Biochemical characterizations reveal different properties between CDK4/cyclin D1 and CDK2/cyclin A

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Abbreviations: ATP, adenosine triphosphate; CDK, cell cycle dependent kinase; FBS: fetal bovine serum; GST: glutathione-S-transferase; MOI: multitude of infection; RB, retinoblastoma protein; RBC: human RB C-terminal protein from amino acid 780 to 928; SDS: sodium dodecyl sulfate

Abstract

CDK2 and CDK4 known promoter of cell cycling catalyze phosphorylation of RB protein. Enzyme specificity between two CDKs that work at a different cell cycle phase is not clearly understood. In order to define kinase properties of CDK2 and CDK4 in complex with cyclin A or cyclin D1 in relation to their respective role in cell cycling regulation, we examined enzymatic properties of both CDK4/cyclin D1 and CDK2/cyclin A *in vitro*. Association constant, K_m for ATP in CDK4/cyclin D1 was found as 418 M, a value unusually high whereas CDK2/cyclin A was 23 M, a value close to most of other regulatory protein kinases. Turnover value for both CDK4/cyclin D1 and CDK2/cyclin A were estimated as 3.4 and 3.9 min⁻¹ respectively. Kinetic efficiency estimation indicates far over one order magnitude less efficiency for CDK4/cyclin D1 than the value of CDK2/cyclin A (9.3 pM⁻¹ min⁻¹ and 170 pM⁻¹ min⁻¹ respectively). In addition, inhibition of cellular CDK4 caused increase of cellular levels of ATP, even though inhibition of CDK2 did not change it noticeably. These data suggest cellular CDK4/ cyclin D1 activity is tightly associated with cellular ATP concentration. Also, analysis of phosphorylated serine/threonine sites on RB catalyzed by CDK4/

cyclin D1 and CDK2/cyclin A showed significant differences in their preference of phosphorylation sites in RB C-terminal domain. Since RB is known to regulate various cellular proteins by binding and this binding is controlled by its phosphorylation, these data shown here clearly indicate significant difference in their biochemical properties between CDK4/cyclin D1 and CDK2/cyclin A affecting regulation of cellular RB function.

Keywords: CDK2/cyclin A; CDK4/cyclin D1; cell cycle; K_m for ATP; p16; RB phosphorylation site

Introduction

Cell cycle dependent protein kinase (CDK) family proteins possess a serine/threonine protein kinase activity and phosphorylate serine or threonine residue in front of proline with a consensus sequence of (S/T) P (X) (K/R) (Endicott *et al.*, 1999). Their major cellular role is suggested to be involved in the positive control of cell cycle progress. Various cell cycle dependent kinase family proteins; CDK1, CDK2, and CDK4 have been identified (Meyerson *et al.*, 1992; Morgan, 1995). Each cell cycle dependent kinase needs to form a heteroduplex with a specific cyclin protein to become an active kinase (Sherr, 1994). Major cyclin proteins include cyclin B1, cyclin A, cyclin E and D type cyclins. Each cyclin protein appears at a specific cell cycle phase, thus activates its partnering CDK (Sherr, 1994; Pavletich, 1999). CDK4 forms a complex with type D cyclins such as D1 and D2 and the cellular amount of cyclin D1 reaches to the highest level when cells are in Mid-G1, thus CDK4/cyclin D1 activity exists in mid G1 phase. Active CDK4/cyclin D1 is necessary to move cells from G0/G1 phase to S phase (Baldin *et al.*, 1993; Sherr, 1994). Therefore, the inhibition of CDK4/cyclin D1 activity renders cells arrested in G0/G1 phase (Bible *et al.*, 1996; Sherr *et al.*, 1999). When cells escape from proliferative phase to enter terminally differentiated stage, cyclin D1 protein disappears (Kiyokawa *et al.*, 1994; Rao *et al.*, 1994). Various mitogenic signaling elevates cyclin D1 expression, because cellular cyclin D1 level is sensitive to the mitogenic signaling such as ras signaling (Aktas *et al.*, 1997). Cellular inhibition of CDK4/ cyclin D1 activity is also achieved by its inhibitory proteins, *i.e.* p16, p21 and p27 (Sherr, 1999). Among these, p16 is specific to CDK4, whereas p21 and p27

are known to inhibit most CDKs such as CDK1, CDK2 and CDK4 (Endicott *et al.*, 1999; Sherr *et al.*, 1999). In almost 40% of human cancers, p16 gene mutation is observed. For this reason, it is classified as one of the important tumor suppressors (Liggett *et al.*, 1998).

