or any other microbial eukaryotes examined thus far. These led to the hypothesis that TLKs are specific for multicellular organisms and probably function in some fundamental aspects of development common to both plants and animals (Silljé et al., 1999).

Two close homologs of TLK were, however, identified in *Trypanosoma brucei*, a unicellular parasite that causes sleeping sickness in humans and nagana in cattle. This organism is deeply branched in the phylogenetic tree and classified to be more ancient than the yeasts (Baldauf, 2003; Bapteste and Gribaldo, 2003). It is transmitted between the mammalian host and the insect vector in three multiplying forms, the bloodstream and two insect (procyclic and epimastigote) forms, displaying distinctive cellular morphology and biological features. The trypanosome cell cycle has the usual sequential G_1, S, G_2 and M phases (McKean, 2003) but differs from other eukaryotes by having a unique mitochondrial DNA complex, the kinetoplast, whose division is mediated by basal body segregation (Woodward and Gull, 1990). A delicate coordination between the progression of the nuclear cycle and basal body/kinetoplast cycle is thus important for cytokinesis (Ploubidou et al., 1999). In the procyclic form, initiation of cytokinesis is dependent primarily on the basal body/kinetoplast cycle (Ploubidou et al., 1999; Li and Wang, 2003; Hammarton et al., 2003). Inhibition of mitosis in the procyclic form does not totally block cytokinesis and results in the formation of anucleate cells with a single kinetoplast. In the bloodstream form, however, inhibition of mitosis prevents cytokinesis but does not affect additional rounds of organelle replication

(Hammarton et al., 2003; Tu and Wang, 2004; Li and Wang, 2006).

An Aurora kinase homolog, AUK1, is essential for spindle formation, chromosome segregation and cytokinesis in both bloodstream and procyclic forms of *T. brucei* (Tu et al., 2006; Li and Wang, 2006), suggesting that, like the Aurora kinase Ark1 in *Schizosaccharomyces pombe*, AUK1 has the functions of both the Aurora A and Aurora B kinases possessed by the higher eukaryotes (Petersen et al., 2001). AUK1 displays a subcellular localization pattern typical of a chromosomal passenger – that is, being concentrated in the nucleus during prophase but translocated to the spindle midzone in late anaphase (Tu et al., 2006; Li and Wang, 2006). This localization is mediated by the formation of a chromosomal passenger complex between Aurora B kinase and several other proteins such as Survivin, Borealin/Dasra and INCENP, the substrate activator of Aurora B kinase, in metazoa (Ducat and Zheng, 2004). However, homologs of these proteins are absent from the trypanosome genome (Z.L. and C.C.W., unpublished). Furthermore, homologs of the majority of the kinetochore components, which are all conserved among yeasts, plants and mammals and some of which are substrates of Aurora B kinase, are also all absent from the genome of *T. brucei* (Berriman et al., 2005). These raise the interesting question of how the Aurora kinase AUK1 from *T. brucei* is regulated in the absence of its regulatory proteins.

As *T. brucei* possesses two homologs of TLK, which acts as a substrate activator of Aurora B kinase in *C. elegans* and phosphorylates the chromatin assembly factor Asf1 in metazoa and plants, we analyzed their potential involvement with AUK1 and the Asf1 homologs in regulating cell cycle progression in *T. brucei*. We observed that, despite the 89% sequence identity between TLK1 and TLK2, only TLK1 cooperates with AUK1 to regulate spindle assembly and chromosome segregation and maintains the S-phase progression by regulating Asf1A and Asf1B. This is the first time, to our knowledge, that a functioning TLK has been identified in a unicellular microorganism.

Results

Identification of TLK homologs in *T. brucei*

Two close homologs of TLK were identified in *T. brucei* (Tb927.4.5180 and Tb927.8.7220) and designated TLK1 and TLK2, respectively. They reside on different chromosomes and are 89% identical in their amino acid sequences. They also showed a 29% identity to the *Arabidopsis* Tousled (supplementary material Fig. S1) and 30% identity to human TLKs (data not shown). Both TLKs possess the putative nuclear localization signal sequences in their N-terminal portions (supplementary material Fig. S1A, gray box), three coiled-coil motifs (Lupas et al., 1991) that are required for oligomerization of Tousled in *Arabidopsis* (Roe et al., 1997) (supplementary material Fig. S1A, striped box) and a well-conserved catalytic domain near the C-terminus (supplementary material Fig. S1A, black box). The catalytic domain of TLK possesses an unusual ATP-binding motif, GxGxxS (position 327-332 in TLK1 and 2, supplementary material Fig. S1B), instead of the canonical GxGxxG motif possessed by other kinases (Hanks et al., 1988). Both TLK1 and TLK2 are expressed in trypanosomes, but the mRNA level of TLK2 is less than 10% of the level of mRNA for TLK1 (Fig. 1A, insets).

TLK1 is required for spindle assembly and chromosome segregation in *T. brucei*

To investigate whether the two TLKs play any role in *T. brucei*, RNAi was performed to knockdown individual gene expression by targeting the divergent 3′-UTR sequences in TLK1 and TLK2. Northern blots using 5′-UTR sequences indicated a ~95% reduction of mRNA after RNAi induction for 24 hours (Fig. 1A, insets). TLK1 knockdown resulted in a significant depression of cell growth, but TLK2 deficiency has no apparent effect (Fig. 1A), suggesting that TLK1 could be the only functional homolog in *T. brucei*.

Flow cytometry showed that, while TLK2 RNAi did not appreciably affect the cell cycle progression (Fig. 1B, right panel), TLK1 RNAi resulted in a significant enrichment of S-phase and G_2/M-phase cells (Fig. 1B, left panel). Within 24 hours of RNAi, the G_1 cell population decreased from ~67% to ~12%, accompanied by a corresponding increase of G_2/M-phase cells from ~13% to ~64%, suggesting that TLK1 RNAi led to a mitotic defect. In addition, however, the number of S-phase cells also increased from ~19% to ~51% within the first 16 hours of RNAi but decreased to ~24% after 24 hours, suggesting that TLK1 deficiency retards S-phase progression but does not totally stop it. TLK1 might thus also play a role in promoting S-phase progression in addition to regulating mitosis.

TLK1 RNAi enriched the cells with one nucleus and two kinetoplasts (‘1N2K’) from ~20% to ~60% of the total population after 24 hours (Fig. 1C). The 1N2K cells were apparently defective in chromosome segregation, as monitored with the L1C6 antibody (Fig. 1D, left panel), which stains the nucleolus and serves as a cellular marker for chromosome segregation in *T. brucei* (Ogbadoyi et al., 2000; Durand-Dubief and Bastin, 2003; Li and Wang, 2006). The control 1N2K cell has a segregating nucleolus, whereas the nucleolus merely becomes slightly enlarged in the TLK1-deficient cell (Fig. 1D, left panel), suggesting an interrupted mitosis probably caused by defective chromosome segregation. TLK1 RNAi, however, did not lead to defects in duplication/segregation of basal bodies or flagella (Fig. 1D, right panel), indicating that only mitosis was defective. A KMX-1 antibody was used to stain the spindle in the control and RNAi cells, and the results showed that, while almost 70% of the 1N2K cells and ~80% of the 2N2K control cells possessed the spindle structures, no spindle was detected in any of the 1N2K cells or among the majority (~90%) of 2N2K cells when TLK1 was knocked down (Fig. 1E). Thus, TLK1 is probably required for spindle assembly.

