Serine 13 Is the Site of Mitotic Phosphorylation of Human Thymidine Kinase*

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It has been reported that the polypeptide of thymidine kinase type 1 (TK1) from human and mouse cells can be modified by phosphorylation. Our laboratory has further shown that the level of human TK phosphorylation increases during mitotic arrest in different cell types (Chang, Z.-F., Huang, D.-Y., and Hsue, N.-C. (1994) J. Biol. Chem. 269:21249–21254). In the present study, we demonstrated that a mutation converting Ser13 to Ala abolished the mitotic phosphorylation of native TK1 expressed in Ltk− cells. Furthermore, we expressed recombinant proteins of wild-type and mutant human TK1 with fused FLAG epitope in HeLa cells, and confirmed the occurrence of mitotic phosphorylation on Ser13 of hTK1. By using an in vitro phosphorylation assay, it was shown that wild-type hTK1, but not mutant TK1A13, could serve as a good substrate for Cdc2 or Cdk2 kinase. Coexpression of p21waf1/cip1, which is a universal inhibitor of Cdk kinases, in Ltk− fibroblasts also suppressed mitotic phosphorylation of hTK1 expressed in this cell line. Thus, Cdc2 or related kinase(s) is probably involved in mitotic phosphorylation on Ser13 of the hTK1 polypeptide. We also found that mutation on Ser13 did not affect the functional activity of hTK1. As the sequences around Ser13 are highly conserved in vertebrate TK1s, we speculate that phosphorylation of Ser13 may play a role in the regulation of TK1 expression in the cell cycle.

Thymidine kinase (TK)1 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. In eukaryotic cells, there are two TK isoenzymes: TK1 and TK2, also called cytosolic and mitochondrial TK, respectively. The amount of cytosolic TK1 is increased significantly in cells during transition from G1 to S phase (1, 2), whereas the expression level of TK2 is low and is increased significantly in cells during transition from G1 to S phase (1, 2), whereas the expression level of TK2 is low and is controlled in a cell cycle-independent manner (3, 4). TK1 is highly expressed in dividing or malignant cells, but is absent in quiescent cells (5–8), and its expression is stringently regulated in normal cells, but not in malignant cells (9, 10).

Studies on TK1 regulation have provided a good model for understanding the molecular events that coordinate progression through the cell cycle. It is well documented that the transcriptional and translational activation of TK1 gene expression leads to elevation of its activity in the G1/S phase (11–18). In addition, other lines of evidence have shown that the C-terminal region of the TK1 polypeptide may determine its stability in cells in different growth states, indicating that cell cycle-dependent degradation is also involved in the regulation of TK1 expression (19–21). Thus, it is clear that multiple levels of control regulate TK1 expression in eukaryotic cells.

Our laboratory has previously reported that TK1 can be phosphorylated in human promyeloleukemia cells in response to growth stimulation (22). The phosphorylated form of TK1 was also detected by isoelectric focusing gel analysis of TK1 purified from mouse Ehrlich ascites tumor cells (23). The regulation of TK1 phosphorylation was investigated further during the cell cycle. When cells were M phase-arrested by treatment with nocodazole, a microtubule-depolymerizing drug, TK1 became hyperphosphorylated in HL-60, K562, and HeLa cells (24). Phosphoaminoacid analysis of immunoprecipitated human TK1 polypeptide indicated that serine is the residue involved in mitotic phosphorylation (22, 24). Amino acid sequence analysis reveals that several potential phosphorylation sites exist for a variety of serine/threonine protein kinases, including cAMP-dependent protein kinase at serine 194, cyclin-dependent kinase(s) at serine 13 and serine 231, and protein kinase C at serine 30. In this study, we examined the specific phosphorylated site for human TK1 in mitotically arrested TK-deficient mouse Ltk− fibroblasts and human carcinoma HeLa cells, and we identified the kinase responsible for the mitotic phosphorylation of hTK1. The physiologic role of the mitotic phosphorylation of hTK1 was also investigated.

EXPERIMENTAL PROCEDURES

Cell Cultures and M-phase Arrest—TK-deficient mouse Ltk− connective fibroblasts and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. For M-phase arrest, nocodazole (NOC) was added to subconfluent Ltk− cells at a final concentration of 0.5 μg/ml for 17 h.

