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Activation of *Xenopus* Chk1 by mutagenesis of threonine-377

Sophie X. Wang, William G. Dunphy*

Division of Biology 216-76, Howard Hughes Medical Institute, California Institute of Technology, 1200 East California Blvd., Pasadena, CA 91125, USA

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Abstract ***Xenopus*** **Chk1 (Xchk1) is required for the checkpoint-associated delay of the cell cycle in frog egg extracts containing unreplicated or UV-damaged DNA. Phosphorylation of Xchk1 at multiple sites in the SQ/TQ domain (residues 314–366) in response to unreplicated or UV-damaged DNA results in elevation of its kinase activity. We have found that mutagenesis of Thr-377 in the conserved Thr–Arg–Phe (TRF) motif of Xchk1 also leads to a substantial increase in kinase activity. Thr-377 does not appear to be a site of phosphorylation in Xchk1. These findings suggest that Thr-377 may play a role in suppressing the activity of Xchk1.** **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Chk1; Phosphorylation; Mitosis; Cdc25; Checkpoint control

1. Introduction

In eukaryotic cells, the entry into mitosis is controlled by maturation or M-phase promoting factor (MPF). In vertebrates, MPF consists of the protein kinase Cdc2, a B-type cyclin, and the Suc1/Cks protein [1,2]. During interphase, the activity of MPF is normally suppressed by inhibitory phosphorylations on the Thr-14 and Tyr-15 residues of Cdc2. These inhibitory phosphorylations are carried out by the kinases Myt1 and Wee1. When the conditions are appropriate for mitosis, the dual-specificity phosphatase Cdc25 dephosphorylates these key sites and thereby generates the active form of MPF.

Consistent with its crucial role in cell division, the activity of MPF is subject to stringent regulation during the cell cycle [2]. For example, MPF cannot become active, and thus cell division cannot occur, if the genomic DNA has not been replicated properly or has suffered damage. These regulatory processes have been designated the DNA replication and DNA damage checkpoints, respectively [3–5]. Largely from genetic studies in budding and fission yeast, it is understood that these checkpoint pathways consist of sensor proteins that detect unreplicated/damaged DNA and effector proteins that regulate downstream targets such as cell cycle control factors. In the case of fission yeast, for example, the kinases Chk1 and Cds1 are important mediators in these regulatory circuits [4,5]. Both Chk1 and Cds1 possess close structural homologs in budding yeast, humans, *Xenopus,* and other species [6–12]. A variety of studies have indicated that Chk1 and Cds1 negatively regulate Cdc25 by phosphorylating it on one or more 14-3-3 binding sites, depending on the organism [6,8,13–16].

We have been studying the functional properties of *Xenopus* Chk1 (Xchk1) by using extracts from *Xenopus* eggs. Previously, our laboratory demonstrated that Xchk1 is essential for an appropriate cell cycle delay in response to unreplicated or UV-damaged DNA [8]. Xchk1 becomes highly phosphorylated in the presence of unreplicated or UV-damaged DNA. This process involves both phosphorylation in the conserved SQ/TQ domain of Xchk1 and autophosphorylation [8,17,18]. The checkpoint-triggered phosphorylation of Xchk1 brings about an increase in its kinase activity [17]. Furthermore, this phosphorylation is required for a normal checkpoint response [18]. In this study, we have found that various mutants in a conserved region of Xchk1 containing Thr-377 display highly increased kinase activity. The potential implications of these findings for the structure and regulation of Xchk1 are discussed.

2. Materials and methods

2.1. Production of recombinant Xchk1 proteins

Wild-type and mutant His6-Xchk1 proteins were purified from baculovirus-infected Sf9 cells as described [8]. Plasmids encoding His6-Xchk1 proteins were constructed in pFastBacHTa vectors (Gibco BRL). ^{35}S-labeled Xchk1 proteins were synthesized using the appropriate pBS-Xchk1 plasmids as templates in the TNT in vitro transcription/translation system (Promega Corp.) in the presence of [^{35}S]Translabel (ICN Biomedicals) as described [8].