CDK2 is a homologous protein to CDK4 with about 47% homology in amino acid sequence. CDK2 forms a complex with cyclin E or cyclin A to be an active kinase complex (Morgan, 1995). Cyclin E protein is transiently appeared when cells are in G1 to S phase transition (Koff *et al.*, 1992). Cyclin A level is highest when cells are in S phase, meaning that CDK2 kinase activity is a major active cell cycle dependent kinase in S phase (Guadagno *et al.*, 1993). CDK2 was found to be included in a protein complex *in vivo* with cyclin A-Rb-E2F (Nevins *et al.*, 1991; Brechot, 1993). In addition, CDK2/cyclin A effectively phosphorylates Rb protein *in vitro* and this phosphorylation prevents E2F binding to Rb (Suzuki-Takahashi *et al.*, 1995). This suggests one of *in vivo* roles of CDK2/cyclin A activity is to free the transcription E2F from Rb by phosphorylating Rb protein.

RB protein, a tumor suppressor protein is a major *in vivo* target for CDK kinase activity (Goodrich *et al.*, 1991; Lin *et al.*, 1991; Weinberg, 1995). RB protein possesses 10 serine/threonine residues with possible CDK phosphorylation consensus sequence and most these sequences exist in its C-terminal (Weinberg, 1995). Phosphorylation of RB C-terminal prevents transcription factor E2F from binding to RB protein, thus activating transcription activity of E2F (Stevens *et al.*, 2003). E2F increases the transcription of genes promoting DNA replication and cell growth. Besides E2F, so far more than 10 different proteins are shown to bind to RB protein and their binding is interfered when RB is phosphorylated by CDKs (Wang *et al.*, 1993; Welch *et al.*, 1993). All CDKs are known to phosphorylate RB protein *in vitro* (Weinberg, 1995). Cell cycle is very tightly controlled and its deregulation leads to cancer (Sherr, 1996). In many cancers, overexpressions of cyclin proteins and loss of CDK inhibitory proteins were observed (Sicinski *et al.*, 1995; Serrano *et al.*, 1996; Shin *et al.*, 2000; Nam *et al.*, 2001). In this reason, chemical inhibitors for CDKs have been developed and are being tested for the treatment of various human cancers (Fry *et al.*, 2001; Soni *et al.*, 2001; Tan *et al.*, 2002).

Many studies on cell cycle dependent kinases have been done largely for their roles associated with cell cycle regulations at cellular level. However their detailed biochemical studies have not been sufficiently exploited yet. As a step to understand detailed biochemical events related to specific cell cycle phases, we need more knowledge of biochemical differences between CDKs. In this report, we carried out com-

parative study on the enzymatic properties of the purified CDK4/cyclin D1 and CDK2/cyclin A.

Materials and Methods

Construction of expression vectors and generation of baculovirus

Human cDNAs for CDK4, CDK2, cyclin D1 and cyclin A were obtained by RT-PCR using mRNA purified from human fibroblast cells. Each cDNA was constructed as a baculoviral expression vector using pBacPak 8 vector (Clontech) by subcloning full length each cDNA into multicloning site on the vector. For the convenient affinity purification of expressed proteins, glutathione-S-transferase gene was fused to N-terminal of CDK4 gene and CDK2 gene was tagged with hexahistidine at its N-terminal. Each Baculovirus was generated using baculovirus generation Kit from ClonTech by cotransfecting the corresponding expression vector along with baculoviral genomic DNA into sf9 cells. Each viral titer was infected into sf9 cells and the expression of the corresponding protein was confirmed by western blotting using specific antibodies. Human p16 cDNA was obtained by RT-PCR and its bacterial expression vector was generated by subcloning its full length cDNA on pET15b bacterial expression vector with hexa histidine tagging on its N-terminal. Human RB C-terminal (a.a. 780 to 928) expression vector was also generated by subcloning on pET15b vector with hexa histidine tagging on its N-terminal.

