Interaction of human thymidine kinase 1 with p21^{Waf1}

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The overexpression of the cyclin-dependent kinase (CDK) inhibitor p21^{Waf1} can inhibit cell proliferation, which is mediated by direct binding to CDK and proliferating-cell nuclear antigen. In this study, we demonstrated that human cytosolic thymidine kinase 1 (TK1) polypeptide can form a complex with p21^{Waf1}. The C-terminal domain of p21^{Waf1} appeared to interact with the TK1 polypeptide, but, despite the inhibitory function of p21^{Waf1}, their association did not alter TK1 functional activity. However,

overexpression of TK1 overcame p21^{Waf1}-mediated growth suppression and blocked the association of CDK2 with p21^{Waf1}, suggesting that TK1 interferes with the inhibitory function of p21^{Waf1}. Based on these results, we here propose that the molecular function of p21^{Waf1} in cells can be perturbed through its interaction with another cellular protein, TK1.

Key words: cell cycle, cell growth, protein interaction.

INTRODUCTION

Cell-cycle progression requires the activation of different cyclin-dependent kinases (CDKs) to initiate biochemical events at the right time and in the right sequence. The activity of these enzymes can be regulated by transient associations with cyclins, reversible phosphorylation reactions and binding of a class of regulators known as CDK inhibitors [1–3]. One of these inhibitory proteins is p21^{Waf1}, which was identified by virtue of its induction by p53 [4], its association with cyclin–CDK complexes [5,6] and its inhibitory effect on DNA synthesis in human senescent fibroblasts [7].

In general, excessive amounts of p21^{Waf1} can inhibit CDK activity and cause growth arrest [8–10]. p21^{Waf1} forms a quaternary complex with cyclins, CDKs and proliferating-cell nuclear antigen (PCNA) [11]. It acts to regulate CDK activity and to inhibit DNA replication directly *in vitro* through its interaction with PCNA [12,13]. In addition, its negative regulation of cell growth has also been found to be through interaction with transcription factor E2F, and thus repression of E2F-mediated transcription [14]. Furthermore, p21^{Waf1} may modulate DNA methylation by disrupting interaction between PCNA and DNA-(cytosine-5) methyltransferase [15]. Recently, it has been shown that cytoplasmic p21^{Waf1} can act as an inhibitor of apoptosis by binding to and inhibition of the apoptosis signal-regulating kinase 1 [16]. Taken together, it seems that p21 in an excess amount plays a negative role when interacting with a number of cellular proteins. However, another line of evidence has shown that human papillomavirus (HPV)-16 E7 oncoprotein interacts with p21^{Waf1} to block p21^{Waf1}-mediated inhibition of CDK activity [17,18]. Thus interacting with an oncogenic viral protein can perturb the inhibitory function of p21^{Waf1}.

Human cytosolic thymidine kinase 1 (TK1) is a salvage-pathway enzyme for dTTP formation. The expression of TK1 is stringently controlled in normal cells during the cell cycle, reaching its peak in the S-phase [19,20]. As TK1 is undetectable in non-dividing cells but highly expressed in tumour cells, de-regulation of TK1 expression may be associated with un-

controlled growth in tumour cells [21–24]. Human TK1 can be phosphorylated in cells during the mitotic phase. We have previously reported that mitotic phosphorylation can be blocked by overexpression of p21^{Waf1} [25]. Further, we have found that TK1 can be co-immunoprecipitated with p21^{Waf1}. In this study, we demonstrate that the C-terminal domain of p21^{Waf1} interacts directly with TK1, and that overexpression of TK1 overcomes p21^{Waf1}-mediated inhibition of cell proliferation by blocking the association of p21^{Waf1} with CDK2.

