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人類胸腺嘧啶激酶蛋白分解之機制探討

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執行單位：台灣大學醫學院

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Abstract

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 proteolysis is highly conserved in all eukaryotes and yeast cell is an excellent model system for protein degradation study, here we report using 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 serine 13 is the site of mitotic phosphorylation of hTK in HeLa cells (Chang, Z.-F., Huang, D.-Y., and Chi, L.M. (1998) J. Biol. Chem. 273. 12095-12100). Here, we further provide evidence that the replacement of serine 13 by alanine (S13A) renders hTK to become stabilized in S. pombe and S. cerevisiae as well. Most interestingly, we demonstrated that degradation of hTK is impaired in S. cerevisiae carrying temperature-sensitive mutation in proteasomal gene prel-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, we proposed that phosphorylation of serine13 probably contributes to the degradation signal for hTK via the SCF-mediated proteolytic pathway.

Keywords: proteolysis, mitosis, proteasome, cell cycle

INTRODUCTION

Thymidine kinase (TK) is an enzyme that catalyzes 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. 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. Our laboratory has previously shown that TK is phosphorylated in human promyeloleukemia cells in response to growth stimulation. When cells were M-phase arrested by treatment of NOC (nocodazole), a microtubule-depolymerizing drug, hTK became hyperphosphorylated in HL-60, K562 and HeLa cells. We have further established that serine 13 is involved in mitotic phosphorylation of hTK by cyclin dependent kinase in mitotically blocked HeLa cells. As hTK is degraded when cells are released from the G2/M blockade, we speculate that phosphorylation may play a role in mitotic degradation of hTK. Given that yeast does not contain endogenous TK, and that CDKs and the protein turnover machinery among all eukaryotes are highly conserved, we used the *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. Formation of ubiquitin-protein conjugates requires the action of E1, E2 and E3 enzymes. We now know that the SCF complex (Skp1-Cullin-1/CDC53-F-box) and the APC/cyclosome (anaphase-promoting complex) are two ubiquitin ligases (E3) that play a crucial role in the eukaryotic cell cycle control. As various temperature-sensitive mutants of *S. cerevisiae* that can be conditionally impaired in components of proteasome, SCF, and APC/C complex are available, in this report we used this genetic system to demonstrate that SCF-mediated pathway contributes to hTK degradation and that serine-13 is involved in its degradation. We also used *Schizosaccharomyces pombe* to show that hTK degradation rate is increased in the mitotic phase and that phosphorylation on serine-13 plays a critical role in its mitotic degradation. The results obtained in this study suggest the feasibility to use yeast genetics to search for the regulatory F-box protein controlling degradation of TK during the cell cycle.

RESULTS AND DISCUSSION

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

A. 

<table>
<thead>
<tr>
<th>cdc25-22 (JP 68)</th>
<th>parental strain (JP73)</th>
</tr>
</thead>
<tbody>
<tr>
<td>36°C</td>
<td>25°C, + thiamine</td>
</tr>
<tr>
<td>4 hr</td>
<td>1 2 3 4 time (hr)</td>
</tr>
</tbody>
</table>

(B) The parental (JP73) and cdc25-22 *S. pombe* (JP68) strains were transformed with pREP1 plasmid expressing hTK. The half-life of hTK was measured by shifting cells to 36°C for 4 hr (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. (B) The relative degradation rate of hTK polypeptide was determined by subjecting the immunoblotting membrane for densitometric scanning.
Figure 2. Serine 13 residue of hTK is important for its degradation in mitotic fission yeasts.

Expression of the hTK wild type (wt), S13A, or S13D mutant 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.

Figure 3 Expression and degradation of hTK in asynchronized and synchronized *Saccharomyces cerevisiae*.

(A) Parental strain of *S. cerevisiae* was transformed with plasmid expressing hTK wild type and S13A mutant. Human TK was induced for 1 hr by galactose addition and at time =0 was repressed by addition of glucose. Cells were harvested at the indicated times, and the abundance of hTK was determined by immunoblotting. The relative half-life of hTK (wt v.s. S13A) was determined by densitometric scanning and shown on the right panel.  (B) Yeasts expressing hTK(wt) and S13A mutant were arrested in the S phase by adding hydroxyurea (HU, 200mM) to the growth medium during galactose induction. At time=0 cells were washed to remove hydroxyurea and glucose was added to repress the GAL 1 promoter to monitor hTK degradation as described above.
Figure 4 Proteasomal component is required for hTK degradation in *Saccharomyces cerevisiae*.

Proteasomal component is required for hTK degradation in *Saccharomyces cerevisiae*. Degradation of hTK in parental (Jy80) and *pre-1* (Jy81) mutant strains was measured as described in the legend to Figure 3.

Figure 5 SCF components are required for hTK degradation in *Saccharomyces cerevisiae*.

Expression and degradation of hTK in parental (JY92), *cdc53-1*(JY91), *cdc34-2*(Jy244), and *cdc16-123* mutant (JY91) strains were performed as described in the legend to Figure 3.
Figure 6 The degradation of hTK in *cdc4*-defective *Saccharomyces cerevisiae*.

<table>
<thead>
<tr>
<th>parental strain (Y80)</th>
<th>Glc (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raf</td>
</tr>
<tr>
<td>TK</td>
<td></td>
</tr>
<tr>
<td>cdc2</td>
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Expression and degradation of hTK in *cdc4*-defective and its parental strains (Y80) were performed as described in the legend to Figure 3.

According to the results obtained in this yeast study system, we hypothesize that mutation of serine13 to alanine may attenuate the degradation signal, resulting in accumulation of hTK in yeast when hTK expression is shut-off by addition of glucose or thiamine in budding or fission yeast, respectively. Although yeast does not contain its endogenous TK, interestingly, our results showed that the machinery in yeast is valid for operating cell cycle-dependent proteolysis of the ectopic expressed hTK. Most interestingly, we found SCF-mediated pathway is the operating system responsible for hTK degradation in *S. cerevisiae*. Whether this is true for mammalian cell remains further investigation. Nevertheless, it should be emphasized that serine13 is the site of mitotic phosphorylation of hTK in HeLa cells and that phosphorylation is the critical signal for the substrate recognition by the SCF complex among all eukaryotes. In this study, our results demonstrate that mutation on serine13 renders hTK more stable in both budding and fission yeasts, at least consistently indicating the critical role of serine 13 in regulating the protein level of hTK expression. Although it has been shown that depletion of N-terminus 40 amino acid stabilizes hTK at mitosis when stably expressed in murine Ltk fibroblasts, 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 (data not shown). As hTK is phosphorylated and degraded in a cell cycle-dependent manner, it is logical to assume that phosphorylation on serine13 contributes to the signal for hTK degradation through the ubiquitin/proteasomal dependent pathway.

However, whether hTK degradation requires signals in addition to phosphorylation on serine 13 remains to be investigated. We did observe that S13A TK mutant still underwent degradation in *S. cerevisiae*, but with a slower rate as compared with its wild type. Therefore, it is likely that 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 that either budding or fission yeast does not contain 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.

**参考文献**