The subcellular localizations of TLK1 and TLK2

TLK1, TLK2 and their kinase-dead mutants TLK1-K349R and TLK2-K349R were overexpressed as hemagglutinin (HA)-tagged proteins in *T. brucei*. A slower-migrating band was detected in the TLK1-overexpressing cells (Fig. 2A, lane 2) but not in the TLK1-K349R, TLK2 or TLK2-K349R overexpressing cells (Fig. 2A, lanes 4, 6 and 8) in western blots. Treatment of the cell lysates with lambda protein phosphatase (λPPase) resulted in the elimination of this slower-migrating protein (Fig. 2B, lane 3), indicating that it might be

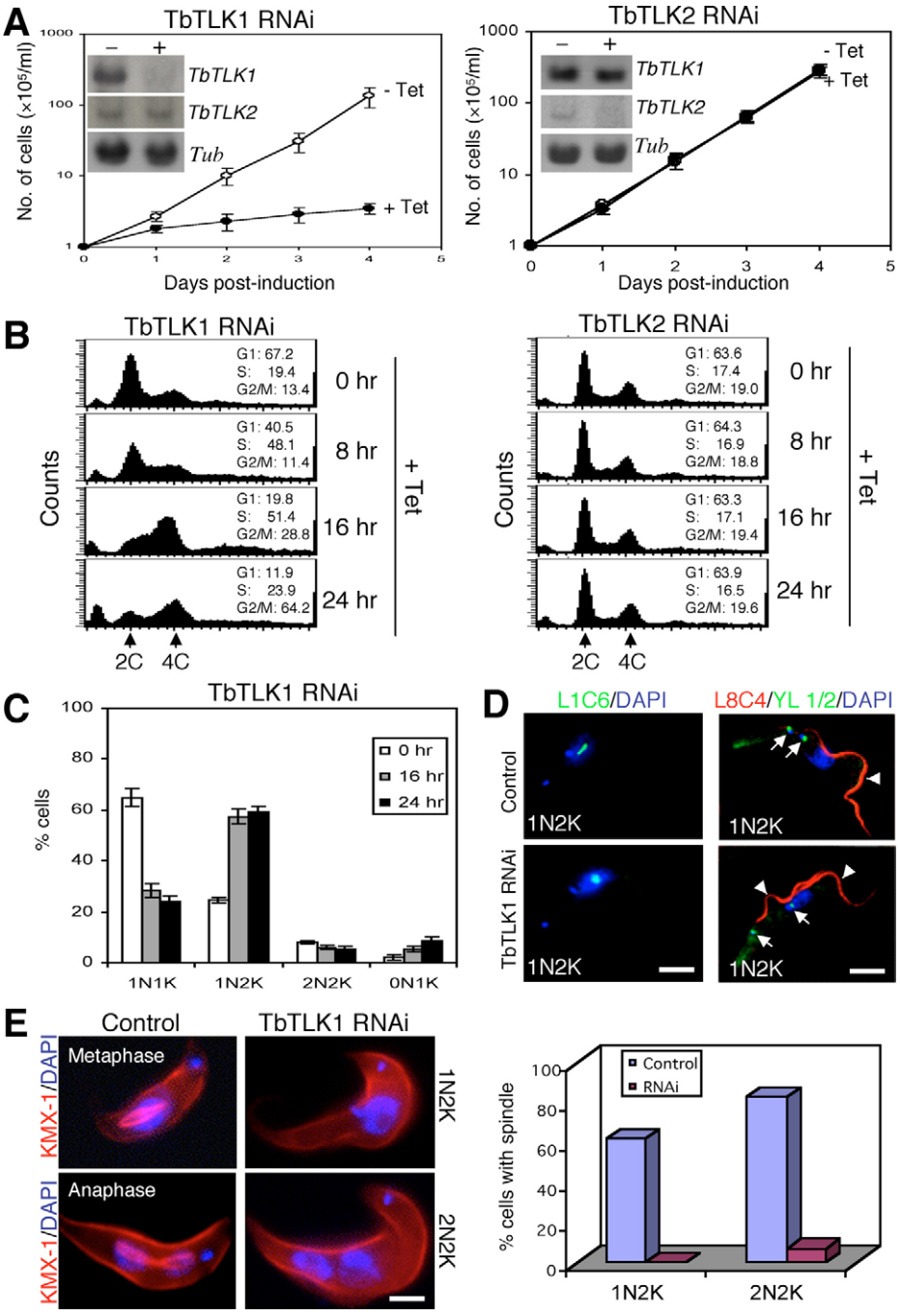

Fig. 1. RNAi silencing of TLK1 and TLK2 in the procyclic form of *T. brucei*. (A) Clonal cell lines harboring the respective TLK1 and TLK2 RNAi constructs were cultivated with (+Tet) or without (–Tet) tetracycline and monitored for cell growth. The insets show the levels of mRNA, monitored by northern blot, in cells before (–) and after (+) RNAi. α-Tubulin (*Tub*) was included as a loading control. (B) Flow cytometry analysis of DNA contents in TLK1 or TLK2 RNAi cells. Insets show the percentages of cells at G_1-, S- or G_2/M-phases. (C) The control and TLK1 RNAi cells after 16 and 24 hours RNAi induction were tabulated for different numbers of nuclei (N) and kinetoplasts (K). The data are presented as the mean percentage±s.e. of ~200 cells counted from three independent experiments. (D) Control and TLK1 RNAi cells after 24 hours induction were stained with L1C6 antibody to monitor the nucleolus, and with L8C4 and YL1/2 antibodies to label the flagella and basal bodies, respectively, and counterstained with DAPI to show the nucleus and kinetoplast. Arrows point to the basal bodies, and arrowheads indicate the flagella. (E) Spindle structures in the control and TLK1 RNAi cells after 24 hours of induction. Cells were stained with the KMX-1 antibody for the spindle and DAPI for DNA. Percentages of cells with the spindles were determined among ~200 1N2K and 2N2K cells in three separate experiments. Bars, 2 μm.

a phosphorylated form of TLK1. As TLK1 is an in vitro substrate of AUK1 (see below), we asked whether this slower-migrating band is TLK1 phosphorylated by AUK1. TLK1 and TLK2 were expressed in AUK1 RNAi cells (Tu et al., 2006), and western blots showed that the slower-migrating band was still present in the TLK1-overexpressing cells (Fig. 2C, lane 4) while AUK1 was knocked down (supplementary material Fig. S2A). It could therefore be the autophosphorylated form of TLK1 in view of its absence from the kinase-dead TLK1-K349R-overexpressing cells (Fig. 2A, lane 4; Fig. 2B, lanes 4 and 5). However, we cannot rule out the possibility that the slower-migrating band is a phosphorylated product of another nuclear protein kinase, because the unphosphorylated TLK1-K349R fails to localize to the nucleus (see below).

We next investigated the subcellular localization of wild-type and kinase-dead TLK1 and TLK2. TLK1 is localized to the nucleus throughout the cell cycle. But it also forms a focal point in the interphase nucleus and two separated focal points at the positions of the two spindle poles during mitosis (Fig. 2D, arrows). When AUK1 is deficient (supplementary material Fig. S2A), the overexpressed TLK1 is still localized to the nucleus, but forms only one focal point (Fig. 2E), possibly owing to a failure in spindle assembly (see Fig. 2E, KMX-1 staining). The TLK1-K349R mutant fails to localize to the