EXPERIMENTAL

Cell cultures

TK-deficient mouse L-tk⁻ connective fibroblasts were obtained from the ATCC and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Plasmid construction

The construction of plasmids pGex-TK1, pCMV-TK1 and pET-TK1, and preparation of recombinant proteins, GST-TK1 and His-tagged TK1 fusion proteins were described previously [25,26]. Plasmids pGST-p21^{Waf1} and pCMV-p21^{Waf1} were kind gifts from Dr S. J. Elledge (Baylor College of Medicine, Houston, TX, U.S.A.) and Dr Y. Xiong (University of North Carolina at Chapel Hill, NC, U.S.A.), respectively. A PCR was carried out by using pCMV-p21^{Waf1} as a template and two primers to generate a 378 bp DNA fragment with a p21^{Waf1} C-terminal deletion (490–567 nt). Primer 1, 5'-CAAGGCCTGCCGCCG-CC-3', spanned positions 120–137 of the coding strand of p21^{Waf1} cDNA. Primer 2, 5'-CGGAATTCACTGAGAGTCTCC-3', contained nt 478–489 of the non-coding strand of p21^{Waf1} cDNA, in which the underlined TCA indicates the introduced stop codon. pGST-p21^{Waf1} (C-del) was generated by ligation of an *AatI*/*EcoRI* fragment of this PCR product into *AatI*/*EcoRI*-digested pGST-p21^{Waf1} plasmid. The PCR product was also digested with *EcoRI* and treated with Klenow enzyme,

Abbreviations used: TK, thymidine kinase; CDK, cyclin-dependent kinase; GST, glutathione S-transferase; PCNA, proliferating-cell nuclear antigen; HPV, human papillomavirus; Ni-NTA, Ni²⁺-nitrilotriacetate.

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followed by *BspI* digestion. The resultant 334 bp DNA fragment was then ligated into *BspI/EcoRV*-digested pCMV-p21^{Waf1} to generate pCMV-p21^{Waf1} (C-del).

Transient transfection of L-tk⁻ fibroblasts

L-tk⁻ cells were seeded and transfected with a mixture of Lipofectamine (Gibco Life Technologies) and plasmid DNA. After transfection for 24 h, cells were washed and lysed in 50 mM Tris/HCl buffer (pH 7.5) containing 1% Triton X-100, 250 mM NaCl, 50 mM NaF, 1 mM β -mercaptoethanol, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin and 1 mM PMSF. The lysates were centrifuged at 10000 *g* for 15 min and the supernatants were then subjected to immunoprecipitation or immunoblot analysis.

Immunological procedures

Lysates containing equal amounts of proteins (200 μ g) were incubated with antiserum against GST-TK1 (where GST is glutathione S-transferase) or CDK2 antibody (Santa Cruz Biotechnology) for 2 h. Immune complexes were then adsorbed on to Protein A-Sepharose (Pharmacia) and washed five times with the lysis buffer containing 0.1% deoxycholate and SDS. For pull-down assays of GST- and His-tagged fusion proteins, glutathione-agarose beads (Sigma) and Ni²⁺-nitrilotriacetate (Ni-NTA) beads (Qiagen) were used, respectively. Cell lysates or immunoprecipitates were separated by SDS/PAGE (12% gel) followed by electrophoretic transfer on to PVDF membrane (Millipore). After blocking with 5% skimmed powdered milk, the membrane was incubated with the indicated antibody for 2 h. The antibodies and their dilutions were: rabbit polyclonal anti-CDK2 (Santa Cruz Biotechnology), 1:5000; monoclonal anti-human p21 (Santa Cruz Biotechnology), 1:3000; polyclonal anti-human p27 (Transduction Laboratories), 1:5000, and rabbit anti-TK1, 1:1000. Alkaline phosphatase-conjugated goat anti-rabbit IgG or anti-mouse IgG antibodies (Promega) or horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG antibodies (Amersham) were used for the detection of the primary antibodies. Alkaline phosphatase colour development or enhanced chemiluminescence detection for the horseradish peroxidase reaction were performed according to the manufacturer's instructions.