Site-directed Mutagenesis—The Scal-BamHI restriction fragment containing cDNA of the human TK1 (hTK1) gene (25) was treated with Klenow enzyme and cloned into the EcoRV site of pBluescript SK− (Stratagene), yielding pBtTK. pBtTK was used as a template for site-directed mutagenesis as described by Deng and Nickoloff (26). The following primers were synthesized: primer 1, CTGGCCGGCCGGGGGAGGAGGACGACCAGGCCGGAG; and primer 2, CAAGAAGGCACCAGGCCGAG. The primers were designed with the underlined nucleotides being changed to convert Ser13 to Ala and Ser231 to Pro, respectively. One primer (5′-TAGAGCTATGAGAACCAGGC-3′) that contains a mutation in the BamHI site unique to pBluescript SK− (27) was also synthesized and was included in the process of site-directed mutagenesis for the selection of the mutated plasmds. The DNA sequence of each mutated

* This work was supported in part by Grants CMRP 436 from the Chang Gung University and NSC87-2314-B-002-165 from the National Science Council, Taiwan, Republic of China. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶The abbreviations used are: TK, thymidine kinase; TK1, TK type 1; hTK, human TK; Ltk−, TK-deficient mouse connective fibroblast; NTA, nitrolaotic acid; DMEM, Dulbecco’s modified Eagle’s medium; NOC, nocodazole; CMV, cytomegalovirus; PMSF, phenylmethylsulfonyl fluoride; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline.

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plasmid used for this study was confirmed byideoxyribonucleotide sequencing.

**Construction of Expression Plasmids**—For transient transfection of expression plasmids into Ltk− mouse fibroblasts, cDNAs encoding hTK1 and some of its mutants were cloned into the XhoI site of an expression vector, pCMV, in a sense orientation. The hTK1 expression plasmids were constructed by inserting the EcoRI/ClaI restriction fragment containing cDNA of the hTK1 from pBSTK without or with a desired site-specific mutation downstream of the cytomegalovirus (CMV) immediate-early promoter. For stable expression in HeLa cells, human TK1 cDNA and its mutant, TK1Ala13, were each cloned into the pCMV vector (New England Biolabs, Beverly, MA) at a blunted XhoI/SmaI site, resulting in a fusion of a sequence encoding FLAG epitope (MDT KD DDDD K) plus 40 residues from the polylinker to the 5′end of the hTK1 coding sequence, by which pFLAG-TK1 and pFLAG-TK1Ala13 were generated.

**Transient Transfection of Ltk− Fibroblasts**—Ltk− cells (2 × 10⁶) were seeded and transfected with a mixture of 18 µg of LipofectAMINE (Life Technologies, Inc.) and 3 µg of plasmid DNA per 6-cm dish. On the day after transfection, cells were washed and then treated with or without NOC for 17 h. Next, cells were then pulse-labeled for 2 h at 37 °C with [³²P]orthophosphate (500 µCi/ml, Amersham Corp.) in phosphate-free medium. Cells were lysed in a lysis buffer containing 50 mM Tris-HCl buffer (pH 7.5), 1% Nonidet P-40, 150 mM NaCl, 50 mM NaF, 1 mM β-mercaptoethanol, 0.1 µg/ml leupeptin, 5 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The microsomes of each sample were precipitated by 10% trichloroacetic acid, and the amount of radioactivity present in the trichloroacetic acid-insoluble portion was determined with a liquid scintillation counter. Lysates containing equal trichloroacetic acid-insoluble counts were then incubated with anti-serum against GST-TK1 for 2 h. Immunocomplexes were adsorbed onto protein-A-Sepharose (Pharmacia Biotech, Inc.) and washed five times with lysis buffer containing 0.1% deoxycholate and 0.2% SDS. Samples were analyzed by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) for autoradiography, followed by immunodetection using the antisera against GST-TK1.

**Stable Transfection of HeLa Cells**—HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Cells were cotransfected with 5 µg of the expression plasmid, pFLAG-TK1 or pFLAG-TK1Ala13, and 0.25 µg of pRSVneo by the LipofectAMINE method. Two days after transfection, cells were trypsinized and plated in the medium containing 0.1% deoxycholate and 0.2% SDS. Samples were analyzed by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) for autoradiography, followed by immunodetection using the antisera against GST-TK1.

