Degradation of human thymidine kinase is dependent on serine-13 phosphorylation: involvement of the SCF-mediated pathway

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The expression level of human thymidine kinase (hTK) is regulated in a cell-cycle-dependent manner. One of the mechanisms responsible for the fluctuation of TK expression in the cell cycle can be attributed to protein degradation during mitosis. Given the facts that cell-cycle-dependent protein degradation is highly conserved in all eukaryotes and yeast cells are an excellent model system for protein-degradation study, here we report on the use of Saccharomyces cerevisiae and Schizosaccharomyces pombe to investigate the degradation signal and mechanism required for hTK degradation. We found that the stability of hTK is significantly reduced in mitotic yeasts. Previously, we have observed that Ser-13 is the site of mitotic phosphorylation of hTK in HeLa cells [Chang, Huang and Chi (1998) J. Biol. Chem. 273, 12095–12100]. Here, we further provide evidence that the replacement of Ser-13 by Ala (S13A) renders hTK stable in S. pombe and S. cerevisiae. Most interestingly, we demonstrated that degradation of hTK is impaired in S. cerevisiae carrying a temperature-sensitive mutation in the proteasomal gene pre1-1 or the Skp1-Cullin-1/CDC53-F-box (SCF) complex gene cdc34 or cdc53, suggesting the contribution of the SCF-mediated pathway in hTK degradation. As phosphorylation is a prerequisite signal for SCF recognition, our results implied that phosphorylation of Ser-13 probably contributes to the degradation signal for hTK via the SCF-mediated proteolytic pathway.

Key words: cell cycle, mitosis, proteasome, proteolysis.

INTRODUCTION

Thymidine kinase (TK) is an enzyme that catalyses the transfer of the terminal phosphate of ATP to the 5'-hydroxyl group of thymidine to form dTMP, which is the salvage pathway for dTTP synthesis. The amount of TK is significantly elevated in cells during transition from G1 to the S phase through the transcriptional and post-transcriptional processing controls [1,2]. Diverse experimental evidence has indicated that increases in both transcriptional and translational efficiencies are important for TK induction during G1–S transition [3–10]. The elevated level of TK is then rapidly decreased by degradation during mitosis in order to return to lower levels in the next G1 phase of the cell cycle. It has been shown that deletion of the C-terminal 40 amino acids of human TK (hTK) completely abolishes cell-cycle regulation and stabilizes the protein throughout the cell cycle [11–13]. Since then the molecular mechanism by which hTK is degraded during the cell cycle has not been made clear. Particularly, it is still uncertain whether hTK degradation is via a proteasomal pathway.

Our laboratory has previously shown that TK is phosphorylated in human promyeloleukemia cells in response to growth stimulation [14]. When cells were M-phase-arrested by treatment with nocodazole, a microtubule-depolymerizing drug, hTK became hyperphosphorylated in HL-60, K562 and HeLa cells [15]. We have further established that Ser-13 is involved in mitotic phosphorylation of hTK by cyclin-dependent kinase (CDK) in mitotically blocked HeLa cells [16]. As hTK is degraded when cells are released from the G1/M blockade, we speculate that phosphorylation may play a role in mitotic degradation of hTK. However, the role of phosphorylation in hTK degradation still remains elusive, since the progress of this study has been hampered by the fact that it has been difficult to detect hTK degradation when hTK is artificially overexpressed under the control of a constitutive cytomegalovirus (‘CMV’) or simian virus 40 (‘SV40’) early promoter in mammalian cells. It has also been shown that the expressed level of hTK mRNA in mammalian cells can affect its regulation at the protein level [17]. In addition, we found that it is difficult to consistently control the artificial expressed level of TK mRNA in mammalian cells. Given that yeast does not contain endogenous TK, and that CDKs and the protein turnover machinery among all eukaryotes are highly conserved, we used Saccharomyces cerevisiae and Schizosaccharomyces pombe as the study systems to explore the mechanism responsible for hTK degradation in the cell cycle.