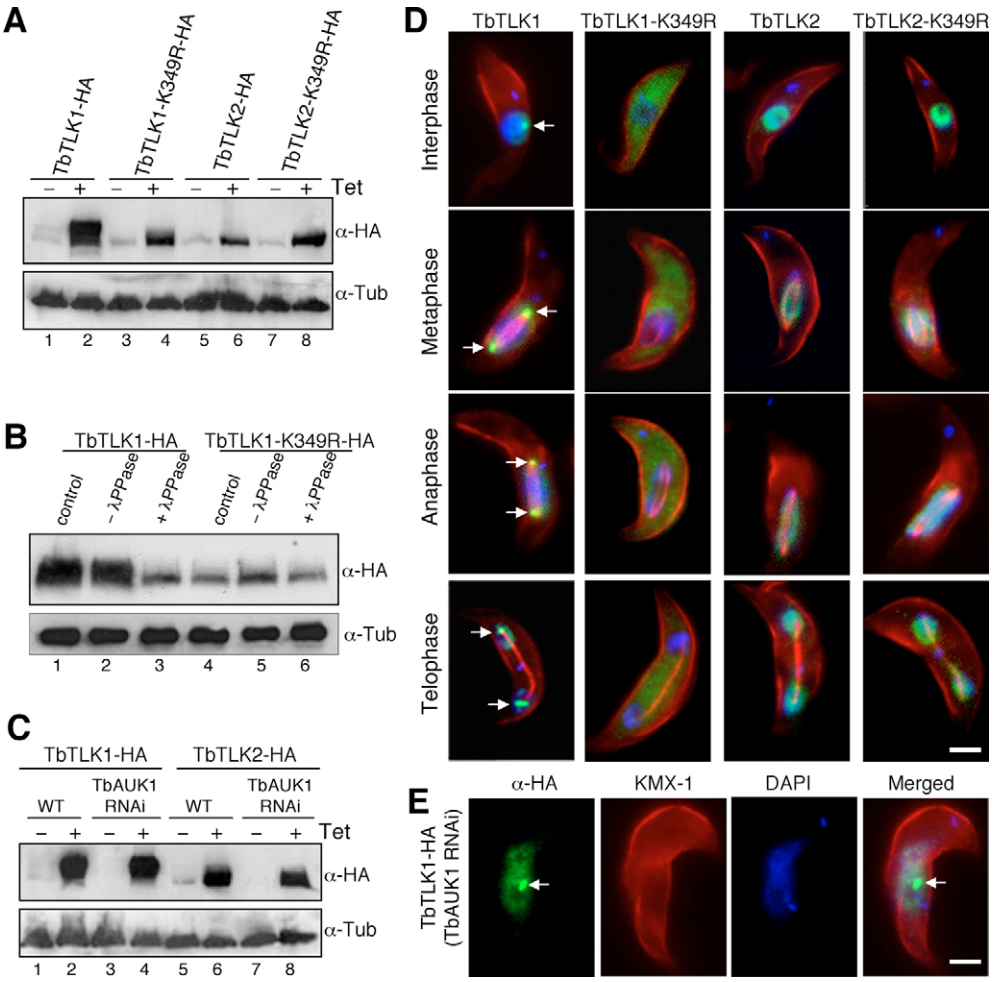

Fig. 2. Subcellular localizations of TLK1 and TLK2 in *T. brucei*. (A) Cells expressing HA-tagged wild-type and kinase-dead TLK1 and TLK2 were boiled in SDS sample solution and immunoblotted with mAb against ('α') HA and mAb against α-tubulin. (B) Cell lysates were dialyzed and incubated with (+ λPPase) or without (– λPPase) lambda protein phosphatase, and blotted with mAb against HA and mAb against α-tubulin. (C) HA-tagged TLK1 and TLK2 were expressed in 29-13 (WT) cells or in cells harboring the AUK1 RNAi construct (AUK1 RNAi). Lysates from un-induced (– Tet) and tetracycline-induced (+ Tet) cells were immunoblotted with mAb against HA and mAb against α-tubulin. (D) Subcellular localizations of wild-type and kinase-dead TLK1 and TLK2. Cells were co-stained with KMX-1 antibody for the spindle (red) and FITC-conjugated antibody against HA for HA-tagged proteins (green) and DAPI for DNA (blue). Arrows point to the brightly stained TLK1 spots, which correspond to the positions of spindle poles. (E) Subcellular localization of TLK1-HA in the AUK1 RNAi cell. The cell was co-stained with KMX-1 and antibodies against HA and counterstained with DAPI. The arrow points to a focal point of TLK1 inside the nucleus. Bars, 2 μm.

nucleus and is dispersed throughout the cytoplasm (Fig. 2D), suggesting that the kinase activity of TLK1 is essential for its nuclear localization. As the mutant is not phosphorylated in vivo (Fig. 2A), TLK1 probably has to be phosphorylated in order to localize to the nucleus. Alternatively, TLK1 has to localize to the nucleus in order to be phosphorylated. TLK2, although unphosphorylated in vivo (Fig. 2A), is localized to the nucleus but not located in foci at the mitotic spindle poles (Fig. 2D). The TLK2-K349R mutant has a similar localization to the nucleus (Fig. 2D), suggesting that localization of TLK2 to the nucleus does not require its kinase function or phosphorylation. It might have a nuclear targeting signal different from that of TLK1 (see below).

Overexpression of wild-type or kinase-dead TLK1 or TLK2 had no apparent effect on cell proliferation or cell cycle progression (supplementary material Fig. S2B,C). The lack of a dominant-negative effect from TLK1-K349R is probably attributable to its localization in the cytoplasm and therefore cannot compete with the functions of wild-type TLK1 in the nucleus (Fig. 2D). The lack of dominant-negative effect from TLK2-K349R, even though it is localized to the nucleus (Fig. 2D), is consistent with the lack of phenotypes from TLK2 RNAi (Fig. 1A,B), which can be attributed to the lack of function in TLK2 rather than its relatively low level of expression.

In order to alleviate the concern that overexpressed proteins might lead to aberrant interactions or localizations, we expressed also HA-tagged TLK1 and TLK2 in situ behind the original promoters of TLK1 and TLK2, respectively, in the same cell lines that overexpress also HA-tagged TLK1 and TLK2 (supplementary material Fig. S3A). Thus, in the absence of tetracycline, the cells express the HA-tagged TLK1 and TLK2 at the endogenous level. When tetracycline is added, however, the cells overexpress these HA-tagged proteins.

We found that the slower-migrating (presumably phosphorylated) band of TLK1 was also detected at the endogenous level but not TLK2 (supplementary material Fig. S3B, compare lanes 1 and 3). Similarly, the tagged TLK1 at its endogenous level is localized to the two spindle poles in addition to the nucleus, whereas TLK2 is only distributed in the nucleus (supplementary material Fig. S3C). A comparison of the levels between the overexpressed and endogenously expressed proteins indicated that TLK1 was only 1.9-fold overexpressed and TLK2 2.6-fold overexpressed (supplementary material Fig. S3B). Given that overexpression does not lead to mislocalization of either protein (compare Fig. 2D with supplementary material Fig. S3C), an approximately twofold overexpression might not cause any detectable aberrant effect.

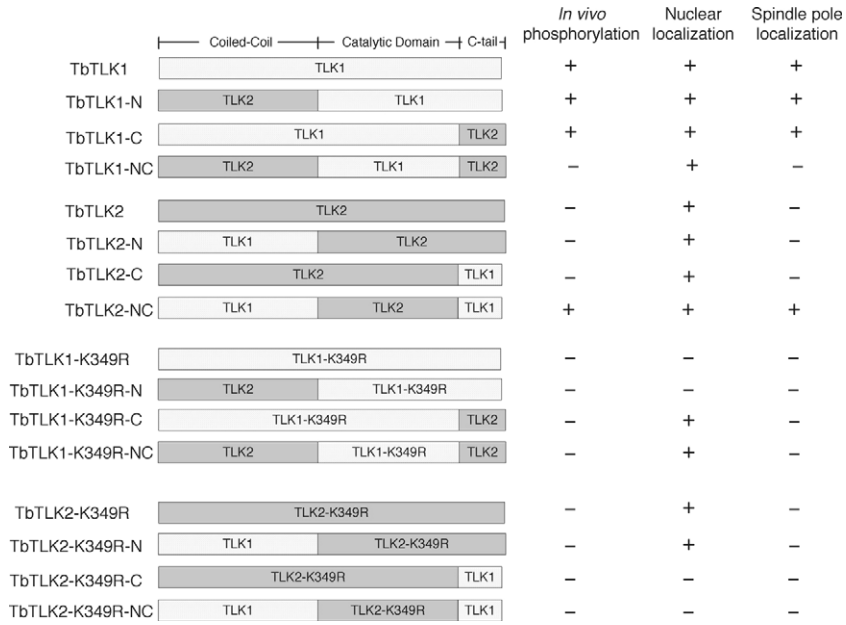

Fig. 3. Domains in TLK1 and TLK2 molecules required for in vivo phosphorylation and subcellular localization. Chimeric proteins constructed by exchanging the N- or the C-termini between TLK1 and TLK2 or their kinase-dead mutants were expressed as HA-tagged proteins in *T. brucei*. Cells were boiled in SDS sampling solution and immunoblotted with mAb against HA for the slower-migrating band as evidence for in vivo phosphorylation. The cells were also fixed, immunostained with mAb against HA and counterstained with DAPI for localizing the HA-tagged chimeric proteins in cells (also see supplementary material Fig. S4).

The domains in TLKs required for in vivo phosphorylation and subcellular localization

The distinctions between TLK1 and TLK2 in their in vivo phosphorylation and subcellular localizations prompted us to examine the differences between the primary structures of the two proteins as a potential basis for the discrepancies. TLK1 and TLK2 are 89% identical in primary sequences, and the most significant differences exist in the N- and C-termini (supplementary material Fig. S1B). The two N-termini contain putative nuclear localization sequences (NLSs) and coiled-coil motifs but differ in the distribution of serine and threonine residues. No known motif has been identified in the C-termini, but the TLK2 C-terminus is much enriched with serine and threonine residues. The 300 amino acids in the N-terminus, which contains the putative NLSs and the coiled-coil motifs, and the 43 amino acids in the C-terminus were exchanged between TLK1 and TLK2 and between their kinase-dead mutants (Fig. 3). The chimeric proteins were tagged with a triple HA epitope and expressed in *T. brucei*. Western blots were performed to look for the slower-migrating band as an indication of in vivo phosphorylation, and immunofluorescence was carried out to determine the subcellular localizations. The results (Fig. 3 and supplementary material Fig. S4) showed that TLK1 could no longer be phosphorylated or localized to the spindle poles when both its N- and C-termini were replaced by those from TLK2, but it could still be imported into the nucleus. TLK1-K349R could not be phosphorylated, imported into the nucleus or localized to the spindle poles unless its C-terminus was that of TLK2, which localized it to the nucleus. TLK2 and TLK2-K349R are both imported into the nucleus but cannot be phosphorylated or localized to the spindle poles unless the catalytic domain of TLK2 was flanked between the N- and C-termini of TLK1. TLK2-K349R can localize to the nucleus as long as its own C-terminus is present.