Expressions and purifications of recombinant proteins

GST-CDK4/cyclin D1 protein complex was obtained as follows. Sf9 cells were maintained in TNM-FH medium containing 10% FBS in culture dish. Baculovirus carrying GST-CDK4 gene and baculovirus of cyclin D1 were coinfecting into sf9 cells with 10 MOI and left for 2 days before harvest for protein purification. Infected 10⁹ cells were disrupted in 20ml of 20 mM Tris (pH 8.0), 0.1 M NaCl, 5 mM DTT, 0.5 mM EDTA, protease inhibitor cocktail (Roche), 10 mM NaF by sonication. After centrifugation at 12,000 rpm for 30 min using Sovall 34 rotor, the supernatant was loaded in Glutathione conjugated sepharose bead column (Amersham). Unbound proteins were washed out with the column buffer of 20 mM Tris (pH 8.0), 0.1 M NaCl, 5 mM DTT, 0.5 mM EDTA and then bound protein was eluted using the column buffer containing 0.2 M of reduced glutathione (Sigma). This eluate was diluted five fold to reduce NaCl concentration to 20 mM and applied to 1 mM bead volume of mono Q column (Pharmacia-LKB). Bound proteins

were eluted by increasing concentrations of NaCl. For CDK2/cyclin A proteins, viruses carrying hexa histidine tagged CDK2 and cyclin A gene were co-infected into sf9 cells with 10 MOI. Cells were harvested after two days and 10^9 cells were disrupted in 20 ml of 20 mM Tris (pH 8.0), 0.5 M NaCl, 1 mM DTT, 0.5 mM EDTA, protease inhibitor cocktail, 10 mM NaF by sonication. CDK2/cyclin A complex where CDK2 has hexa histidine tagging in its N-terminal, was purified using Ni^{2+} affinity column according to the protocol provided by the manufacturer (Novagen). The expression of human RB C-terminal protein from amino acid 780 to 928 (referred as RBC) and p16 protein were performed in BL21 *E. coli* cells transformed by the expression vectors. Each gene has hexahistidine tagging on its N-terminal. Proteins were purified using Ni^{2+} -affinity column chromatography (Novagen) according to the manufacturer's manual.

Standard enzyme assay

Twenty μl of standard enzyme assay mixture for CDK4/cyclin D1 as well as CDK2/cyclin A contains 100 ng of CDK enzyme, 10 μg of purified RB C-terminal protein of a.a. 780-928 (RBC), 10 μM ATP, 0.2 μCi $\text{p}^{32}\text{-}\gamma\text{-ATP}$ and 5 mM MgCl_2 in 20 mM Tris-HCl (pH 8.0). Enzyme reaction was carried for 10 min and stopped by adding half volume of 30% phosphoric acid. During 10 min reaction time, the reaction rate remains constant. The stopped mixture was spotted in P81 phosphocellulose paper (Whatman) and washed five times with 20 mM Phosphate buffer (pH 6.0) containing 100 mM NaCl. The bound p^{32} -radioactivity in each spot was quantitated using BAS counter (KODAK).

Measurement of K_m for ATP and turnover rate

K_m for ATP was measured using standard assay method except with the serial variation of ATP concentration. In the variation of ATP concentration, the ratio between cold and $\text{p}^{32}\text{-}\gamma\text{-ATP}$ remains constant. The radioactivity on RB C-terminal protein (RBC) from BAS counting was used as the relative enzyme activity and reciprocal plotting method was used to determine K_m value. To determine turn over rate, ATP concentration of 1 mM was used to confer the saturated concentrations of ATP in the enzyme reaction mixture. For other factors, the standard enzyme assay condition described above was used. After 10 min reaction, the same volume of 2 $\times$ lamelli buffer was added to the reaction mixture with subsequent boiling. The samples were separated on 15% SDS-PAGE gel electrophoresis and stained with Coomassie brilliant blue. The RBC protein band with p^{32} radioactivity was excised and the radioactivity on the band was

measured using scintillation counter. This measured radioactivity on RB protein band was compared to the total radioactivity added in initial enzyme reaction mixture. From the ratio of radioactivity on RB protein band to the total p^{32} radioactivity in the initial reaction mixture, we could calculate the total mole number of phosphate transferred to RB protein during reaction. This is based on the fact that the ratio of radioactivity on RB protein band versus the total p^{32} radioactivity in the initial reaction mixture is exactly the same with the ratio of transferred phosphate moles on RB versus the initial ATP moles. The turn over rate was described as pico moles of phosphate transferred to RB C-terminal per min per pico mole of enzyme.