Mutations in Xchk1 were created with the QuikChange™ site-directed mutagenesis kit (Stratagene) using the following oligonucleotides: T377A (*Bss*HII): 5′-CGTTTGGTCAAAAGGATGGCGCG-CTTCTTTACAAAAGTGAATGCC-3′ and 5′-GGCATTCACTTT-TGTAAAGAAGCGCGCCATCCTTTTGACCAAACG- 3′; T377E (*Spe*I): 5′-GTTTGGCAGCGACTAGTCAAAAGGATGGAGAGG-TTCTTTAC-3′ and 5′-GTAAAGAACCTCTCCATCCTTTTGACT-AGTCGCTGCCAAAC-3′; and ΔKRMTRFF (*Bss*SI): 5′-GTTTGG-CAGCGTCTCGTGACAAAAGTGAATGCC-3′ and 5′-GGCATT-CACTTTTGTCACGAGAGGCTGCCAAAC-3′. Shown in the parentheses are the restriction sites generated by silent mutations in the coding region of the primers that allow for the identification of the mutant clone. All mutations have been confirmed by DNA sequencing analysis.

2.2. Egg extracts and isolation of nuclear fractions

Xenopus egg extracts were prepared as described [19]. Reticulocyte lysates containing ^{35}S-labeled Xchk1 proteins were added to interphase egg extracts supplemented with 100 μg/ml cycloheximide. Demembranated *Xenopus* sperm (3000 nuclei/μl of extract) were added to the extracts in the absence or presence of 50 μg/ml aphidicolin. To isolate the nuclear fraction, 50 μl of egg extract was loaded (after incubation at 23°C for 100 min) onto 0.5 ml of a pre-chilled sucrose

*Corresponding author. Fax: (1)-626-795 7563.
E-mail: dunphy@cco.caltech.edu

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solution (1 M sucrose in 80 mM KCl, 25 mM K-gluconate, 10 mM Mg-gluconate, 20 mM HEPES–KOH, pH 7.5) and then centrifuged at $6600\times g$ for 5 min in a swinging bucket rotor in an Eppendorf centrifuge (model 5417C, Eppendorf Scientific, Inc.). The pellets were resuspended gently in the same buffer and recentrifuged at $6600\times g$ for 5 min. The supernatants were discarded and the final pellets were boiled in SDS gel loading buffer and analyzed by SDS–PAGE.

2.3. Kinase assays

His6-Xchk1 proteins were incubated with GST-Cdc25(254–316)-WT [8] in 20 μl of kinase buffer (5 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml ovalbumin, 5 μCi [γ-^{32}P]ATP, and 50 μM ATP). The kinase mixtures were incubated at 23°C for 15 min before SDS gel loading buffer was added.

2.4. In vivo ^{32}P-labeling and isolation of His6-Xchk1 proteins

Baculovirus-infected Sf9 cells were incubated in normal growth medium for 40–45 h before harvesting. The harvested cells were washed three times in phosphate-free minimal essential medium (MEM) (Gibco BRL). The washed cell pellet was then resuspended in the same medium containing ^{32}P-labeled orthophosphate (0.5 mCi/ml) and 1.5% dialyzed fetal bovine serum (Gibco BRL). After incubation at 23°C for 4.5 h, the cells were collected and washed twice in HEPES-buffered saline (HBS) as described [8]. The labeled recombinant Xchk1 proteins were isolated using nickel agarose beads as described [8]. Phosphoamino acid analysis and phosphopeptide mapping were performed as described [20].