TK activity assay

Immune complexes were suspended in a buffer containing 50 mM Tris/HCl (pH 7.9), 2.5 mM MgCl₂, 5 mM ATP, 0.1% BSA, 90 μ M thymidine, 5 mM NaF, 2 μ Ci of [³H]thymidine (25 Ci/mmol, Amersham) and 1 mM β -mercaptoethanol in a final volume of 100 μ l. The reaction mixtures were then incubated for 10 min at 37 °C. [³H]Thymidine nucleotide formation was determined as described elsewhere [27].

RESULTS

Co-immunoprecipitation of p21^{Waf1} with TK1 polypeptide

To test the ability of TK1 polypeptide to associate with p21^{Waf1} and another CDK2 inhibitor, p27^{Kip1} [28], expression vector pCMV-TK1 was co-transfected with pCMV-p21^{Waf1} or pCMV-p27 into TK-deficient L-tk⁻ mouse fibroblasts. Antiserum against human TK1 was used to immunoprecipitate the transfected cell lysates. The immunoprecipitates were separated by

Figure 1 Co-immoprecipitation of human TK1 with the CDK inhibitor, p21^{Waf1}, from cell extracts overexpressing TK1 and p21^{Waf1} polypeptides

(A) L-tk⁻ cells (5×10^5) were transfected with control vector plasmid pCDNA3 (6 μ g of DNA) alone, or with pCMV-TK1 (3 μ g) together with 3 μ g of pCDNA3, pCMV-p21^{Waf1}, pCMV-p27 or pCMV-p21^{Waf1} + pCMV-p27 as indicated. After each transfection, cells were refreshed with 10% fetal bovine serum for 24 h. Cells were lysed and the lysates (20 μ g of proteins) were separated by SDS/PAGE (12% gel), followed by immunodetection with antibodies against human TK1, p21^{Waf1} and p27 to indicate the ectopic expression of TK1, p21^{Waf1} and p27. Lysates from the same set of experiments were immunoprecipitated (I.P.) by antiserum against TK1. Immunoprecipitates were then separated by SDS/PAGE (12% gel) and transblotted on to PVDF membrane. Immunoblot was probed with antibodies against p21^{Waf1} and p27. (B) GST-TK proteins were expressed in *Escherichia coli* and bound to glutathione-agarose beads as described previously [26]. Beads carrying 20 μ g of GST-TK protein were each incubated with cell extracts (300 μ g of proteins) of L-tk⁻ that had been transfected with control vector (C), pCMV-p21^{Waf1} or CMV-p27 for 24 h. After 30 min of incubation, the beads were washed four times with PBS buffer containing 1% Triton X-100. Proteins eluted by reduced glutathione (5 mM) were boiled in Laemmli sample buffer, separated by SDS/PAGE (12% gel) and transferred to PVDF paper. The immunoblot was probed with antibody against p21^{Waf1} or p27, accordingly. (C) Beads carrying 100 μ g of GST or 20 μ g of GST-TK proteins were incubated with cell extracts (300 μ g of proteins) of L-tk⁻ expressing p21^{Waf1}, eluted and analysed by immunoblotting as described above. For comparison, different proportions of 20 μ g of GST-TK were detected on the same immunoblot, as shown in 'INPUT'.

SDS/PAGE and analysed by immunoblot using antibodies against p21^{Waf1} and p27. It appeared that p21^{Waf1}, but not p27, was precipitated by antiserum against human TK1 (Figure 1A). Similar results were also obtained from co-transfection experiments in which pFLAG-TK1 was used to express FLAG-tagged TK1 recombinant protein and M2 monoclonal antibody specific for the FLAG epitope was used for co-immunoprecipitation (results not shown).