It is well established that ubiquitin-dependent proteasomal proteolysis plays a pervasive role in protein destruction in a cell-cycle-dependent manner [18,19]. Formation of ubiquitin–protein conjugates requires the action of E1, E2 and E3 enzymes [20,21]. We now know that the SCF complex (Skp1-Cullin-1/CDC53-F-box) and the anaphase-promoting complex (APC)/cyclosome are two ubiquitin ligases (E3) that play a crucial role in eukaryotic cell-cycle control [22–26]. As various temperature-sensitive mutants of S. cerevisiae that can be impaired conditionally with respect to components of the proteasome, SCF and APC/cyclosome complexes are available [27–29], in this report we used this genetic system to demonstrate that the SCF-mediated pathway contributes to hTK degradation and that Ser-13 is

Abbreviations used: APC, anaphase-promoting complex; CDK, cyclin-dependent kinase; hTK, human TK; SCF complex, Skp1-Cullin-1/CDC53-F-box; TK, thymidine kinase.

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involved in its degradation. We also used *S. pombe* to show that the hTK degradation rate is increased in the mitotic phase and that phosphorylation on Ser-13 plays a critical role in its mitotic degradation. The results obtained in this study suggest the feasibility of using yeast genetics to search for the regulatory F-box protein controlling degradation of TK during the cell cycle [30–32].

**EXPERIMENTAL**

**Materials**

Anti-hTK polyclonal antibody was prepared as described previously [15]. Anti-cdc2 polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Hydroxyurea, thiamine, RNase A, proteinase K, propidium iodide and the glass beads for yeast-cell-extract preparation were purchased from Sigma (St. Louis, MO, U.S.A.). Amino acid mixture supplement was obtained from Clontech (Palo Alto, CA, U.S.A.).

**Plasmids constructions and site-directed mutagenesis**

For *S. pombe* experiments, cDNA encoding hTK and some of its mutants were amplified by PCR using pBSTK with or without Ser-13 mutation as the templates [16] and subcloned into the *SalI* site of expression vector pREP1 under control of the thiamine-repressible *mttl* promoter [33,34]. For *S. cerevisiae* experiments, hTK expression plasmid was constructed by inserting the *BamHI* restriction fragment containing the hTK cDNA from pREP1-TK (wild-type) and pREP1-TK (S13A) into the pB566 expression vector. For hTK expression in *pre1*-defective and its parental strain, the *KpnI/SacI* fragment containing cDNA of the hTK from pB566-TK was subcloned into the pRS425 expression vector, yielding pRS425-TK. The expression of hTK in pB566-TK and pRS425-TK was under control of the GAL1 promoter [35].

For Ser-13 mutation, site-directed mutagenesis was performed as described by Chang et al. [16]. pBSTK was used as a template for site-directed mutagenesis. The following primers were synthesized: primer 1, 5'-CGCCGGCGGCCCCCCGCAAGACCCC-GGGGG-3', and primer 2, 5'-CTGGCCGGTGACCCCCACCC-AAGACCGGGGG-3'. The primers were designed with the underlined nucleotides being changed to convert Ser-13 into Ala and Asp, respectively. The DNA sequence of each mutated plasmid used for this study was confirmed by dideoxynucleotide sequencing.

**Yeast strains and media**

All *S. pombe* and *S. cerevisiae* strains used in the study are listed in Table 1. *S. pombe* (cdc25-22) and its parental strain were kindly provided by Dr Jin-Yuan Su (National Yang-Ming University, Taipei, Taiwan, Republic of China). *S. cerevisiae* mutants and corresponding parental strains were kindly given by Dr Stephen J. Elledge (Baylor College of Medicine, Houston, TX, U.S.A.) and Dr Yu-Li Jung (Tzu-Chi University, Hualien, Taiwan, Republic of China). *S. pombe* yeasts were cultured in YES medium [3% glucose/0.5% (w/v) yeast extract/amino acids mixture] at 25°C. Transformed clones were cultured in minimal EMM medium (Bio101). *S. cerevisiae* yeasts were cultured in complete YPAD medium [1% (w/v) yeast extract/2% (w/v) bactopeptone/100 μg/ml adenine/2% (w/v) glucose] at 25°C. Transformed clones were cultured in minimal SD medium [yeast nitrogen base without amino acids/2% (w/v) raffinose] supplemented with appropriate amino acids.