Thus, the catalytic domains in TLK1 and TLK2 appear to be similar, if not identical. But the domain requires also the flanking of both N- and C-termini from TLK1 in order for the protein to be phosphorylated and localized to the spindle poles. The TLK2 C-terminus can import both TLK1 and TLK2, with or without catalytic activity, into the nucleus. This C-terminus thus probably possesses a nuclear targeting signal. But when the two proteins are catalytically active, this particular C-terminus is not needed for nuclear import and can be replaced by that from TLK1. We are thus unravelling a rather complex structure-function relationship in the two proteins. But it is clear that the structural basis making TLK1 a functional and TLK2 a nonfunctional protein lies in the minute discrepancies between their N- and C-terminal sequences. In addition, the probable correlation between in vivo phosphorylation and spindle pole localization suggests that the phosphorylated TLK1 is probably concentrated in the spindle poles.

TLK1 is a substrate of AUK1

As the phenotype from TLK1 RNAi is similar to that from AUK1 RNAi (Tu et al., 2006), and, as TLK-1 in *C. elegans* interacts with Aurora B kinase (Han et al., 2005), we investigated whether the two TLK forms interact with AUK1. GST-AUK1 was capable of pulling down [^{35}S]-labeled TLK1 or TLK2 (Fig. 4A, lane 2), whereas both GST-TLK1 and GST-TLK2 could pull down TLK1 and TLK2 (Fig. 4A, lanes 3 and 4). In the yeast two-hybrid assays, however, only TLK1 could oligomerize and interact with AUK1 (Fig. 4B), despite the fact that both TLK1 and TLK2 proteins were expressed with the latter present at a somewhat higher level (supplementary material Fig. S5). To test whether TLK1 interacts with AUK1 in *T. brucei*, HA-tagged TLK1 or TLK2 was expressed in *T. brucei* cells harboring PTP-tagged AUK1 (Schimanski et al., 2005). Immunoprecipitation was performed with a polyclonal antibody (pAb) against HA to bring down TLK1 or TLK2. The immunoprecipitates were then blotted with a monoclonal antibody (mAb) against protein C that recognizes AUK1-PTP. AUK1 was co-immunoprecipitated with TLK1 but not with TLK2 (Fig. 4C, upper panel). In a reciprocal scheme, only TLK1 was brought

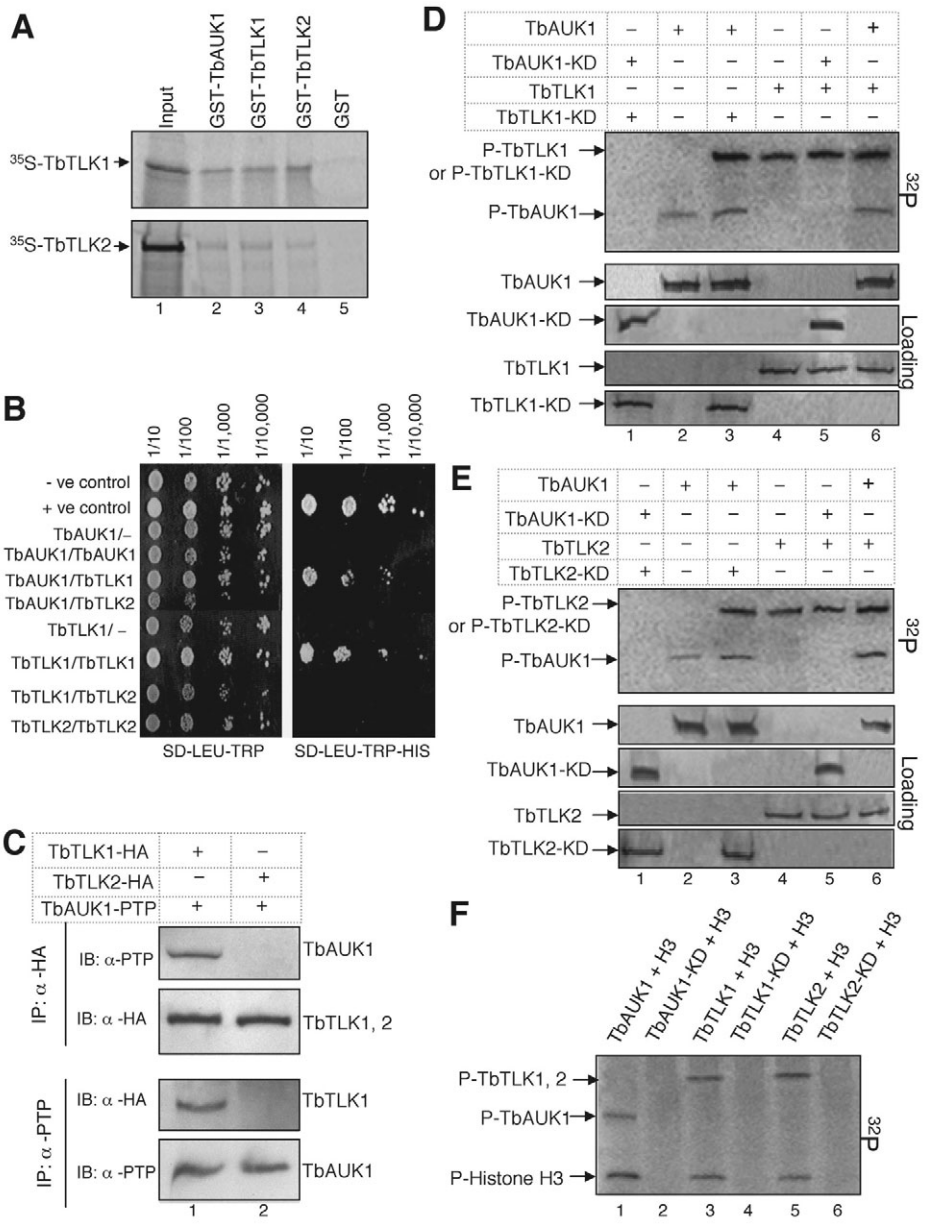

Fig. 4. TLK1 interacts with AUK1 in vivo and is phosphorylated by AUK1 in vitro. (A) In vitro GST pulldown assays. (B) Yeast two-hybrid assays. (C) Co-immunoprecipitation of TLK1 with AUK1 from *T. brucei* cells. TLK1-HA and TLK2-HA were each expressed in *T. brucei* cells harboring endogenous AUK1 tagged with a PTP epitope. Cells were lysed in IP lysis buffer and immunoprecipitation was performed with a pAb against HA, followed by immunoblotting with a mAb against HA or a mAb against protein C that recognizes the PTP epitope. Reciprocally, immunoprecipitation was carried out with a mAb against protein C to bring down AUK1-PTP and then blotted with a pAb against HA and a mAb against protein C, respectively. Note that no slower-migrating protein band was observed in the TLK1-HA lane because the cells were lysed in IP lysis buffer and incubated on ice for 30 minutes before boiling in SDS sample solution. The incubation might have dephosphorylated the phosphorylated protein. (D,E) AUK1 phosphorylates TLK1 (D) and TLK2 (E) in vitro. The wild-type and kinase-dead (KD) mutants of AUK1, TLK1 and TLK2 were expressed as GST-fusion proteins in *E. coli*, purified and mixed in various combinations for kinase assays. (F) In vitro phosphorylation of histone H3 by the purified wild-type and kinase-dead mutant (KD) TLK1, TLK2 and AUK1 recombinant proteins.

down with AUK1 (Fig. 4C, lower panel). Thus, only TLK1 interacts with AUK1 in vivo. (It should be pointed out that the cell lysates in this experiment were kept on ice for 30 minutes before immunoprecipitation, whereas, in the previously described western blots (Fig. 2A-C), the lysates were immediately boiled in SDS sampling solution. The incubation might result in dephosphorylation of the phosphorylated proteins in the former experiment.)