To ensure that co-immunoprecipitation of TK1 and p21^{Waf1} is a result of specific associations involving the p21^{Waf1} and TK1

Figure 2 C-terminal domain of p21^{Waf1} binds to TK1 polypeptide

L-tk⁻ cells (5×10^5) were transfected with control vector plasmid pCDNA3 (6 μ g of DNA) alone, or with pCMV-TK1 (3 μ g) together with 3 μ g of pCDNA3, pCMV-p21^{Waf1} or pCMV-p21^{Waf1}(C-del) as indicated. Cells were lysed and the lysates (20 μ g of proteins) were separated by SDS/PAGE (12% gel), followed by immunodetection with antibodies against human TK and p21^{Waf1} to indicate the ectopic expression of human TK, p21^{Waf1} and C-terminus-deleted p21^{Waf1}. Co-immunoprecipitation (I.P.) was then carried out with the lysates by antibody against human TK, followed by immunoblot analysis with antibodies against human TK and p21^{Waf1} as described for Figure 1.

polypeptides, we then performed *in vitro* interaction by incubating GST-TK-glutathione-agarose with lysates of L-tk⁻ cells over-expressing p21^{Waf1} or p27. After washing the glutathione beads in each incubation mixture, proteins associated with glutathione-agarose were separated by SDS/PAGE. The gel was then immunoblotted for detection of p21^{Waf1} or p27 (Figure 1B). It appeared that bacterially expressed GST-TK could interact with p21^{Waf1}, but not p27, expressed in the L-tk⁻ cell extract. GST beads could not pull down p21^{Waf1} expressed in the lysates, confirming further that p21^{Waf1} expressed in L-tk⁻ cells can form a complex with the TK1 moiety of the GST-TK fusion protein (Figure 1C).

C-terminal domain of p21^{Waf1} is involved in complex formation with TK1

The interaction regions of p21^{Waf1} with CDK2 and PCNA were found to be in the N- and C-termini, respectively [29]. It is known that the N-terminus of p27 shares amino acid sequence similarity with p21^{Waf1}, and here we found that p27 could not form a complex with TK1. Therefore, we examined whether the C-terminal domain of p21^{Waf1} is responsible for complex formation with the TK1 protein. An expression vector containing a p21^{Waf1} mutant with a 26-amino-acid deletion at its C-terminus was constructed. L-tk⁻ cells were transfected with pCMV-TK1 in the presence of the expression vector for the wild-type or C-terminus-deleted mutant of p21^{Waf1}. As shown in Figure 2, only wild-type p21^{Waf1} was detected in the immunoprecipitate obtained with TK antiserum. Thus the 26 amino acids that were deleted from the C-terminal region of p21^{Waf1} are indeed involved in complex formation with the TK1 polypeptide.

Direct interaction *in vitro* of TK1 and p21^{Waf1}

To determine whether p21^{Waf1} interacts directly with TK1 polypeptide, we used bacterially produced and purified GST-p21^{Waf1},

Figure 3 Direct interaction between p21^{Waf1} and TK1 *in vitro*

(A) GST-p21^{Waf1} and GST-p21^{Waf1} (C-del) proteins were purified, resolved by SDS/PAGE (12% gel) and visualized by staining with Coomassie Brilliant Blue R-250. The positions of GST-p21^{Waf1} and GST-p21^{Waf1} (C-del) are indicated by arrows. (B) Fusion proteins (0.25, 0.5 and 1 μ g) of GST-p21^{Waf1} or GST-p21^{Waf1} (C-del) were incubated with Ni-NTA-His-tagged TK1 (containing 2 μ g of His-tagged TK1 protein) and Ni-NTA beads, respectively. After extensive washing with a buffer containing 10 mM Tris/HCl, pH 7.6, 150 mM NaCl, 50 mM NaF, 2 mM EDTA, 0.1% SDS and 0.1% deoxycholate, beads were eluted, boiled in Laemmli buffer and separated by SDS/PAGE (12% gel) for immunoblot analysis by p21^{Waf1} antibody. Lanes 1–4, proteins associated with Ni-NTA-His-tagged TK1 beads; lanes 5–8, proteins associated with Ni-NTA beads.

and His-tagged TK1 fusion proteins to perform *in vitro* interaction assays. His-tagged TK1 adsorbed on Ni-NTA-agarose beads was incubated with various amounts of GST-p21^{Waf1} or GST-p21^{Waf1} (C-del). As a control, Ni-NTA-agarose beads were mixed with the same GST fusion proteins. After extensive washing, all samples were subjected to immunoblotting analysis with p21^{Waf1} antibody. As shown in Figure 3, GST-p21^{Waf1} could be pulled down with Ni-NTA-agarose beads containing His-tagged TK1 protein under conditions *in vitro*. Consistently, the purified C-terminus-deleted p21^{Waf1} fused with GST protein was incapable of interacting with His-tagged TK1 protein. Based on these results, it is likely that TK1 can interact directly with the C-terminal domain of p21.