**Motic Arrest and Flow Cytometry of Stable Cell Lines**—For mitotic arrest of stably transfected cell lines, 1 × 10⁶ cells/10-cm dish were treated with thymidine (2 mM) in DMEM containing 10% fetal bovine serum for 20 h, then released for 8 h, and followed by addition of 2 mM thymidine for another 20 h. After this double thymidine block, cells were treated with NOC (50 ng/ml) for 16 h. For release from mitotic arrest, after NOC treatment cells were washed with PBS twice and refreshed with DMEM containing 10% fetal bovine serum. Immediately after harvesting, cells were frozen in 70% ethanol and stored at −20 °C until analysis. At this time, the cells were thawed and stained with propidium iodide as described elsewhere (27). Flow analysis was performed on a Becton Dickinson FACScan flow cytometer, and the data were interpreted by use of the ModiFit cell cycle analysis program (version 2.0). At least 10,000 cells were analyzed for each sample.

**Immunoblotting**—Twenty micrograms of cytosolic protein were resolved on 12% SDS-PAGE, followed by electrophoretic transfer to PVDF membrane (28). After blocking with 5% powdered milk, the membrane was incubated with antisera against GST-TK1 (1:1500) for 2 h, and treated for 1 h with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Promega) or with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Amersham). The alkaline phosphatase color development or ECL detection for the horseradish peroxidase activity present in the trichloracetic acid-insoluble portion was determined with a liquid scintillation counter. Lysates containing equal trichloroacetic acid-insoluble counts were then incubated with anti-sera against GST-TK1 for 2 h. Immunocomplexes were adsorbed onto protein-A-Sepharose (Pharmacia Biotech, Inc.) and washed five times with lysis buffer containing 0.1% deoxycholate and 0.2% SDS. Samples were analyzed by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) for autoradiography, followed by immunodetection using the antisera against GST-TK1.

**RESULTS**

Mouse TK-deficient Ltk− cells were transfected with expression vectors encoding human wild-type or mutant types of TK1. Two mutants each contain a single amino acid substitution (Ser13 → Ala or Ser194 → Pro) that generates catalytically active TK1 with similar levels of TK activity when expressed in proliferating Ltk− cells. After transfection, cells were either maintained in the proliferating state or treated overnight with NOC for mitotic blocking. Cells were incubated with [³²P]orthophosphate for immunoprecipitation by hTK1 antisera (24). In proliferating Ltk− cells, phosphorylation of ectopically expressed TK1 was weakly detectable for the wild-type as well as the two mutants (Fig. 1A). In NOC-treated Ltk− cells, the phosphorylation level of wild-type hTK1 was greatly enhanced (Fig. 1B). However, the intensity of phosphorylation...
for ectopically expressed mutant hTK1 (Ser13 → Ala) was reduced significantly, even though a greater amount of hTK1 was immunoprecipitated in this cell lysate (Fig. 1B). The phosphorylation intensity of another mutant hTK1 (Ser194 → Pro) in NOC-treated L-cells was essentially similar to that of the wild-type hTK1. Thus, serine 13 appears to be an important site contributing to an increased phosphorylation level of hTK1 expressed in NOC-treated Ltk− cells.

To learn whether mitotic phosphorylation on Ser13 of hTK1 also occurs in human cells, we established HeLa cell lines stably expressing FLAG-tagged wild-type TK1 or mutated TK1Ala13. For this purpose, HeLa cells were cotransfected with pCMV-FLAG-TK1 or pCMV-FLAG-TK1Ala13, separately, with pRSV-neo as a selection marker. FT and FN cell lines, which stably expressed FLAG-TK1 and FLAG-TK1Ala13, respectively, were established by G418 selection. The expression of FLAG-TK1 in proliferating and mitotically arrested FT cells was measured by immunoprecipitation with monoclonal antibody M2 against the FLAG epitope, followed by Western blotting with antiserum against hTK1. Isoforms of FLAG-TK1, displaying a mobility shift, were detected in mitotically arrested cells (Fig. 2A). Alkaline phosphatase treatment of immunoprecipitated FLAG-TK1 from the cell lysate of mitotically arrested HeLa (FT) cells abolished the up-shifted band, indicating that phosphorylation of FLAG-TK1 decreases its electrophoretic mobility during separation on SDS-PAGE (Fig. 2A). Clearly, alterations in the phosphorylation state of FLAG-TK1 were detectable by immunoblot analysis with antibody to TK1. Because hTK1 can be present as homodimer or homotetramer (29, 30), in FT cells endogenous TK1 of a molecular size of 24 kDa could also be detected by hTK1 antiserum in the FLAG-TK1 immunoprecipitates recognized by the M2 antibody. We noted that detection of a mobility shift of endogenous TK1 during mitotic arrest was rather unclear. The presence of mobility shift for FLAG-TK1, but not TK1, expressed in mitotically blocked cells might be due to the introduction of several negative charges of residues in the FLAG and fusion region, which somehow allowed a clear separation of the phosphorylated isoform of FLAG-TK1 in SDS-PAGE.