3. Results

3.1. Mutagenesis of Thr-377 stimulates hyperphosphorylation of Xchk1

As described previously, Xchk1 in *Xenopus* egg extracts undergoes hyperphosphorylation during a checkpoint response to unreplicated DNA (induced by treatment with the DNA polymerase inhibitor aphidicolin) or UV-damaged DNA [8]. This phosphorylation occurs in the conserved SQ/TQ domain of Xchk1 (see Fig. 1A). An example of the phosphorylation of ^{35}S-labeled Xchk1 that is triggered by aphidicolin is depicted in Fig. 1B (compare lanes 1 and 2). This checkpoint-associated phosphorylation is abolished in the Xchk1-4AQ mutant in which the four Ser/Thr residues (e.g. Thr-314, Ser-344, Ser-356, and Ser-365) in the SQ/TQ motifs of Xchk1 have all been mutated to alanine [17,18] (see also Fig. 1B, lanes 1–4). In parallel with these studies, we prepared a mutant of Xchk1 containing alanine instead of threonine at position 377 (Fig. 1A). Thr-377 lies in a region of Xchk1 that is highly conserved in the Chk1 family of proteins [8], which we will refer to as the TRF (Thr–Arg–Phe) motif. We observed that the ^{35}S-labeled Xchk1-T377A protein underwent an upshift of electrophoretic mobility in both the absence and presence of aphidicolin, though the phosphorylation was greater in the presence of aphidicolin (Fig. 1B, lanes 7 and 8). Similar results were obtained with a related mutant