Effect of associated p21^{Waf1} on TK1 activity

Given that the interaction of p21^{Waf1} with CDKs or PCNA causes their functional inhibition, we examined whether interaction of p21^{Waf1} with TK1 could affect the activity of TK1. For this purpose, L-tk⁻ fibroblasts were transfected with a constant amount of pCMV-TK1 with various amounts of pCMV-p21^{Waf1}. After transfection for 24 h, cells were harvested for TK1 immunoprecipitation in the total lysates. The TK1 immune complexes were then subjected to TK activity assay and immunodetection of TK1 and p21^{Waf1} (Figure 4). The results revealed that TK activity was not decreased by increased amounts of p21^{Waf1} associated with the TK1 immune complexes.

Effect of TK1 on p21^{Waf1}-mediated inhibition of cell proliferation

To test the biological role of this interaction, we examined whether TK1 can affect the inhibitory function of p21^{Waf1} on cell proliferation. L-tk⁻ fibroblasts were transfected with pCMV-p21^{Waf1} in the absence or presence of pCMV-TK1. After trans-

Figure 4 Effect of p21^{Waf1} on the functional activity of TK1

L-tk⁻ cells (3×10^5) were transfected with pCMV-TK1 (1 μg) together with various amounts of pCMV-p21^{Waf1} (1–4 μg) as indicated. After transfection for 24 h, cell lysates (20 μg of protein) were separated by SDS/PAGE (12% gel), followed by immunodetection with antibodies against p21^{Waf1} to indicate the ectopic expression of p21^{Waf1}. The same cell lysates, each containing 100 μg of protein, were also immunoprecipitated by TK1 antibody, followed by immunoblot analysis and TK activity assay. The panel below the graphs shows the TK activity present in the same immune complex.

Table 1 Effect of TK1 expression on p21-mediated growth inhibition

L-tk⁻ cells (4×10^5) were transfected with pCMV-p21, pCMV-p21 (C-del), pCMV-TK1 and control vector as indicated. After transfection for 48 h, cells were trypsinized and cell number was counted in duplicate with Trypan Blue exclusion. Data are means $\pm$ S.E.M. from three independent experiments. Growth inhibition was calculated by comparing the cell-number difference relative to the culture transfected with the control vector only. *Significant difference from cells transfected with pCMV-p21^{Waf1} ($P < 0.05$; unpaired Student's *t* test). wt, wild type.

Control vector (μg)	CMV-TK1 (μg)	CMV-p21 ^{Waf1} (μg)	Cell number ($\times 10^4$)	Growth inhibition (%)
4.0	0.0	0.0	105.0 $\pm$ 6.61	0.0
3.0	1.0	0.0	84.7 $\pm$ 10.5	19.33
1.0	0.0	3.0 (wt)	57.5 $\pm$ 13.92	45.24
0.0	1.0	3.0 (wt)	85.8 $\pm$ 12.58	18.29*
1.0	0.0	3.0 (C-del)	52.5 $\pm$ 1.0	50.01
0.0	1.0	3.0 (C-del)	51.3 $\pm$ 4.0	51.14

fection for 48 h, cell number was counted for each culture. As compared with the control vector, transfection with p21^{Waf1} alone caused a more than 40% decrease in cell number. In cells transfected with both p21^{Waf1} and TK1 expression plasmids, total cell number was increased by almost 30% when compared with the culture transfected with p21^{Waf1} alone (Table 1). In other words, TK1 markedly reversed p21^{Waf1}-mediated inhibition of cell proliferation. In contrast, expression of TK1 did not exert an effect on growth inhibition mediated by the C-terminus-deleted mutant of p21^{Waf1}, which is incapable of interacting with TK1. Since TK1 expression vector alone did not stimulate cell proliferation, it is likely that the interaction between p21^{Waf1} and TK1 plays a role in overcoming p21^{Waf1}-mediated growth inhibition.