Measurement of cellular ATP concentration

HepG2 cells were grown and maintained using RPMI containing 10% fetal bovine serum. Using 6 well culture dish, 2×10^5 cells were plated on each well and grown overnight. Next day, 2 μM of Flavopiridol (IC_{50} for CDK4/cyclin D1: 70 nM) (kindly provided by Dr. SH Kim) and 20 μM of Roscovitine (IC_{50} for CDK2/cyclin A: 500 nM) (purchased from SIGMA) were treated on the HepG2 cells respectively. For measuring intracellular amount of ATP, we used ATP measuring kit (from SIGMA) that is based on the luciferase activation by depending on the amount of cellular ATP. Cells were lysed using the lysis buffer provided in the kit and 10 μl was used to measure the luciferase activity using luminometer. The luciferase activity of each sample represents the relative amount of cellular ATP.

Phospho-aminoacid analysis

Four μg of RBC (human RB C-terminal) protein was subject to phosphorylation by CDK2/cyclin A or CDK4/cyclin D1 in a standard reaction condition including 5 μCi of $\text{p}^{32}\text{-ATP}$ and separated in 12% polyacrylamide gel electrophoresis and transferred to PVDF (polyvinylidene difluoride) membranes. The portion of membrane containing RBC protein was excised and hydrolyzed in 0.2 ml of 6 N HCl at 110°C for 1.5 h. Samples were then centrifuged at 12,000 g for 10 min, and the supernatants were dried using Speed Vac. The dried pellets were solubilized in a mixture of cold phosphoaminoacid standards (Ser(P), Thr(P), and Tyr(P), 25 nmol each) and analyzed by two dimensional thin layer cellulose electrophoresis, using 750 V for each dimension. The each radio activity from phospho-serine, phospho-threonine and phospho-tyrosine spots was quantitated using BAS phosphoimager (Kodak).

Results

Expression and purification of CDK4/cyclin D1 and CDK2/cyclin A using baculoviral system

CDK4 and CDK2 are known to form a specific complex with cyclin D1 and cyclin A respectively to become enzymatically active (Morgan, 1995). To obtain active CDK4/cyclin D1 heterocomplex protein, the sf9 cells were coinfecting with the baculoviruses carrying full length CDK4 gene tagged with GST gene in its N terminal along with the viruses carrying a full length cDNA of cyclin D1. Fusion of C-terminus of glutathione S transferase gene to CDK4 gene for the affinity purification of expressed protein resulted in the increased solubility of CDK4 protein after overexpression in sf9 cells. Without fusion to GST, CDK4 protein tends to form an inclusion body after being overexpressed (data not shown). The two expressed proteins were expected to form a complex within cells (Kato *et al.*, 1994). The purified protein using glutathione bead affinity column chromatography, contained both CDK4 protein and cyclin D1 (Figure 1A). In the Mono Q column chromatography, GST-CDK4/cyclin D1 complex protein was eluted as a single symmetrical peak at 0.4 N NaCl (Figure 1B). This result indicates a tight association between CDK4 and cyclin D1. CDK2/cyclin A protein was similarly obtained after cotransfection of baculovirus carrying CDK2 and cyclin A. Hexahistidine tagging in N-terminal CDK2 gene enabled the purification of CDK2/cyclin A complex protein over 90% homogeneity by a single Ni^{2+} affinity purification (data not shown). This purified protein was used for subsequent biochemical characterizations.