We next tested whether AUK1 phosphorylates TLK1 or TLK2 or vice versa. An in vitro kinase assay was performed with purified recombinant wild-type and kinase-dead TLK1, TLK2 and AUK1 in different combinations. All the wild-type proteins were catalytically active and capable of autophosphorylation, whereas all the kinase-dead mutants were catalytically inactive but capable of being phosphorylated by an active kinase (Fig. 4D,E). AUK1 can phosphorylate TLK1 and TLK2 (Fig. 4D,E, lane 3), but TLK1 and TLK2 are incapable of phosphorylating AUK1 (Fig. 4D,E, lane 5), suggesting that TLK1 and TLK2 are both substrates of AUK1 in vitro. As TLK1 interacts with AUK1 in vivo (Fig. 4C), it could also be the in vivo substrate of AUK1. Given that *C. elegans* TLK-1 is a substrate activator of Aurora B kinase (Han et al., 2005), TLK1 could also be a substrate activator of AUK1, but we were unable to test this owing to the lack of specific substrates of AUK1 at present.

TLKs phosphorylate histone H3 in vitro

Histone H3 can be phosphorylated in vitro by human TLK1B (Li et al., 2001) and *Arabidopsis* Tousled kinase (Ehsan et al., 2004), but not by TLK-1 from *C. elegans* (Han et al., 2005), suggesting that, in some systems, histone H3 is not a substrate of Tousled-like kinase. We assayed TLK1 and TLK2 for activity in phosphorylating histone H3 in vitro and found both active (Fig. 4F, lanes 3 and 5), suggesting that TLK1 might be

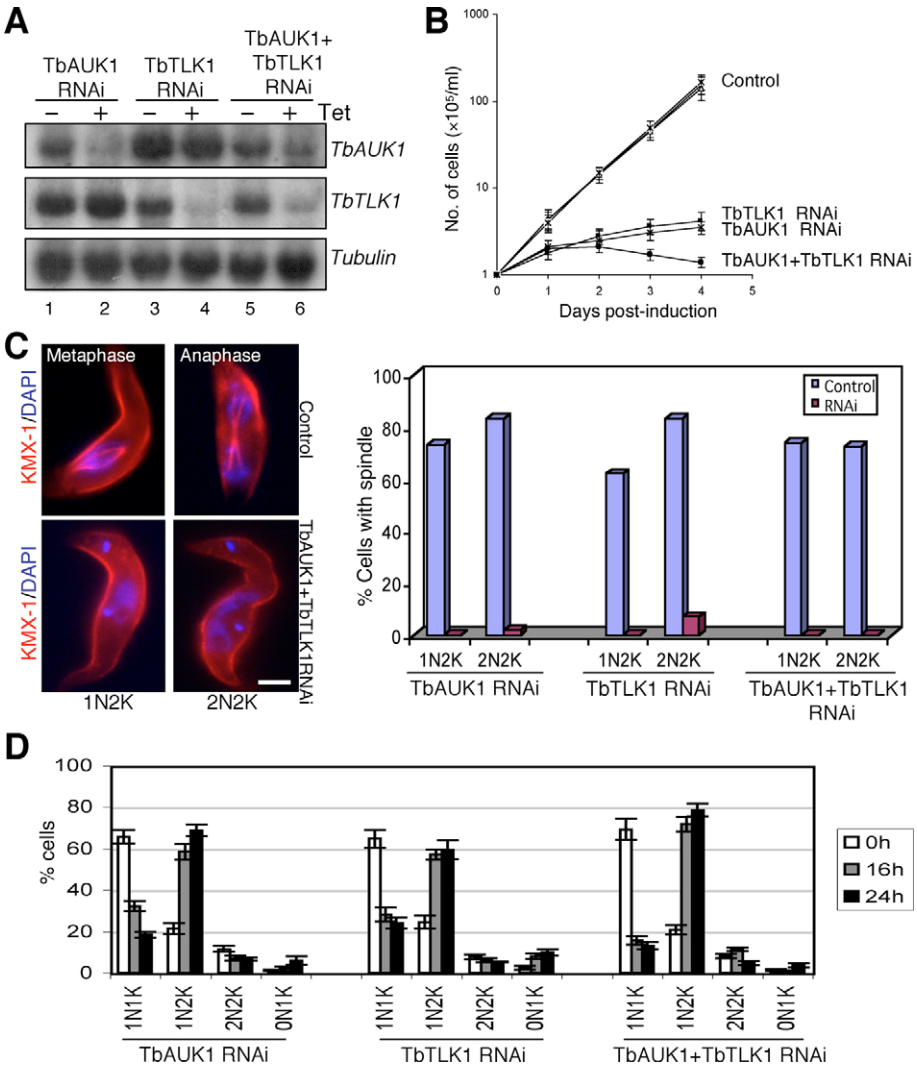

Fig. 5. Effect of TLK1 and AUK1 double knockdown on spindle formation and chromosome segregation in *T. brucei*. The effect of TLK1 and AUK1 double knockdown was compared with those from TLK1 or AUK1 single knockdowns. (A) Northern blot with AUK1, the 5′-UTR of TLK1 or α-tubulin as probes. (B) Comparison of growth of TLK1 and AUK1 double knockdown cells with those of the single knockdown cells. (C) Effect of AUK1 and TLK1 double knockdown on spindle formation. The 1N2K and 2N2K cells were labeled with the KMX-1 antibody (left panel) and examined for spindle structures. The data are presented as the mean percentage±s.e. of ~200 cells counted from three independent experiments (right panel). (D) Double knockdowns of TLK1 and AUK1 enriched the 1N2K cells by 10-20% in comparison with those from the single knockdowns. The data are presented as the mean percentage±s.e. of ~200 cells from three separate experiments. Bar, 2 μm.

involved in modifying the chromatin structure through phosphorylating histone H3 in *T. brucei*.

Functional interaction between TLK1 and AUK1 in *T. brucei*

To test the potential functional interaction between TLK1 and AUK1 in *T. brucei*, AUK1 and TLK1 double knockdown led to the reduction of respective mRNA levels by ~80-90% (Fig. 5A, lanes 2, 4 and 6). The double knockdown inhibited cell growth more extensively than individual knockdown of AUK1 or TLK1 (Fig. 5B), suggesting an additive effect of double knockdown. Furthermore, AUK1 and TLK1 double knockdown resulted in a complete loss of spindle structure in all the 1N2K and 2N2K cells (Fig. 5C) and further enriched the 1N2K cells by another 10-20% after 24 hours (Fig. 5D). These results, together with the in vivo interaction between TLK1 and AUK1, suggest that TLK1 and AUK1 act together to regulate spindle assembly and chromosome segregation.

TLK1 phosphorylates the chromatin assembly factors Asf1A and Asf1B in regulating S-phase progression in *T. brucei*

The chromatin assembly factor Asf1, a histone H3/H4 chaperone protein involved in DNA replication and DNA repair, has been identified as the substrate of TLK in human, *Drosophila* and *Arabidopsis* (Silljé and Nigg, 2001; Carrera et al., 2003; Ehsan et al., 2004). As TLK1 knockdown led to an S-phase delay in *T. brucei*, we asked whether any Asf1 homolog is present in *T. brucei* and whether it is the substrate of TLK1. Two Asf1 homologs (Tb927.1.630 and Tb927.8.5890), designated Asf1A and Asf1B, were identified, and both exhibited significant identities to Asf1 proteins from *S. cerevisiae*, *Drosophila* and human (supplementary material Fig. S6). To test whether Asf1A and/or Asf1B play a role in S-phase progression in *T. brucei*, the individual, or both, genes were knocked down by RNAi. The mRNA level of Asf1A was reduced ~87% in the single RNAi cells and ~95% in the double RNAi cells. The mRNA level of Asf1B was reduced ~95% in both the single and double RNAi cells (Fig. 6A). Knockdown of Asf1A and Asf1B led to a growth depression of ~64% and ~43%, respectively, whereas the double knockdown exhibited a growth depression of ~70% after 4 days of RNAi (Fig. 6B). Flow cytometry showed that knockdown of Asf1A, Asf1B or both all led to a gradual enrichment of S-phase cells (Fig. 6C). Specifically, the S-phase cells increased from ~15% to ~55% in Asf1A knockdown, from ~18% to ~36% in Asf1B