Disruption of the association of p21^{Waf1} with CDK2 by TK1

Next we asked whether the interaction of TK1 and p21^{Waf1} affects the amount of p21^{Waf1} in its association with CDK2, so that TK1 can overcome p21-mediated growth suppression. L-tk⁻ fibroblasts were transfected with pCMV-p21^{Waf1} or pCMV-p21^{Waf1} (C-del) in the absence or presence of pCMV-TK1. Antibody against CDK2 was used to immunoprecipitate the cell

Figure 5 Overexpression of TK1 can abrogate association of p21 with CDK2

L-tk⁻ cells (5×10^5) were transfected with a control vector plasmid, pCMV-TK1, pCMV-p21^{Waf1} or pCMV-p21^{Waf1} (C-del) (3 μg each) in different combinations to make a final expression-plasmid mixture of 6 μg. Cells were lysed and the lysates (20 μg of protein) were separated by SDS/PAGE (12% gel), followed by immunodetection with antibodies against CDK2, TK1 and p21^{Waf1} to indicate the ectopic expression of human TK and p21^{Waf1}. Antibody against CDK2 was added to each lysate (200 μg of protein) for the co-immunoprecipitation experiment (I.P.), followed by immunoblot analysis by antibody against p21^{Waf1} as described in Figure 1.

Figure 6 Association of endogenous TK1 and p21^{Waf1}

HeLa(mf) cells (2×10^6) metabolically labelled with [³⁵S]methionine (200 μCi/ml) for 2 h were immunoprecipitated by preimmune TK antiserum, or CDK2 antibody, separately. The immune complexes were separated by SDS/PAGE (12% gel) and transferred on to a PVDF membrane prior to autoradiography (left-hand panel). The same membrane was then probed with p21^{Waf1} monoclonal antibody (right-hand panel). Abbreviations: Ab, antibody; C, control.

lysates. The immunoprecipitates were separated by SDS/PAGE and analysed by immunoblot using antibody against p21^{Waf1}. It appeared that ectopic expression of p21^{Waf1} was precipitated by antibody against CDK2 in L-tk⁻ fibroblasts. However, the amount of p21^{Waf1} present in the CDK2 immunoprecipitate was less in the lysate overexpressing TK1 (Figure 5). Thus p21^{Waf1} association with CDK2 is interfered with by elevated expression of human TK1 in the cells. As for cells expressing the C-terminus-deleted mutant of p21^{Waf1}, the association between CDK2 and p21^{Waf1} (C-del) was not affected by the expression of TK1.

Interaction of endogenous TK1 and p21^{Waf1}

In an attempt to assess the physiological meaning of the interaction between p21^{Waf1} and TK1, we examined further whether the endogenous amounts of p21^{Waf1} and TK1 could also form a complex. Here we used a HeLa-cell subclone, designated HeLa(mf), because this cell line expresses high levels of TK1 and some p21^{Waf1}. HeLa(mf) cells were metabolically labelled with [³⁵S]methionine for the co-immunoprecipitation experiment. We showed that a newly synthesized polypeptide with a molecular size of 21 kDa was included in the TK but not the CDK2 immune complex. By Western-blot analysis using p21^{Waf1} antibody, we confirmed that p21^{Waf1} is present in the TK immune complex (Figure 6).

DISCUSSION

p21^{Waf1} is known to form a complex with cyclin-CDK to exert its inhibitory function on DNA synthesis. In this study, we present the first evidence that p21 can form a complex with human TK1 protein. In spite of the inhibitory function of p21 when interacting with CDKs and PCNA, we did not find the activity of TK1 to be affected significantly when bound to p21^{Waf1}. However, we observed that overexpression of TK1 abrogates the association of ecotopically expressed p21^{Waf1} with endogenous CDK2 present in L-tk⁻ cells and overcomes p21^{Waf1}-mediated growth inhibition, which is the first evidence to demonstrate that the inhibitory function of p21^{Waf1} can be perturbed by interacting with an S-phase cellular protein, TK1.