**Cell-cycle synchronization and FACS analysis**

*S. pombe* temperature-sensitive mutant cdc25-22 cultures were grown to the logarithmic phase (D 

\[D_{600} \approx 0.3–0.4\]) at 25°C prior to cell-cycle arrest. To arrest at the G1/M phase, yeasts were shifted to 36°C for 4 h to disrupt cdc25 phosphatase gene expression. For release from the G1/M phase, cultures were shifted to 25°C, followed by examining septated cell formation to confirm cell-cycle progression. For *S. cerevisiae* synchronization experiments, cultures were grown to a *D* 

\[D_{600} \approx 0.5\] value of 0.5, followed by adding hydroxyurea (200 μM) into the culture medium to arrest cells at the S phase. To re-enter the cell cycle, hydroxyurea-treated yeast cells were washed twice with fresh SD medium and resuspended in SD medium without hydroxyurea.

Yeast cells (5 × 10⁶) were collected and centrifuged at 1000 g and 4°C for 5 min. Cell pellets were resuspended in 500 μl of buffer A [0.5 M EDTA/0.3%, (w/v) NaN₃] followed by centrifugation at 1000 g at 4°C for 5 min. Cell pellets were then resuspended in sterilized H₂O and fixed in cold ethanol at a final concentration of 70% (v/v) at 4°C overnight, after which cells

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Figure 1 Degradation rate of hTK expressed in S. pombe is increased in the mitotic phase

(A, B) For both cdc25-22 mutant and wild-type yeasts, cells were grown at 36 °C for 4 h and shifted to 25 °C. The percentage of cells with septum formation was determined by observation using dark-field microscopy. (C) cdc25-22 S. pombe (JP68) strain cells were transformed with the pREP1 plasmid expressing hTK. The half-life of hTK was measured by shifting cells to 36 °C for 4 h to inactivate the mutant cdc25 gene, followed by switching to 25 °C and adding thiamine to acutely repress de novo expression from the nmt1 promoter. Degradation of hTK was monitored by immunoblotting with hTK antibody. The same membrane was blotted with cdc2 kinase antibody to confirm equal loading of protein. (D) Expression and degradation of hTK in wild-type S. pombe (JP73) strain were performed as above. (E) The relative abundance of hTK polypeptide was determined by subjecting the immunoblotting membrane to densitometric scanning and normalized by the cdc2 signals in the same sample.

Protein expression, repression and protein stability experiments

For repression of pREP1-TK expression, cells were grown in EMM medium by adding thiamine (4 μM) to inactivate the nmt1 promoter. Cell-cycle analysis was performed using a Becton Dickinson FACScan flow cytometer, and the data were analysed by use of CellQuest software.

were pelleted and washed twice with buffer containing 50 mM sodium citrate (pH 7.2) and resuspended in 1 ml of sodium citrate (50 mM). Incubation of the cell suspension solution with RNase A at a final concentration of 250 μg/ml at 50 °C was carried out for 1 h, followed by addition of proteinase K to a final concentration of 100 μg/ml for another 1 h. Cells were then washed twice with 50 mM sodium citrate prior to incubation with propidium iodide (20 μg/ml) in the dark for 10 min. Cell-cycle analysis was performed using a Becton Dickinson FACScan flow cytometer, and the data were analysed by use of CellQuest software.
Figure 2 Degradation of hTK in cdc25-22 S. pombe (JP68) in the S phase

cdc25-22 S. pombe (JP68) strain cells expressing hTK were arrested at G1/S phase by adding hydroxyurea (HU, 200 mM) to the growth medium when incubated at 25 °C for 8 h. Cells were then released from G1/S arrest by washing out the hydroxyurea, followed by addition of thiamine in the fresh growth medium to repress the GAL1 promoter to monitor hTK degradation at the indicated time points.