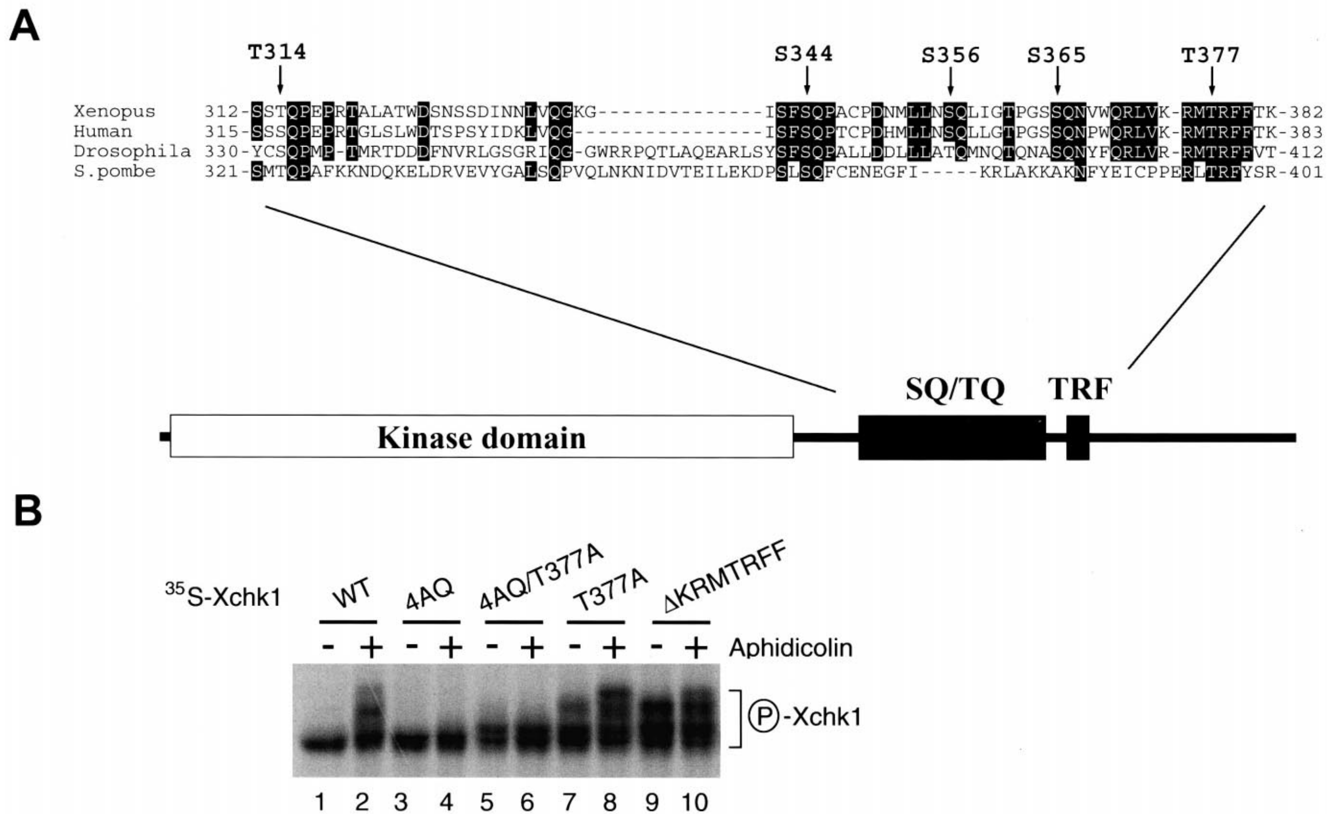

Fig. 1. Mutagenesis of threonine-377 in the conserved TRF motif of Xchk1. A: Sequence alignment of the Chk1 proteins from *Xenopus*, humans, *Drosophila* (grapes), and *Schizosaccharomyces pombe* in the SQ/TQ and TRF regions (top). Schematic representation of the locations of the kinase domain, SQ/TQ domain, and TRF motif in Xchk1 (bottom). The Thr-314, Ser-344, Ser-356, Ser-365, and Thr-377 residues in Xchk1 are denoted with arrows. B: Modification of various ^{35}S-labeled Xchk1 proteins in *Xenopus* egg extracts. ^{35}S-labeled Xchk1 proteins (WT, 4AQ, 4AQ/T377A, T377A, and ΔKRMTRFF) were incubated in interphase egg extracts containing 3000 sperm nuclei/μl in the presence (+) or absence (−) of 50 μg/ml aphidicolin. An aliquot (50 μl) of each extract was centrifuged through a sucrose cushion to isolate the nuclear fractions (see Section 2), which were then subjected to SDS–PAGE and autoradiography.

(Xchk1-ΔKRMTRFF) in which residues 374–380 were deleted (Fig. 1B, lanes 9 and 10). Finally, phosphorylation due to disruption of Thr-377 was strongly reduced but not completely abolished in a combination mutant (Xchk1-4AQ/T377A) in which Thr-377 as well as Thr-314, Ser-344, Ser-356, and Ser-365 were all changed to alanine (Fig. 1B, lanes 5 and 6).

3.2. *Mutagenesis of Thr-377 leads to a strong stimulation of Xchk1 activity*

In order to assess whether Thr-377 plays a role in kinase activity, we prepared His6-Xchk1-T377A in baculovirus-infected Sf9 cells. Next, we compared the kinase activity of His6-Xchk1-T377A and wild-type His6-Xchk1. As indicators of kinase activity, we measured the ability of these proteins to phosphorylate themselves (i.e. autophosphorylation) and the exogenous substrate GST-Cdc25(254–316)-WT. As shown in Fig. 2A, His6-Xchk1-T377A was considerably more active (10–15-fold) than wild-type Xchk1. A form of Xchk1 containing both the mutations T377A and N135A (which eliminates the catalytic function of Xchk1) [8], did not possess detectable kinase activity (Fig. 2B). This observation implies that the increased activity of His6-Xchk1-T377A is an intrinsic feature of this mutant and not, for example, due to association with another kinase activity. As shown in Fig. 3, the kinase activity of Xchk1 was also highly elevated in a mutant in which seven residues containing Thr-377 were deleted (ΔKRMTRFF). Finally, alteration of Thr-377 to a negatively charged amino acid (e.g. glutamic acid) to yield the Xchk1-T377E mutant likewise resulted in increased activity (Fig. 3).