Distinct from parental HeLa cells, FT and FN stable cell lines, expressing FLAG-TK1 and FLAG-TK1Ala13, respectively, required a double thymidine block followed by NOC treatment for mitotic arrest. To compare the expression patterns of FLAG-TK1 and FLAG-TK1Ala13 in different phases of the cell cycle, we harvested FT and FN cells in proliferating, mitotic arrest, and recovering from mitotic arrest states for fluoroscein-activated cell sorting analysis to assure that the two cell lines had a similar cell cycle distribution for each treatment condition. The parallel cell lysates obtained under these conditions were subjected to Western blotting (Fig. 2B). It appeared that the steady-state level of FLAG-TK1 was relatively low in the proliferating FT cells and increased in G2/M arrested cells. After release from mitotic blocking, the intensity of the up-shifted band of FLAG-TK1 detected in mitotically blocked cells decreased. In contrast, the steady-state levels of FLAG-TK1Ala13 expressed in FN cells remained rather constantly elevated, without the detection of an up-shifted band in different phases of the cell cycle. This result not only confirmed the presence of mitotic phosphorylation on Ser13 but also indicated that conversion of Ser13 to Ala affects the fluctuation of hTK1 expression in different phases of the cell cycle.

For a better understanding of the biochemical nature of the mitotic phosphorylation of human TK1, we performed phosphorylation reactions in vitro. Both GST and GST-TK1 produced in E. coli and isolated on glutathione-agarose were used as substrates for the phosphorylation reaction carried out with the cell extracts. The GST-TK1 fusion protein was intensively phosphorylated in the reaction performed in the extracts prepared from mitotically arrested HeLa cells (Fig. 3A). In contrast, phosphorylation of GST-TK1 was not detected in the reaction carried out with the proliferating extract. Because GST was not well phosphorylated in either cell extract, the TK1 moiety of GST-TK1 was specifically phosphorylated in the mitotic extract. The phosphoaminoacid analysis of in vitro phosphorylated GST-TK1 revealed that serine is the only residue that is phosphorylated by the mitotic extract (Fig. 3A). For the phosphorylation reaction of GST-TK1 in the mitotic extract, we also observed a prominent phosphorylated band at a molecular size of 26 kDa. Because this phosphorylated band did not appear in other reactions shown in Fig. 3, A and B, we speculated that a molecule present in the mitotic extract formed complex with GST-TK1 on glutathione-agarose and became phosphorylated in the reaction.
Our previous results of in vivo labeling experiments showed that mitotic phosphorylation of TK1 in HeLa cells is reduced significantly by staurosporine treatment at a concentration of 50 nm (24), which is the dosage range for Cdc2 kinase inhibition (31). Staurosporine at a 50 nm concentration could also abolish in vitro phosphorylation of GST-TK1 by extracts of noc-treated HeLa cells (data not shown). Moreover, the sequence around Ser13 of hTK1 is compatible with a Cdc2 kinase consensus site. These facts prompted us to examine whether hTK1 polypeptide is a direct substrate for Cdc2 kinase. It has been demonstrated that p13, a fission yeast protein, can bind avidly to active forms of Cdc2 and Cdk2 kinases (32–34). Therefore, Cdc2 and Cdk2 kinases can be specifically immobilized by p13-agarose from the cell extracts of the mitotically blocked HeLa. The p13-agarose after incubation with the mitotic extract of HeLa cells was used for the in vitro kinase reaction of GST-TK1. Sepharose CL-4B was mixed with the same cell extract and used as a control. Both p13-agarose and Sepharose CL-4B were analyzed by Western blotting and detected by monoclonal antibodies against Cdc2 or Cdk2 kinase (upper panel). Separate sets of p13 and Sepharose CL-4B beads were incubated with GST or GST-TK1 glutathione-agarose for in vitro kinase reaction as described above (lower panel).