Figure 3 Ser-13 of hTK is important for its degradation in mitotic fission yeasts

Expression of the hTK wild-type (wt), or S13A or S13D mutants under the control of the nmt1 promoter in S. pombe (cdc25-22) (JP68) and measurement of hTK degradation were performed as described in the legend to Figure 1.
densitometric scanning to determine hTK abundance and normalized against the internal control cdc2 signals.

RESULTS

Mitotic degradation of hTK in *S. pombe*

All yeast strains used for this study are listed in Table 1. A temperature-sensitive mutant of *S. pombe* (cdc25-22) [33,34] that can be arrested in the G$_s$/M phase at 36 °C was used to examine whether the degradation rate of hTK is increased during mitosis in yeast. When cells were incubated at 36 °C for 4 h, all *S. pombe* (cdc25-22) cells became elongated without septum formation, indicating G$_s$/M arrest. Following a temperature shift to 25 °C for 2 h, we observed septum formation in these cells, representing mitosis progression, which reached a peak level of 95–100% for *S. pombe* (cdc25-22) as compared with 5–25% throughout the same experimental period for the wild-type yeasts (compare Figures 1A and 1B). We then used this system to examine whether there is an increase in hTK degradation rate at the mitotic phase in yeast. The expression of hTK in *S. pombe* was under the control of the thiamine-repressible *nmt1* promoter. We found that hTK is accumulated in *S. pombe* (cdc25-22), which were grown at 36 °C for 4 h (Figure 1C, lane 1). Upon shift to 25 °C, thiamine was added to the medium to repress new expression of hTK, and hTK proteolysis was monitored by immunoblotting analysis. We observed that the level of hTK significantly decreased in *S. pombe* (cdc25-22) within 2 h (Figure 1C, lanes 2–5), concomitant with the timing of cell septation (Figure 1A). As for wild-type *S. pombe* that cannot be synchronized in the G$_s$/M phase under these experimental conditions, the half-life of hTK was more than 3 h (Figure 1D). Because the regulation of cdc2 kinase is mainly controlled by its

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Figure 4  Expression and degradation of hTK in asynchronized and synchronized *S. cerevisiae*

Parental strain of *S. cerevisiae* was transformed with a plasmid expressing wild-type (wt) or S13A mutant hTKs. (A) Expression of hTK was induced for 1 h by adding galactose (Gal) to the medium and was repressed by addition of glucose (Glc) at time 0. Cells were harvested at the indicated times for immunoblotting analysis. (B) The relative half-life of hTK (wild-type versus S13A) was determined by densitometric scanning of the immunoblots from (A) as described in the legend to Figure 1. (C) Yeasts expressing wild-type hTK and the S13A mutant were arrested in the S phase by adding hydroxyurea (HU, 200 mM) to the growth medium during galactose induction. At time 0 cells were washed to remove hydroxyurea and glucose was added to repress the *GAL1* promoter to monitor hTK degradation as described above. (D) The relative half-life of hTK (wild-type versus S13A) was determined by densitometric scanning of immunoblots from (C) as described in the legend to Figure 1. Raf, raffinose.
phosphorylation status and its associated proteins, but not by its own expressed level in the cell cycle, we used cdc2 kinase as the internal control for all the immunoblotting experiments. The level of cdc2 kinase was similar in the yeasts during all experimental conditions, indicating that hTK degradation in cdc25-22 yeasts is a rather specific event. By densitometric scanning, the half-life of hTK in S. pombe at 25°C in these mitotic cells (cdc25-22) was 90 min, and 200 min for asynchronized wild-type cells that contain a mixture of G1, G1/S, S, G2, and G2/M populations (Figure 1E). We also arrested S. pombe (cdc25-22) in the G1/S phase by hydroxyurea treatment for 8 h at 25°C. By washing out hydroxyurea to resume the S-phase progression, we found that about 70% of cells entered the G2 phase in 90 min, as judged by cell elongation. We then tested whether hTK was degraded during the S-phase progression under these experimental conditions. It appeared that hTK is very stable within this period of S-phase progression (Figure 2), which is in contrast with the clear reduction of hTK during mitotic progression. Thus, similar to that in mammalian cells, the rate of hTK degradation in S. pombe can be significantly increased in the mitotic phase.