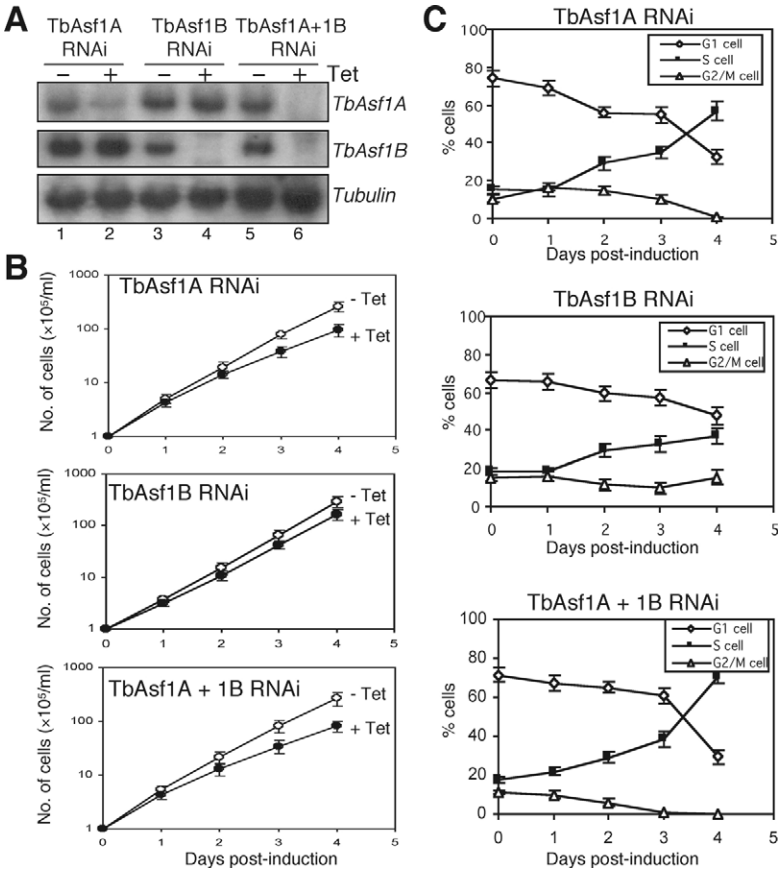

Fig. 6. The two Asf1 homologs are required for promoting S-phase progression in *T. brucei*. (A) Single and double knockdowns of Asf1A and Asf1B in *T. brucei*. A northern blot was performed with Asf1A, Asf1B or α-tubulin sequences as probes. (B) Effects of the single and double knockdowns on cell growth. (C) Effects of the single and double knockdowns on cell cycle progression. The percentages of cells at G_1-, S- and G_2/M-phases were determined and plotted against time post induction.

knockdown, and from ~18% to ~70% in the double knockdown after 4 days of RNAi (Fig. 6C), suggesting that both Asf1A and Asf1B might be required for S-phase progression in *T. brucei*.

To test whether the two Asf1 proteins interact with TLK1 or TLK2, we performed in vitro pulldowns and found that both TLK1 and TLK2 can interact with Asf1A and Asf1B in vitro (Fig. 7A, lanes 2 and 3). To test the in vivo interactions among these proteins, TLK1 and TLK2 were tagged with a Ty epitope (Bastin et al., 1996) and expressed in *T. brucei* cells that expressed also Asf1A-3HA or Asf1B-3HA (Fig. 7B). Immunoprecipitation was performed with a mAb against Ty to precipitate TLK1 or TLK2 (Fig. 7B) and followed by immunoblotting with a pAb against HA that recognizes Asf1A-HA and Asf1B-HA. Both Asf1A and Asf1B co-immunoprecipitated with TLK1 (Fig. 7B, lanes 1 and 2) but not with TLK2 (Fig. 7B, lanes 3 and 4). Conversely, TLK1, but not TLK2, co-immunoprecipitated with Asf1A and Asf1B (Fig. 7B, lanes 1 and 2). These results suggest that only TLK1 interacts with Asf1A and Asf1B in vivo. Kinase assays showed that both Asf1A and Asf1B could be phosphorylated by either TLK1 or TLK2 in vitro (Fig. 7C, lanes 1 and 2, 5 and 6). In view of the in vivo interactions between TLK1 and the two Asf1 proteins (Fig. 7B), both Asf1A and Asf1B are probably the in vivo substrates of TLK1.

To test the potential functional interaction between TLK1 and the two Asf1 proteins, a TLK1, Asf1A and Asf1B triple knockdown was performed, and the outcome was compared with the results from Asf1A and Asf1B double knockdown and TLK1 single knockdown (Fig. 7D,E). Significant reduction (~95%) of the respective mRNAs was achieved within 24 hours of RNAi (Fig. 7D). Flow cytometry showed that ,while double knockdown of Asf1 proteins enhanced the S-phase cell population only slightly within the initial 24 hours (Fig. 7E and see also Fig. 6C), knockdown of TLK1 led to a significant increase of S-phase cells from ~30 to ~50% after 16 hours but dropped to ~25% after 24 hours of RNAi induction (Fig. 7E; also see Fig. 1B). However, TLK1, Asf1A and Asf1B triple knockdown resulted in an increase in the S-phase cells from ~30% to ~60% after 8 hours of RNAi, which was maintained at a similar level up to 16 hours and then dropped to ~40% after 24 hours (Fig. 7E). This further enrichment of S-phase cells by the triple knockdown suggests that the three proteins might function in the same pathway in promoting S-phase progression, which most likely occurs through phosphorylation of Asf1A and Asf1B by TLK1.

Discussion

Tousled-like kinases in a unicellular eukaryote

In this study, we identified and characterized two TLK homologs (TLK1 and TLK2) in the microbial eukaryote *T. brucei*. Although they are 89% identical in their amino acid sequences and share considerable similarities in the putative nuclear localization sequences, coiled-coil motifs and the catalytic domain (supplementary material Fig. S1), several lines of evidence indicate that only TLK1 plays an essential role in regulating chromosome segregation and promoting S-phase progression. First, RNAi of TLK1, but not TLK2, resulted in growth inhibition, transient enrichment of S-phase cells and massive accumulation of G_2/M-phase cells (Fig. 1A,B). Second, only TLK1 is phosphorylated in vivo and localized to the spindle poles during mitosis (Fig. 2). Finally, TLK1, but not TLK2, interacts with AUK1 and the chromatin assembly factors Asf1A and Asf1B in vivo (Figs 4 and 7). Despite the lack of any apparent in vivo functions, however, TLK2 is expressed, albeit at a lower level than TLK1 (Fig. 1A), and possesses in vitro activities similar to those of TLK1. Among the in vitro assays, TLK2 possesses kinase activity on histone H3 (Fig. 4F), exhibits weak interactions with AUK1, Asf1A and Asf1B (Figs 4 and 7), can be phosphorylated by AUK1 (Fig. 4E) and is capable of phosphorylating Asf1A and Asf1B (Fig. 7C). These in vitro features should make TLK2 a bona fide structural and functional homolog of TLK. Its failure in vivo is most likely attributed to its N- and C-termini that have to be replaced with those from TLK1 in order to be phosphorylated in vivo and localized to the spindle poles, the two essential features for TLK1 to function in vivo. Two TLK