In this report, the C-terminal domain of p21^{Waf1} was defined as the region that interacts with TK1 polypeptide under conditions both *in vivo* and *in vitro* (Figures 2 and 3). We also found that another CDK inhibitor, p27^{Kip1}, could not exhibit interaction with human TK1, even though p21^{Waf1} and p27^{Kip1} belong to the same class of CDK inhibitor. p27^{Kip1} inhibits a variety of cyclin-CDK complexes in a manner similar to p21^{Waf1}, and the N-terminal domains of both p21^{Waf1} and p27^{Kip1}, which contribute to their interaction region with CDK, share amino acid sequence similarity [30–32]. Therefore, it is unlikely that the N-terminal domain mediates the interaction between p21^{Waf1} and TK1 polypeptide. The C-terminal domain of p21^{Waf1} is also known to interact with PCNA and HPV-16 E7 oncoprotein [17,29]. By computer analysis, however, we did not find the amino acid sequences of PCNA and HPV-16 E7 to share any similarity with that of TK1. The interaction domain of TK1 with p21^{Waf1} remains to be determined.

The role of the p21^{Waf1} protein in growth control has been manifested by its up-regulation during terminal differentiation [33–36] and in cultured cells undergoing senescence [7]. In addition, the p21^{Waf1} gene is induced after DNA damage, and its induction plays a necessary role in p53-dependent cell-cycle arrest [37]. It has been demonstrated that p21^{Waf1} is present in the immunoprecipitated complexes of CDKs in non-transformed normal human fibroblasts, but is absent in simian virus 40-transformed fibroblasts and other tumour cell lines, such as HeLa and HEK 293 [6,12]. Therefore, it has been suggested that the lack of p21^{Waf1} protein in the CDK complexes in transformed cells can cause loss of inhibitory control of CDKs, which in turn contributes to de-regulation of growth control in tumour cells. Given that ectopically expressed p21^{Waf1} could no longer associate with CDK2 when TK1 was simultaneously overexpressed (Figure 5), we hypothesize that elevated expression of TK in some tumour cells not only provides dTTP for DNA replication, but also exerts an effect on attenuating the function of p21^{Waf1}, which can be induced under certain circumstances such as DNA damage. If this hypothesis is true, it may explain partially why

p21 was undetected in the CDK complexes in some tumour cells in which TK1 was highly expressed. In this study, we did detect a weak association between endogenous TK1 and p21^{Waf1} in one clone of HeLa cells [HeLa(mf) cells]. However, in other co-immunoprecipitation experiments using extracts from HL-60, U937 and other clones of HeLa cells, we failed to find a consistent condition for the detection of endogenous TK1 polypeptide associated with p21^{Waf1}, because these cells often express very little p21 but high levels of TK1. We also performed a co-immunoprecipitation experiment with serum-stimulated IMR-90 cells, in which the expression of TK1 is highly regulated. However, we found that p21^{Waf1} was readily detectable in asynchronous and serum-deprived IMR-90 fibroblasts, but disappeared in 24 h-serum-stimulated cells. Conversely, TK1 was maximally expressed in 24 h-serum-stimulated IMR-90 fibroblasts (results not shown). It is, therefore, difficult to detect their endogenous association by the co-immunoprecipitation experiment in this normal cell strain. A consistent physiological condition for the proteins to be expressed simultaneously, which would allow us to understand the meaning of this interaction, remains to be determined. In view of the negative role of the viral protein HPV-16 E7 on the inhibitory function of p21^{Waf1}, our results provided another line of evidence that, in addition to the viral protein HPV-16 E7, the cellular protein TK1 can modulate the inhibitory function of p21.

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