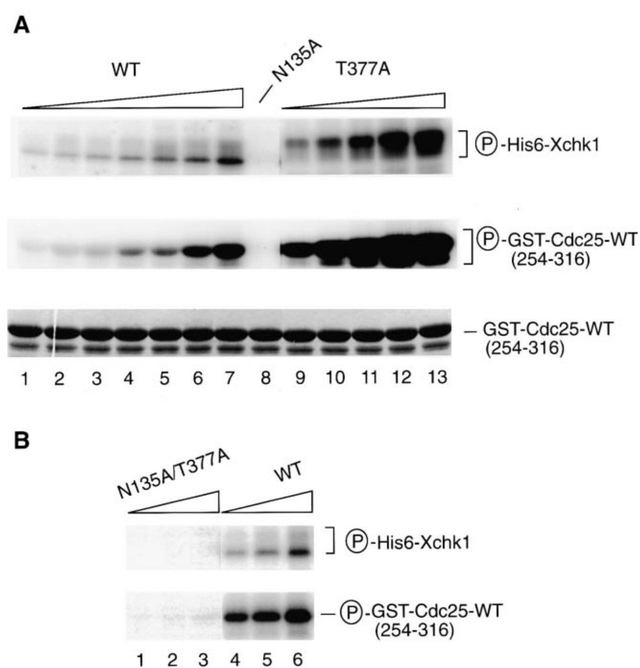

Fig. 2. The T377A mutant of Xchk1 displays increased in vitro kinase activity. A: His6-Xchk1 recombinant proteins (WT, T377A, and N135A) were assayed for Ser-287-specific kinase activity using GST-Cdc25(254–316)-WT as the substrate. The kinase reaction mixtures were then subjected to SDS–PAGE, Coomassie blue staining, and autoradiography. Shown in the figure are the autophosphorylation of the indicated His6-Xchk1 proteins (top), the phosphorylation of GST-Cdc25(254–316)-WT (middle), and the Coomassie blue-stained GST-Cdc25(254–316)-WT protein (bottom). The amounts of the indicated Xchk1 protein used in lanes 1–13 were 25 ng, 50 ng, 100 ng, 200 ng, 200 ng, 500 ng, 1 μg, 1 μg, 50 ng, 100 ng, 200 ng, 400 ng, and 600 ng, respectively. Note that lanes 4 and 5 are duplicates. B: The double mutant His6-Xchk1-N135A/T377A possesses negligible kinase activity. His6-Xchk1 proteins (WT and N135A/T377A) were assayed for autophosphorylation (top) and phosphorylation of GST-Xchk1(254–316)-WT (bottom) as described above in (A). The amounts of the indicated Xchk1 protein used in lanes 1–6 were 62.5, 125, 250, 62.5, 125, and 250 ng, respectively.

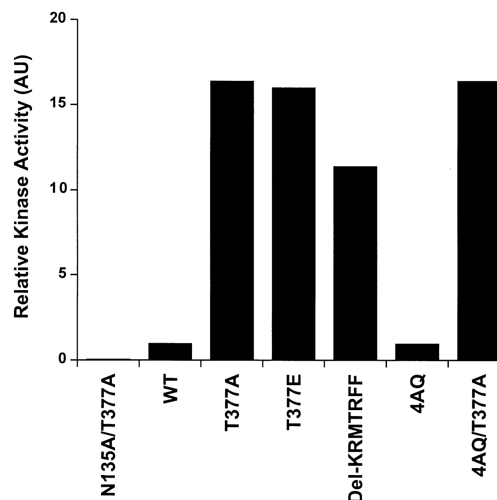

Fig. 3. Further characterization of the role of Thr-377 in the kinase activity of Xchk1. Various His6-Xchk1 proteins (WT, N135A/T377A, T377A, T377E, ΔKRMTRFF, 4AQ, and 4AQ/T377A) were assayed for kinase activity as described above. The histogram depicts phosphorylation of the substrate GST-Cdc25(254–316)-WT.