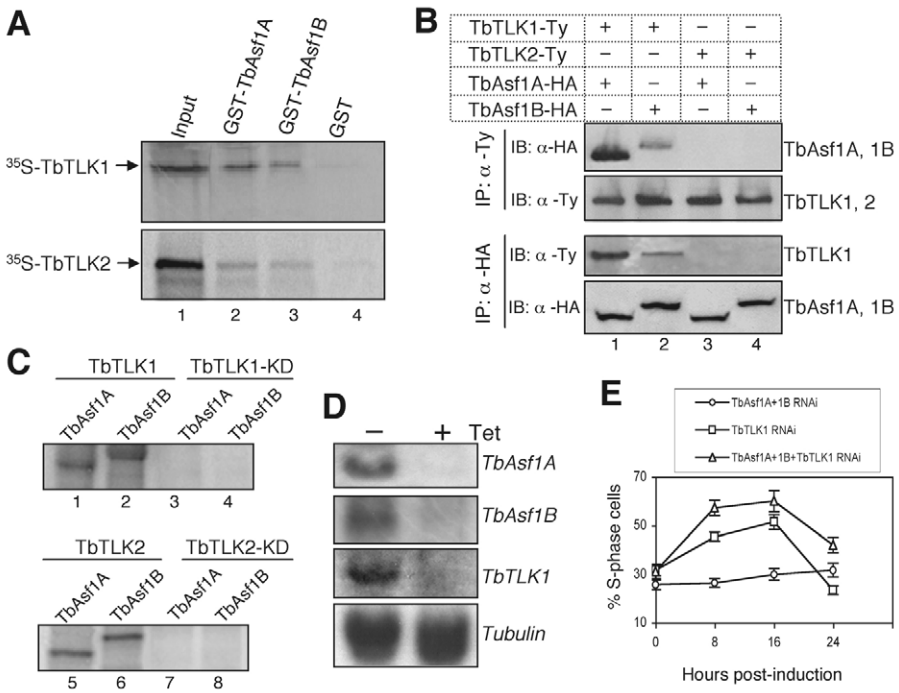

Fig. 7. TLK1, Asf1A and Asf1B act in the same pathway of promoting S-phase progression in *T. brucei*. (A) In vitro GST pulldown assays. (B) Co-immunoprecipitation of TLK1 with Asf1A or Asf1B from *T. brucei*. TLK1-Ty and TLK2-Ty were coexpressed with Asf1A-HA and Asf1B-HA in *T. brucei*. Immunoprecipitation was performed with a mAb against Ty (BB2) and the immunoprecipitates on western blots were immunostained with a pAb against HA and a mAb against Ty, respectively. Conversely, immunoprecipitation was performed with the pAb against HA followed by immunoblotting with the mAb against Ty and the mAb against HA, respectively. Note that no slower-migrating band was observed in the TLK1-HA lane owing to cell lysis in the IP lysis buffer for 30 minutes on ice. (C) In vitro phosphorylation of Asf1A and Asf1B by TLK1 and TLK2. Wild-type and kinase-dead (KD) mutants of TLK1 and TLK2, as well as Asf1A and Asf1B, were expressed as GST fusion proteins in *E. coli*, purified and used for kinase assays in the presence of [γ-^{32}P]ATP. (D) Triple knockdowns of Asf1A, Asf1B and TLK1 in *T. brucei*. A northern blot was performed with Asf1A, Asf1B, the 5′-UTR of TLK1 or α-tubulin as probes. (E) Effect of Asf1A, Asf1B and TLK1 triple knockdowns on S-phase progression. The percentages of S-phase cells during RNAi induction were compared among the cells with triple knockdowns, the double knockdowns of Asf1A and Asf1B, and the TLK1 single knockdown. The data represent three independent RNAi induction experiments using the same cell lines.

homologs, TLK1 and TLK2, were also identified in human (Silljé et al., 1999), and they share 84% identity with each other and up to 50% similarity with the Tousled kinase from *Arabidopsis*. They are equally functional in vivo, with their kinase activities regulated by cell-cycle-dependent phosphorylation and displaying maximal activities during S phase (Silljé et al., 1999). The biological significance behind the differences between TLK1 and TLK2 remains largely unclear for the moment.

TLK1 is phosphorylated by AUK1, the single Aurora kinase in *T. brucei*, and is required for proper chromosome segregation in *T. brucei* (Figs 1, 4 and 5), which resembles the function of TLK-1 in *C. elegans* (Han et al., 2005). TLK1 phosphorylates histone H3 and the two Asf1 homologs from *T. brucei*, and its deficiency leads to a retarded S-phase progression (Figs 1, 4 and 7). These functions have also been observed in the TLKs from human (Silljé and Nigg, 2001; Li et al., 2001) and *Drosophila* cells (Carrera et al., 2003), suggesting an important role of TLK1 in controlling DNA replication and chromosome segregation in *T. brucei*. There are, however, two additional features of TLK1 that have not been observed among the other TLKs from multicellular organisms. These are the focalized localization of TLK1 at the spindle poles during mitosis and its involvement in spindle assembly in *T. brucei.*

TLK1, centrosomes and spindle assembly

One of the most intriguing observations from this study is the targeting of TLK1 to the spindle poles during mitosis, resulting in two brightly stained spots at the two opposite ends of the spindle in immunofluorescence assays (Fig. 2D). This targeting to the spindle poles probably requires phosphorylation of TLK1, which is not catalyzed by AUK1 but by either autophosphorylation or another, currently non-identified, nuclear protein kinase. TLKs are known to localize to the nucleus in animals and plant cells, but have not been found in the centrosomes/spindle pole bodies. However, strong mitotic centrosome staining was observed in addition to nuclear staining when *C. elegans* embryos were stained with antibodies against the AIR-2-phosphorylated TLK-1 (Han et al., 2005). Such centrosome staining could not be obtained with the non-phospho-TLK-1 antibody (Han et al., 2003), and was not eliminated from the *tlk-1* (RNAi) or *air-2* (RNAi) embryos, which led to the tentative conclusion that the centrosome staining in *C. elegans* is probably due to the recognition of an antigen other than phospho-TLK-1 (Han et al., 2005). It is thus still uncertain whether TLK-1 is localized to centrosomes in *C. elegans*.

The centrosome is the main microtubule-organizing center as well as a regulator of cell cycle progression in metazoa. The unicellular *T. brucei*, however, appears to lack the typical structural equivalents of the spindle pole bodies/centrosomes commonly identified in yeasts and metazoa (Ogbadoyi et al., 2000). Instead, trypanosomes possess typical centrioles in the form of basal bodies at the base of flagella associated with the kinetoplasts across the mitochondrial membrane. Homologs of centrin, a highly conserved component of the centrosome, are localized to the basal and Golgi bodies and control their duplications in *T. brucei* (He et al., 2005). The segregation of centrioles/basal bodies is required for kinetoplast segregation and cytokinesis (Robinson and Gull, 1991) but not mitosis in the procyclic form of *T. brucei* (Das et al., 1994). However, *T.*

brucei does possess the spindle-organizing center that is a ring-like structure of ~70 nm in diameter located at the two spindle poles (Ogbadoyi et al., 2000) and consists of γ-tubulin, a conserved component of the microtubule-organizing center and an essential molecule for nucleation and stabilization of the spindles (Scott et al., 1997; He et al., 2005). Our finding that TLK1 is localized to the mitotic spindle poles and required for spindle assembly implies that it might be involved directly in regulating the organization of spindle microtubules. Recently, in a whole-genome RNAi screen of *Drosophila* S2 cells for genes required in spindle assembly, the TLK homolog appeared to be involved in targeting of γ-tubulin to the centrosomes, suggesting a role for TLK in spindle assembly in *Drosophila* (Goshima et al., 2007). However, as TLK1 regulates chromosome segregation in cooperation with AUK1 (Fig. 5), which is also required for spindle assembly in *T. brucei* (Tu et al., 2006; Li and Wang, 2006), TLK1 might regulate spindle assembly by being a substrate activator of AUK1, as was observed in *C. elegans* (Han et al., 2005).

TLK1, the chromatin assembly factors Asf1A, Asf1B and DNA replication

Asf1 proteins are the in vitro substrates of TLKs from human, *Drosophila*, *Arabidopsis* and *C. elegans* (Silljé and Nigg, 2001; Carrera et al., 2003; Ehsan et al., 2004; Han et al., 2005). They function as a histone H3/H4 chaperone that facilitates the DNA replication-dependent chromatin assembly into nucleosomes (Tyler et al., 1999). They were also identified in a complex with the DNA damage checkpoint kinase Rad53 and probably function in chromatin assembly during DNA repair (Emili et al., 2001; Hu et al., 2001). Two Asf1 homologs, Asf1a and Asf1b, were identified in human cells (Silljé and Nigg, 2001) and are phosphorylated by the two TLKs in vivo during regulation of chromatin assembly during DNA replication. The single Asf1 homolog in *Drosophila* was also phosphorylated by the single TLK, and they act together to regulate chromatin structure and endo-replication (Carrera et al., 2003).

In *T. brucei*, TLK1 RNAi led to a slow-down of S-phase progression, whereas knockdown of Asf1A or Asf1B resulted in a gradual accumulation of S-phase cells. There was an additive effect on the S-phase defect when all three proteins were depleted simultaneously (Fig. 7E), suggesting that they act in the same pathway in promoting DNA replication. This conclusion was further supported by the in vivo binding and in vitro phosphorylation of both Asf1 proteins by TLK1. It would seem that a pathway for regulating chromatin assembly identified in metazoa also functions in *T. brucei*.

Overall, our results showed that only one of the two TLK homologs in *T. brucei*, TLK1, is required for spindle assembly and chromosome segregation. The mechanism of action in this aspect is most likely to involve being a substrate activator of the Aurora kinase AUK1. TLK1 regulates also DNA replication by phosphorylating (and perhaps activating) the two histone chaperones Asf1A and Asf1B in *T. brucei*. TLK1 and its multiple functions can find their parallels in metazoa and plants, whereas the presence of two homologs of TLK and two homologs of Asf1 in this unicellular organism is paralleled only in human cells. The potential biological significance behind these features should be further explored.