Development of CDK2/cyclin A and CDK4/cyclin D1 enzyme assay protocol

In order to measure CDK2/cyclin A and CDK4/cyclin D1 enzyme activity quantitatively, we have developed a p81 filter binding assay method using hexa histidine tagged RB C-terminal protein (RBC) as a substrate and it is schematically described in Figure 2. RB C-terminal protein (amino acid; 780 to 928) has a positive charge at neutral pH (PI value; 10.4). Spotting the reaction mixture onto p81 cellulose filter and washing subsequently with 20 mM Phosphate buffer (pH 6.0) containing 0.1 M NaCl retains the RBC protein on the paper whereas unreacted negatively charged free ATP is almost washed away. The degree of phosphorylation of RBC by CDK2 or CDK4 activity does not interfere with the protein binding onto p81 filter paper.

K_m value of CDK4/cyclin D1 for ATP is very high compared to that of CDK2/cyclin A

K_m value for ATP and turn over rate of CDK4/cyclin D1 and CDK2/cyclin A were determined respectively. CDK4/cyclin D1 and CDK2/cyclin A use dual substrates such as ATP and RB protein. In this measurement, we used 20 μM of purified human RB C-terminal protein (a.a. 780 to 928) as a substrate. However with this RB concentration, enzyme activity still did not reach to maximum (data not shown). However, because increase over this RB concentration caused experimental inconveniences, we performed all enzyme kinetic experiments at 20 μM of RBC concentration. From the reciprocal plot (Figure 3A), K_m of CDK2/cyclin A for ATP was measured as 23 μM and this value is similar to K_m for ATP of most other protein kinases involved in signal transductions (Su *et al.*,

Figure 1. Purification of CDK4/cyclin D1. (A) One L culture of sf 9 cells coinfecting with GST-CDK4 and cyclin D1 baculovirus was lysed by sonication and centrifuged. The soluble supernatant was subjected to glutathione-agarose affinity column chromatography. After washing unbound flow-through proteins, the bound protein was eluted with 5 ml of buffer containing 20 mM reduced glutathione. Each protein sample in the course of chromatographic step was added with equal volume of 2×Laemli buffer and electrophoresed in 10% SDS-polyacrylamide gel. The gel was stained with Coomassie brilliant blue. Lysate: total lysate, Supernatant: supernatant after centrifugation. Unbound FT: unbound flow through. Eluate: eluate with 20 mM reduced glutathione. (B) The eluate from glutathione column was applied to Q-sepharose ion exchange column chromatography. The bound protein was gradually eluted by stepwise increase of NaCl concentration. Each fraction was added with equal volume of 2×Laemli buffer and electrophoresed in 10% SDS-polyacrylamide gel. The gel was stained with Coomassie brilliant blue.

1996). However K_m of CDK4/cyclin D1 for ATP showed a value of $418 \text{ } \mu\text{M}$ (Figure 3B). Compared to other cellular regulatory kinases, this is an abnormally high K_m value for ATP. Next, we measured the turnover rate of the two CDK kinases. In the measurement, we used 1 mM of ATP and $20 \text{ } \mu\text{M}$ of RBC protein concentration. The reason why we used ATP concentration of 1 mM is due to the high K_m value for ATP

Figure 2. Schematic representation of p81 filter binding assay for CDK2/cyclin A and CDK4/cyclin D1. Detailed explanation is described within the text.

of CDK4/cyclin D1. In these substrate concentrations, CDK2/cyclin A gives transfer of $3.4 \text{ pmole phosphate per min, per pmole enzyme}$. This turnover rate value of CDK2/cyclin A shown here was similar to the previously reported value of $3.8 \text{ pmole per min per pmole enzyme}$ for *in vitro* activated CDK2/cyclin A (Jeffrey *et al.*, 1995). This means that the purified CDK2/cyclin A enzyme from sf9 cells is in the active state. On the other hands the turn over rate of CDK4/cyclin D1 was measured as $3.9 \text{ pmole per min per pmole enzyme}$. This value is slightly higher than that of CDK2/cyclin A, indicating that CDK4/cyclin D1 we purified is as much an active form as CDK2/cyclin A. However, their K_m values for ATP are different by 18 fold. Kinetic efficiency (turnover number over K_m) estimation indicates over one order magnitude less efficiency for CDK4/cyclin D1 than CDK2/cyclin A ($9.3 \text{ } \mu\text{M}^{-1} \text{ min}^{-1}$ for CDK4/cyclin D1 and $170 \text{ } \mu\text{M}^{-1} \text{ min}^{-1}$ for CDK2/cyclin A). Intracellular ATP concentration that varies from $10 \text{ } \mu\text{M}$ to 1 mM has been shown to be a regulatory factor for p27 protein to regulate cell cycle (Sheaff *et al.*, 1997). Our data suggest that the intracellular ATP concentration could be an important regulatory factor for *in vivo* CDK4/cyclin D1 activity because its K_m value is so high that a high local ATP concentration such as 1 mM is required for most CDK4/cyclin D1 within cells to work.