As described previously, residues in the SQ/TQ domain (e.g. Thr-314, Ser-344, Ser-356, and Ser-365) are required for the checkpoint-associated phosphorylation and activation of Xchk1 [17,18]. To ask whether these residues play a role in the elevated kinase activity of the T377A mutant, we prepared a combination mutant (4AQ/T377A) in which Thr-314, Ser-344, Ser-356, Ser-365, and Thr-377 were all mutated to alanine. As shown in Fig. 3, the His6-Xchk1-4AQ/T377A protein is also approximately 15-fold more active than wild-type His6-Xchk1. This finding indicates that the stimulation of kinase activity that occurs upon mutagenesis of Thr-377 occurs independently of phosphorylation on Thr-314, Ser-344, Ser-356, or Ser-365.

3.3. *Thr-377 does not appear to be a site of phosphorylation on Xchk1*

One explanation for the increased kinase activity of His6-Xchk1-T377A would be that Thr-377 is required for a putative inhibitory phosphorylation of Xchk1. Since recombinant His6-Xchk1-T377A that has been directly purified from baculovirus-infected Sf9 cells is already more active than His6-Xchk1-WT, such an inhibitory phosphorylation would need to have occurred in the Sf9 cells. To address this possibility, we performed the following in vivo labeling experiment. Sf9 cells were infected with baculoviruses encoding His6-Xchk1-WT or His6-Xchk1-T377A. Subsequently, the infected cells were incubated in medium containing ^{32}P-orthophosphate for 4 h prior to purification of the recombinant Xchk1 proteins. Finally, the ^{32}P-labeled His6-Xchk1-WT and His6-Xchk1-T377A proteins were subjected to phosphoamino acid analysis and tryptic phosphopeptide mapping. As shown in Fig. 4A, both His6-Xchk1-WT and His6-Xchk1-T377A

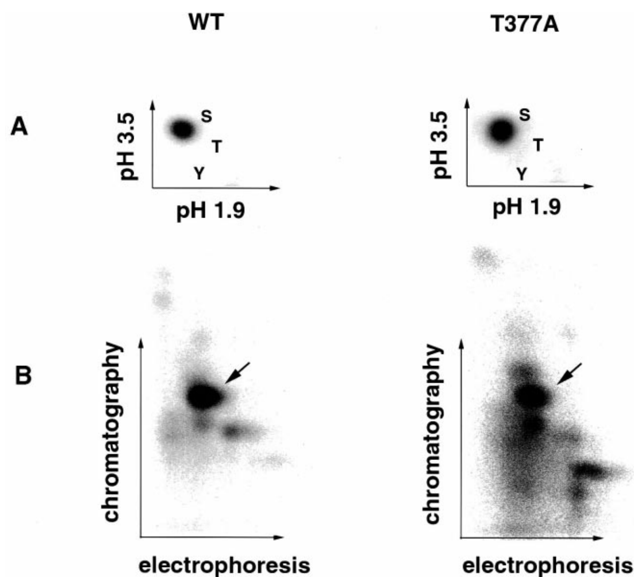

Fig. 4. Phosphorylation of the His6-Xchk1-WT and His6-Xchk1-T377A proteins in vivo in baculovirus-infected Sf9 cells. The indicated His6-Xchk1 proteins were labeled in vivo with ^{32}P, purified, and characterized as described in Section 2. Shown in the figure are the phosphoamino acid analysis (A) and two-dimensional tryptic phosphopeptide map (B) of each protein. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

contained exclusively phosphoserine with no detectable phosphothreonine or phosphotyrosine. In the tryptic phosphopeptide maps of each protein, we observed one major radiolabeled spot (denoted with an arrow in Fig. 4B). In addition to the major spot, the tryptic phosphopeptide map of His6-Xchk1-T377A from ^{32}P-labeled Sf9 cells contained a number of less intensely radioactive spots. These tryptic phosphopeptides most probably contain additional sites of autophosphorylation due to the increased ability of the T377A mutant to phosphorylate itself. Taken together, these findings suggest that Thr-377 is not a site of inhibitory phosphorylation. This conclusion is based on the facts that His6-Xchk1-WT contains no detectable phosphothreonine and does not yield a tryptic phosphopeptide that is absent from the corresponding map of the His6-Xchk1-T377A mutant. However, it is possible that Thr-377 undergoes a modification that cannot be detected by ^{32}P-labeling.