Materials and Methods

Trypanosome cell cultures and RNAi

The procyclic form of *T. brucei* strain 29-13 (Wirtz et al., 1999) was cultured at 26°C in Cunningham's medium supplemented with 10% fetal bovine serum (Atlanta Biological) and 15 μg/ml G418 and 50 μg/ml hygromycin B.

The 3′-untranslated regions (UTRs) of TLK1 and TLK2, which bear the most significant sequence divergences, were cloned into the pZJM vector (Wang et al., 2000) for RNAi. An AUK1 + TLK1 double RNAi construct was made by ligating a fragment of AUK1 with the 3′-UTR sequence of TLK1 into the pZJM vector. For Asf1A and Asf1B single and double knockdowns, fragments of Asf1A and Asf1B were cloned into pZJM either individually or ligated together. For TLK1, Asf1A and Asf1B triple knockdown, the 3′-UTR of TLK1 was cloned into the Asf1A plus Asf1B double RNAi construct. Transfection of the procyclic form was performed as described previously [41]. The transfectants were selected under 2.5 μg/ml phleomycin and cloned on agarose plates (Carruthers and Cross, 1992). RNAi was induced by adding 1.0 μg/ml tetracycline.

Northern blotting

Total RNA was denatured and blotted onto nitrocellulose membranes. Northern blotting was performed as described previously (Li and Wang, 2002). The same blot was used to hybridize with different probes.

Flow cytometry

Flow cytometry analysis of trypanosome cells stained with propidium iodide was carried out as described previously (Li and Wang, 2003). Percentages of cells in each phase of the cell cycle (G_1, S and G_2/M) were determined by ModFit LT V3.0 software (BD Biosciences).

Immunofluorescence microscopy

The following primary antibodies were used: YL1/2 for the basal body (Kilmartin et al., 1982; Sherwin et al., 1987; Sasse and Gull, 1988; Chemicon); L8C4 for the flagellum; L1C6 for the nucleolus; KMX-1 for the spindles (Sasse and Gull, 1988); and FITC-conjugated mAb against HA (Sigma-Aldrich) for HA-tagged proteins. For L1C6 single staining and YL1/2 and L8C4 double staining, cells were fixed with cold methanol. For KMX-1 and anti-HA double staining, cells were fixed with 4% paraformaldehyde. Immunostaining was performed as described previously (Li and Wang, 2006).

Expression of epitope-tagged proteins in *T. brucei* and tagging of endogenous proteins

Genes encoding TLK1, TLK2, Asf1A and Asf1B were each tagged with a triple HA epitope at the C-terminus, cloned into a pLew100-Phleo vector (Wirtz et al., 1999) and transfected into the 29-13 cell line. Stable transfectants were selected under 2.5 μg/ml phleomycin. For expression in AUK1 RNAi cells, HA-tagged TLK1 and TLK2 were cloned into a pLew100-Pac vector for transfection, and the stable transfectants were selected under 1.0 μg/ml puromycin. For immunoprecipitation, The gene encoding AUK1 was cloned into the pC-PTP-Neo vector (Schimanski et al., 2005) and transfected into the 427 cell line, which placed the PTP-tagged AUK1 under its endogenous promoter. Stable transfectants were then selected under 40 μg/ml G418. Subsequently, HA-tagged TLK1 or TLK2 was cloned into the pTSO-Pac vector (Sommer et al., 1995; Li et al., 2002) and transfected into the cells containing AUK1-PTP. For coexpression of TLK1 or TLK2 with Asf1A or Asf1B, the genes encoding TLK1 or TLK2 with a C-terminal Ty-epitope (Bastin et al., 1996) were cloned into pLew100-Pac and transfected into the cells harboring pLew100-Asf1A-3HA-Phleo or pLew100-Asf1B-3HA-Phleo. Stable transfectants were selected under 2.5 μg/ml phleomycin and 1.0 μg/ml puromycin.

Endogenous TLK1 and TLK2 were each tagged at the C-terminus with the HA epitope in the respective overexpressing cell lines using the integrating constructs pC-TLK1-HA-bla and pC-TLK2-HA-bla, which were modified from the PTP construct pC-PTP-Neo (Schimanski et al., 2005) by replacing the PTP module with the HA epitope and the neomycin resistance with blasticidin resistance. Transfectants were selected under 10 μg/ml blasticidin. The transfectants express both the HA-tagged endogenous protein and the tetracycline-inducible ectopically expressed HA-tagged protein, thus allowing direct comparison between the levels of overexpressed and endogenous proteins from the same cells.

Domain swaps between TLK1 and TLK2

To make the chimeric proteins of TLK1 and TLK2, the N-terminal coiled-coil domain (residues 1-300) and/or the C-terminal domain (residues 608-650) of wild-type or kinase-dead (K349R) TLK1 and TLK2 were swapped and cloned into the pLew100-3HA vector. The chimeric proteins were expressed in the procyclic form cells and examined by western blotting and immunofluorescence microscopy with a mAb against HA.

GST pulldown and in vitro kinase assays

Full-length coding sequences of TLK1, TLK1-K349R, TLK2, TLK2-K349R, AUK1, AUK1-K58R, Asf1A, Asf1B and histone H3 were cloned into pGEM-4T-3

vector (Amersham), expressed in *Escherichia coli* BL21 cells and purified through glutathione sepharose 4B beads. For GST pulldown experiments, [^{35}S]-labeled TLK1 or TLK2, produced from the TNT in vitro transcription/translation system (Promega), was incubated with the purified GST-AUK1, GST-TLK1, GST-TLK2, GST-Asf1A, GST-Asf1B or GST, each bound to the glutathione sepharose beads, in the binding buffer (20 mM HEPES, pH 7.6, 150 mM KCl, 2.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 1 mM DTT, 5 mg/ml BSA and 1 mM ATP) and washed six times with the wash buffer (20 mM HEPES, pH 7.6, 150 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT and 1% Triton X-100). The beads were then boiled in SDS sampling buffer, and the supernatant analyzed by SDS-PAGE. The dried gels were exposed to phosphorimager. The intensities of the bands were determined, compared with those of the input bands between TLK1 and TLK2. For the in vitro kinase assay, purified recombinant proteins were mixed together in different combinations in the kinase buffer (10 mM HEPES, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) containing 1 μCi [γ-^{32}P]ATP (Amersham) and incubated at room temperature for 60 minutes. Proteins were separated by SDS-PAGE, and the dried gels were exposed to phosphorimager.

Yeast two-hybrid assay

Full-length genes encoding TLK1, TLK2 and AUK1 were cloned into the pGADT7 vector for expression of proteins fused to the Gal4 activation domain (prey) or into the pGBKT7 vector for expression of proteins fused to the Gal4 binding domain (bait) (Clontech). Yeast strains AH109 (mating type **a**) and Y187 (mating type α) were transformed with the prey or the bait plasmid, respectively, and mated in YPDA media for 24 hours at 30°C, followed by plating on SD-Leu-Trp plates, selecting for the presence of both plasmids. Each mated strain was then spotted in four tenfold serial dilutions onto SD-Leu-Trp and SD-His-Leu-Trp plates, with the latter selecting for clones carrying the bait protein and the prey protein that interact with each other.

Immunoprecipitation, immunoblotting and lambda protein phosphatase treatment

Cells were incubated in the lysis buffer (25 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1% Nonidet P-40, 1 mM dithiothreitol and protease inhibitor cocktail) for 30 minutes on ice and cleared by centrifugation. The lysate was incubated with a mAb against Ty (BB2) or a pAb against HA at 4°C for 1 hour and then precipitated with protein G sepharose beads overnight. For immunoprecipitation with the mAb against protein C, cells were lysed in the buffer containing 150 mM KCl, 20 mM Tris-HCl, pH 7.6, 3 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.1% Tween20 and protease inhibitor. The cleared lysate was incubated with anti-protein C affinity matrix (Roche) at 4°C for 2 hours. The immunoprecipitates thus collected were fractionated by SDS-PAGE, transferred onto PVDF membranes and immunoblotted with mAb against Ty, pAb against HA or mAb against protein C. Immunoblotting was performed as described previously (Li and Wang, 2002). $CaCl_2$ (1 mM) was always included in the blotting and wash buffers for the immunoblotting with mAb against protein C. Cells were boiled in SDS sampling buffer, and the supernatants were dialyzed to remove SDS. The latter were then incubated with lambda protein phosphatase (Upstate) at 37°C for 30 minutes, blotted onto a PVDF membrane and stained with antibody against HA.

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