Ser-13 mutation reduces the rate of hTK degradation in fission yeast

As it has been shown that hTK is phosphorylated by cdc2 kinase on Ser-13 [16] and is degraded faster in the mitotic phase in human cells [8,12], we then examined whether Ser-13 plays a crucial role in mitotic degradation of TK in S. pombe. Remarkably, we found that mutation of Ser-13→Ala (S13A) not only resulted in elevated accumulation of hTK in S. pombe, but also reduced the rate of hTK degradation during mitosis (compare Figures 3A and 3B). As Ser-13 is the site for cdc2 kinase phosphorylation during G1/M arrest, we then asked the question whether a mutation of Ser-13→Asp can mimic a charge change due to phosphorylation to render TK susceptible for degradation. It appeared that Ser-13→Asp mutation also caused an effect similar to that of Ser-13→Ala on TK stability in the mitotic phase (Figure 3C). Therefore, the degradation signal of hTK is not merely due to a change of charge at the position of Ser-13 (Figure 3D).

Degradation of hTK in S. cerevisiae is also sensitive to Ser-13 mutation

To analyse further the proteolysis of hTK in S. cerevisiae, we subcloned hTK into an inducible expression vector under the control of the GAL1 promoter and transformed this construct into S. cerevisiae. TK expression can be induced by galactose and turned off in S. cerevisiae by adding glucose to the medium. The amount of hTK decreased with a half-life of <20 min, whereas the S13A mutant form was significantly more stable, with a half-life of 40 min (Figure 4B), indicating that the half-life of TK is prolonged significantly by the mutation of Ser-13→Ala. To avoid the complication of a difference in cell-cycle position of yeasts expressing wild-type or mutant hTK, we arrested cells in the S-phase by addition of 200 mM hydroxyurea, followed by release from the S-phase arrest and glucose addition. Under these experimental conditions, we still observed that the half-life of hTK was increased by mutation of Ser-13→Ala (Figures 4C and 4D). Thus Ser-13 does play a role in hTK stability.

SCF-mediated proteolytic pathway targets hTK degradation in S. cerevisiae

We next examined the stability of hTK expressed in a number of S. cerevisiae mutants carrying temperature-sensitive mutations in cdc16-123, cdc34-2 and cdc53-1 [21,22] to explore the contribution of APC or SCF in TK degradation. It appeared that the APC-mediated pathway was probably not responsible for hTK degradation, as the half-life of hTK expressed in cdc16-123 mutant was similar to that in parental wild-type cells (Figure 6A). Since it has been reported that pre-1 and cdc16-123 mutants can both be arrested at the metaphase/anaphase transition with high levels of mitotic CDK activity [36,38,39], it is unlikely that the stabilization of hTK in proteasome-defective yeasts was a result of cell-cycle arrest. As for expression in cdc34-2 and cdc53-1 mutants, we found that hTK is no longer degraded at 36°C (Figure 6A) and that the degradation rate of hTK expressed was similar in the three different parental strains JY92, JY236 and Jy242. This result indicated that the SCF complex is probably required for hTK’s proteolysis in S. cerevisiae. It is generally recognized that the SCF complex and APC/cyclosome play a
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Figure 6 SCF components are required for hTK degradation in S. cerevisiae