Fig. 3. In vitro phosphorylation of hTK1 by extracts of noc-treated HeLa cells. A, the GST and GST-TK1 precipitated by glutathione-agarose were incubated with extracts prepared from proliferating and M phase-arrested HeLa cells in the presence of [γ-32P]ATP as described under “Experimental Procedures.” After incubation, GST and GST-TK1 on glutathione-agarose were washed and eluted. The phosphorylated proteins were analyzed on 10% SDS-PAGE and visualized by autoradiography. The positions of GST and GST-TK1, as visualized by Coomassie Blue staining, are indicated. Two-dimensional phosphoaminoacid analysis of GST-TK1 labeled in vitro by extracts of noc-treated HeLa cells is indicated in the right panel. B, Sepharose CL-4B or agarose conjugated with p13 protein was added to the cell extracts from noc-treated HeLa cells and rotated for mixing at 4 °C for 4 h. The adsorbed beads were then washed three times with PBS, boiled in SDS-PAGE loading buffer, and resolved on 10% SDS-PAGE, followed by transblotting to a PVDF membrane. The membrane was then immunodetected by monoclonal antibodies against Cdc2 or Cdk2 kinase (upper panel). Separate sets of p13 and Sepharose CL-4B beads were incubated with GST or GST-TK1 glutathione-agarose for in vitro kinase reaction as described above (lower panel).

Fig. 4. Mutation on serine 13 abrogates in vitro phosphorylation of hTK1 by Cdc2 or Cdk2 kinase. One half microgram (protein) of purified His-tagged TK1 or His-tagged TK1Ala13 was each incubated with p13-agarose or Sepharose CL-4B for the in vitro phosphorylation reaction as described under “Experimental Procedures.” At the end of the reaction, the supernatant of each reaction mixture was mixed with 150 µl of binding buffer (50 mm NaH2PO4, 300 mm NaCl, 10 mm imidazole, 1 mm PMSF, pH 8.0) and 10 µl of Ni-NTA beads. After binding for 10 min, His-tagged proteins adsorbed on Ni-NTA beads were washed three times with binding buffer and suspended in SDS-PAGE loading buffer, 0.1 volume of which was then subjected to 10% SDS-PAGE and transferred to a PVDF membrane for autoradiography (upper panel). After 1 h of x-ray film exposure, the membrane was immunodetected by hTK antiserum for verification of similar amounts of his-tagged TK1 or his-tagged TK1Ala13 in each reaction (lower panel).
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FIG. 5. Decrease of in vivo phosphorylation of hTK1 by p21<sup>wt</sup> <i>cip1</i>. Ltk<sup>−</sup> cells (2 x 10<sup>5</sup>) were transfected with control vector plasmid pCDM8 (2 μg of DNA) alone, or with pCMV-hTK1 (1.5 μg) together with 0.5 μg of pCDNA3 or pCMV-p21<sup>wt/cip1</sup> as indicated. After each transfection, cells were refreshed with 10% fetal bovine serum in the presence of NOC (0.5 μg/ml) overnight, followed by in vivo [<sup>32</sup>P]orthophosphate labeling for 2 h. Cells were lysed and immunoprecipitated as described in the legend to Fig. 1. Cell lysates (30 μg of protein) from the same set of experiments were separated by 10% SDS-PAGE and immunodetected by antibodies against hTK and p21<sup>wt/cip1</sup> to indicate the ectopic expression of hTK1 and p21<sup>wt/cip1</sup>.

**DISCUSSION**

Phosphorylation has been considered to play a central role in regulating the biochemical events that coordinate progression through the cell cycle. In the present study, we investigated the mitotic phosphorylation of hTK1. The data presented here illustrate that a mutation converting Ser<sup>13</sup> to Ala decreases the extent of mitotic phosphorylation of both native TK1 and recombinant FLAG-TK1 expressed in Ltk<sup>−</sup> fibroblasts and HeLa cells, respectively. We designed an in vitro system to show that M-phase kinase was responsible for TK phosphorylation on serine residue(s). We further demonstrated that Cdc2 or Cdk2 kinase could carry out this in vitro phosphorylation of wild-type TK1, but not mutant TK1<sup>Ala13</sup>. Because coexpression of p21<sup>wt/cip1</sup> in Ltk<sup>−</sup> fibroblasts could also suppress the mitotic phosphorylation of hTK1 expressed in this cell line in a cotransfection experiment, our data suggest that cdc2 or related kinase(s) is involved in mitotic phosphorylation at Ser<sup>13</sup> of the hTK1 polypeptide.