3.4. Increased biological activity of His6-Xchk1-T377A in Xenopus egg extracts

In order to characterize the T377A mutation further, we assessed the biological activity of His6-Xchk1-T377A in *Xenopus* egg extracts. As described previously, the addition of wild-type His6-Xchk1 to *Xenopus* egg extracts results in a dose-dependent delay of mitotic initiation [8]. The endogenous concentration of Xchk1 in egg extracts is approximately 2 ng/μl. As shown in Fig. 5A, the addition of 1.5 ng/μl His6-Xchk1-WT elicited a delay of 15–20 min, whereas a similar amount of His6-Xchk1-T377A (1.8 ng/μl) delayed mitotic initiation by approximately 170 min. Similarly, there was a delay of 40 min in the presence of 3 ng/μl His6-Xchk1-WT, while a comparable concentration of His6-Xchk1-T377A (3.6 ng/μl) caused an essentially permanent arrest in interphase. By averaging the cell cycle delay elicited at various kinase concentrations, we determined that the T377A mutant is approximately 8-fold more active than wild-type Xchk1 in this assay (Fig. 5B). Thus, the increased kinase activity of the T377A mutant is also reflected in its increased potency in this biological assay.

4. Discussion

During a checkpoint response to unreplicated or UV-damaged DNA, Xchk1 undergoes a marked hyperphosphorylation, which results in a substantial decrease in electrophoretic mobility [8]. Mutagenesis of the serine and threonine residues in the four conserved SQ/TQ motifs in Xchk1 abolishes this checkpoint-dependent phosphorylation [17,18]. Significantly, these SQ/TQ motifs comprise part of the consensus site for phosphorylation by members of the DNA-dependent protein kinase family (e.g. DNA-PK, ATM, and ATR) [21–24]. In recent studies, we have shown that a *Xenopus* homolog of ATR (Xatr) phosphorylates these sites [18]. Furthermore, phosphorylation of the SQ/TQ domain is involved in the activation of Xchk1 [17]. For this reason, the Xchk1-4AQ mutant is unable to act as an effector in the DNA replication checkpoint [18].

The major finding of this paper is that various mutants in the TRF motif immediately adjacent to the SQ/TQ domain of Xchk1 (e.g. T377A, T377E, and ΔKRMTRFF) exhibit a strong increase in kinase activity. One explanation for this finding would be that Thr-377 represents a site for inhibitory phosphorylation. To address this possibility, we performed phosphoamino acid analysis and tryptic phosphopeptide map-