(A) Expression and degradation of hTK in parental (JJY92), cdc53-1 (JJY240), cdc34-2 (JJY244) and cdc16-123 mutant (JJY91) strains were performed as described in the legend to Figure 4. (B) In parallel, parental and temperature-sensitive mutant cells expressing hTK that used for the above degradation experiment were collected for FACS analysis to confirm cell-cycle progression at the indicated time point after cells shifting to 36 °C. Vertical axes, relative DNA content; left-hand horizontal axes, relative cell numbers. Gal, galactose; Glc, glucose; Raf, raffinose.

critical role in proteolysis of cell-cycle regulators [22–26], and several lines of evidence have revealed that the SCF-complex-component defective temperature-sensitive mutants fail to enter the S phase because they are unable to eliminate the S-phase cyclin/CDK inhibitor Sic1 [40–43]. It is reasonable that prolonged half-life of hTK in cdc34-2 and cdc53-1 temperature-sensitive mutants may be a consequence of cell-cycle arrest at a specific phase by shifting cells to 36 °C. To assess this possibility, we used the FACS analysis to examine whether each mutant strain is arrested at a particular cell-cycle phase under the experimental condition. It appeared that cdc16-11, cdc34-2 and cdc53-1 mutant strain cells were not synchronized at a specific phase within 90 min of non-permissive temperature shift (Figure 6B). Therefore, it seems that the lack of hTK degradation in cdc34-2 and cdc53-1 mutant strains is a result of functional impairment of the SCF complex, rather than the cell-cycle effect at non-permissive temperature.

The F-box protein cdc4 does not contribute to hTK degradation in S. cerevisiae

We next examined hTK stability in the yeast F-box protein mutant strain cdc4-1 [27–29]. hTK was degraded in the cdc4-1 strain similarly to that seen in its parental strain (Figure 7A), suggesting that cdc4-1 is not the F-box protein required for recognition of hTK for SCF. It should be pointed out that the cdc4-1 mutation could also result in G1 arrest due to the fact that cdc4 is indispensable for recognition of phosphorylated Sic-1, a cdk
operating cell-cycle-dependent proteolysis of the ectopically expressed hTK. Most interestingly, we found that the SCF-mediated pathway is the operating system responsible for hTK degradation in *S. cerevisiae* (Figure 6). Whether this is true for mammalian cells requires further investigation. Nevertheless, it should be emphasized that Ser-13 is the site of mitotic phosphorylation of hTK in HeLa cells [16] and that phosphorylation is the critical signal for the substrate recognition by the SCF complex among all eukaryotes [18,19,22–26]. In this study, our results demonstrate that mutation on Ser-13 renders hTK more stable in budding and fission yeasts, at least consistently indicating the critical role of Ser-13 in regulating the protein level of hTK expression. Although it has been shown that deletion of the C-terminal 40 amino acids stabilizes hTK at mitosis when stably expressed in murine Ltk<sup>−</sup> fibroblasts [11–13], it is possible that this region contains the ubiquitination site for E2/E3, which in turn is necessary for its degradation. In our laboratory, we have found that TK can be polyubiquitinated in HeLa cells (results not shown). As hTK is phosphorylated and degraded in a cell-cycle-dependent manner, it is logical to assume that phosphorylation on Ser-13 contributes to the signal for hTK degradation through the ubiquitin/proteasome-dependent pathway.

However, whether hTK degradation requires signals in addition to phosphorylation on Ser-13 remains to be investigated. We did observe that the S13A TK mutant still underwent degradation in *S. cerevisiae*, but at a slower rate than the wild type. Therefore, it is likely that the other site can also contribute to the degradation signal. Nevertheless, our results provide the first evidence that hTK can utilize the cell-cycle-dependent machinery in yeast to carry out its proteolytic process, even though neither budding nor fission yeasts contain the TK gene in their genomes. Most importantly, these results suggest a good possibility that an as-yet-undetermined F-box protein is crucial for controlling TK turnover rate in eukaryotes.

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