We have previously demonstrated that TK1 expressed a 10-fold higher affinity for thymidine in proliferating HeLa cells than in mitotically blocked cells. Because hyperphosphorylation of TK1 in mitotically blocked HeLa cells is accompanied by a decrease in its affinity for its substrate, thymidine, we have proposed that the physiologic function of specific TK1 phosphorylation by M-phase kinase(s) is to decrease its catalytic efficiency to prevent unnecessary synthesis of dTTP in mitotic cells. However, in this study we did not find any significant difference in the kinetic properties of purified his-tagged TK1 and phosphorylated his-tagged TK1 by Cdc2 or Cdk2 kinase (data not shown). Since we did not rule out the possibility that the extent of TK phosphorylation in vitro was different from that in vivo, the functional significance of phosphorylation of hTK1 during mitotic arrest remains to be established. Nevertheless, we found that mutation of Ser<sup>13</sup> rendered a constitutively elevated expression of FLAG-TK1<sup>Ala13</sup>, independent of the growth state of the cells. These results raise the possibility that phosphorylation of Ser<sup>13</sup> by Cdc2 or Cdk2 kinase influences the level of TK1 expression during the cell cycle. The expression of FLAG-TK1, but not FLAG-TK1<sup>Ala13</sup> in HeLa cells was increased significantly after double thymidine block and NOC treatment. Recently, it has been demonstrated that thymidine treatment can inhibit the growth arrest-specific degradation of TK1 protein in transfected Ltk<sup>−</sup> cells (38). Based on this line of evidence, it is possible that the stability of FLAG-TK1<sup>Ala13</sup> expressed in HeLa cells was relatively unaffected by the treatment of double thymidine block. Given that the expression of FLAG-TK1 and that of FLAG-TK1<sup>Ala13</sup> both are controlled by the CMV promoter, it is logical to assume that the difference of their expressed amounts may result from the effect of Ser<sup>13</sup> mutation at the regulatory level of translational efficiency or degradation.

Analysis of phosphorylation sites for Cdc2 kinase substrates reveals a consensus that requires a minimum of a serine or threonine followed by proline. A search of the hTK1 sequence for the presence of this consensus showed only two potential sites: the serines at positions 13 and 231. We have also carried out a point mutation on Ser<sup>231</sup> located at the C terminus to obtain TK1<sup>Ala231</sup> for an in vivo phosphorylation experiment. Our results showed that the phosphorylation levels of TK1<sup>Ala231</sup> in mitotically blocked Ltk<sup>−</sup> cells were not reduced significantly as compared with that of wild-type TK1 (data not shown). We also found that a mobility up-shift was detectable for FLAG-TK1<sup>Ala231</sup>, but not for FLAG-TK1<sup>Ala13,Ala231</sup>, in stable cell lines after mitotic arrest (data not shown). However, we cannot be certain whether Ser<sup>231</sup> is definitely excluded from phosphorylation. According to these results, we concluded that the mitotic phosphorylation site of hTK1 is primarily on Ser<sup>13</sup> by cyclin-dependent kinase(s) during mitotic arrest. What role phosphorylation on Ser<sup>13</sup> of hTK might play during the G<sub>2</sub>/M transition is still unclear.

Previously, it has been reported that deletion of the C-terminal 40 amino acids of hTK1 can result in a dramatic stabilization of TK1 polypeptide in HeLa cells that have been released from mitotic blocking (19, 20). The C-terminal 30 residues of murine TK1 have also been found to be responsible for triggering TK1 degradation during serum deprivation in an L-cell line that stably expresses murine TK1. As the vaccinia virus and related viral TK proteins are highly homologous to hTK1, but lack 16 N-terminal and 42 C-terminal amino acids (39), it has been suggested that viral TKs have not retained the C-terminal region of the protein because cell cycle regulation is not necessary for virus multiplication (19). By comparing the amino acid sequence of TK from different species, it was also noted that the sequences around Ser<sup>13</sup> are highly conserved in vertebrate TK1s. It has been shown that the truncated form of hTK1 with deletion of the C-terminal 40 amino acids still retains its functional activity (19). We found that Ser<sup>13</sup> is also not required for hTK1 activity. Here, we speculate that both the N and C termini of TK1 have evolved to serve as regulatory domains. At present, it is of interest to determine how phosphorylation on Ser<sup>13</sup> and the C-terminal region interact to regulate the fluctuation of TK1 expression during the cell cycle.

**Acknowledgment**—We thank Dr. Yue Xiong for providing p21<sup>wt/cip1</sup> expression vector. We thank Dr. Su-Ming Hsu for editing the manuscript.

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