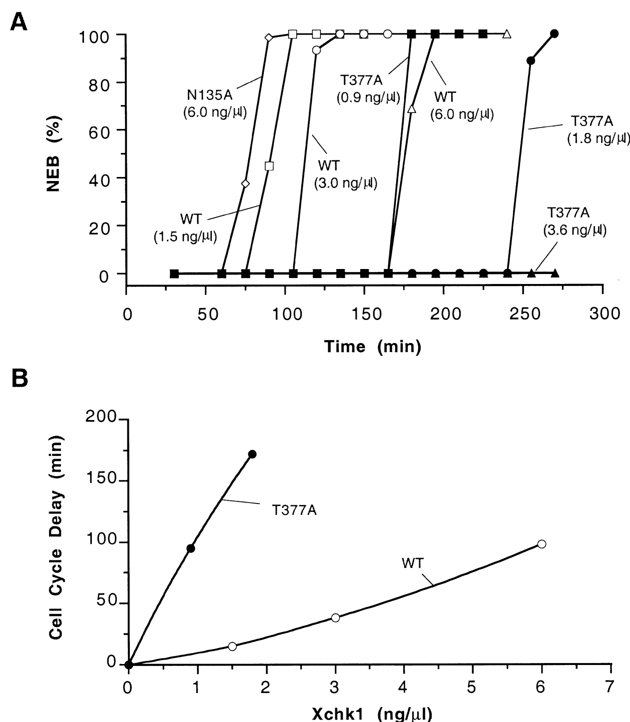

Fig. 5. The T377A mutant of Xchk1 displays increased potency in delaying the cell cycle in *Xenopus* egg extracts. A: Purified His6-Xchk1 recombinant proteins (WT, N135A, and T377A) were added to the M-phase extracts (10% v/v) before activation of the extracts with calcium. The final concentrations of the added proteins are indicated in the figure. The timing of NEB (nuclear envelope breakdown) was monitored by microscopy. B: Dose–response plot of the data from (A).

ping of the wild-type and T377A forms of His6-Xchk1 that had been labeled in vivo with ^{32}P in baculovirus-infected Sf9 cells. These studies indicated that wild-type and T377A His6-Xchk1 contain phosphoserine but no detectable phosphothreonine. Furthermore, the tryptic phosphopeptide map of ^{32}P-labeled, wild-type His6-Xchk1 did not contain a radioactive spot that was missing from the map of the T377A mutant. Thus, it seems unlikely that the difference in kinase activity between wild-type and T377A His6-Xchk1 could be due to phosphorylation on Thr-377.

Other possibilities are that the TRF motif in wild-type Xchk1 is responsible for recruiting a negative regulator or that mutants of Xchk1 with a defective TRF motif possess a higher affinity for a positive regulator, respectively. Such regulators would necessarily be present in Sf9 insect cells, because wild-type and T377A His6-Xchk1 (as well as the T377E and ΔKRMTRFF mutants) assayed directly after purification from baculovirus-infected Sf9 cells show a large difference in kinase activity. However, we have not been able to identify candidates for inhibitory or stimulatory factors in preparations of recombinant Xchk1 from Sf9 cells.

Another type of explanation, which we presently favor, would be that mutagenesis or deletion of Thr-377 leads to a direct alteration (e.g. a structural change) that results in increased kinase activity. For example, a number of kinases (e.g. myosin light chain kinase and calmodulin-dependent protein kinase I) are known to contain autoinhibitory regions that suppress catalytic activity by various mechanisms [25]. Recently, Chen et al. [26] reported the crystal structure of the catalytic domain of human Chk1. Interestingly, Chen et al. found that the catalytic domain of human Chk1 (which resides in the N-terminal half of the protein and thus lacks Thr-377) is about 20-fold more active than full-length human Chk1. It will be interesting to observe the disposition of Thr-377 in the structure of the full-length protein. It is intriguing that the critical residues in the SQ/TQ domain that are required for both the activation and checkpoint function of Xchk1 (e.g. Thr-314, Ser-344, Ser-356, and Ser-365) are quite close to Thr-377 in the linear polypeptide sequence of Xchk1 (see Fig. 1A). Thus, it seems plausible that phosphorylation of Xchk1 in its SQ/TQ domain may relieve an inhibitory constraint on kinase activity that could be imposed by the nearby TRF motif.

The TRF motif is highly conserved in Chk1 proteins from fission yeast, *Drosophila*, humans, *Xenopus*, and other species (Fig. 1A). Since relatively minimal disruptions in this motif lead to a large change in kinase activity, it may be possible to design chemical agents that could enhance the activity of Chk1 by a similar mechanism. Conversely, agents which preserve or potentiate the effect of the TRF motif may suppress the function of Chk1. Such approaches might be employed for the modulation of Chk1 in living cells.

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