Inhibition of cellular CDK4 activity changes the intracellular ATP concentration

Given the interesting possibility that cellular CDK4/cyclin D1 activity depends on cellular ATP concen-

Figure 3. Reciprocal plotting of $1/V$ versus $1/[\text{ATP}]$ for CDK2/cyclin A and CDK4/cyclin D1. (A) With the variation of ATP concentration, CDK2/cyclin A enzymatic activity was measured with triplicate by the standard assay condition and the reciprocal plot of $1/\text{relative activity}$ ($1/V$) vs $1/[\text{ATP}]$ was plotted. At least three measurements of the kinase activity were done. A straight line was obtained by linear regression. The K_m for ATP was calculated from x-intercept. (B) With the variation of ATP concentrations, CDK4/cyclin D1 enzymatic activity was measured with triplicate by the standard assay condition and the reciprocal plot of $1/\text{relative activity}$ ($1/V$) vs $1/[\text{ATP}]$ was plotted and K_m for ATP was obtained from x-intercept.

tration, we next examined a possibility that inhibiting CDK4/cyclin D1 in cells could change cellular ATP concentration. When we treated HepG2 cells with 2 μ M of Flavopiridol, a specific CDK4 inhibitor (Bible *et al.*, 1996), cellular ATP concentration increases up to about 3 fold during 8 h treatment (Figure 4). This increase occurs ahead of cell cycle arrest that is observed normally from 12 h after Flavopiridol treat-

Figure 4. Measurement of cellular amount of ATP. Hep G2 cells were treated with 2 μ M of Flavopiridol (filled circle) or 20 μ M of roscovitine (open circle) for indicated times. Cells were harvested in 500 μ l of lysis buffer and 10 μ l was used to measure the luciferase activity using the ATP measuring kit from Sigma as described by the manufacturer. Three measurements were done and the luciferase activity was plotted at each treated time with standard deviation. The luciferase activity of each lysate represents the relative ATP concentration in the cell lysate.

ment (Bible *et al.*, 1996). At this time, it is not clear why inhibition of CDK4 makes cellular amount of ATP increased. On the other hand, inhibition of cellular CDK2 activity by treating HepG2 cells with its specific inhibitor, Roscovitine (IC_{50} : 500 nM) for 8 h did not give any considerable change of cellular ATP concentration (Figure 3). Along with the fact that K_m of CDK4/Cyclin D1 for ATP is 418 μ M, this data emphasize the possibility that regulation of cellular CDK4/cyclin D1 activity is tightly associated with the cellular concentration of ATP.

Preferential phosphorylation sites in RB C-terminal are different between CDK4/cyclin D1 and CDK2/cyclin A

CDK4/cyclin D1 and CDK2/cyclin A belong to proline directed kinases, that phosphorylate serine or threonine ahead of proline residue and are shown to share a preferential consensus peptide substrate sequence, (S/T) P X (R/K) (Endicott *et al.*, 1999). The phosphorylation of RB C-terminal domain is known to free transcription factor E2F from RB binding, thus activates E2F dependent gene expressions (Draus *et al.*, 2001; Stevens *et al.*, 2003).

Even though RB C-terminal phosphorylation by CDKs has been well known so far, detailed differences in this phosphorylation by each CDK are barely known. In order to exploit possible differences in the phosphorylation of RB C-terminal sites by CDK4/cyclin D1 and CDK2/cyclin A, we performed phospho-amino acid analysis on RB C-terminal peptide (a.a. 780 to 928) after enzymatic reaction with these CDKs. There

A

SPIPHIPRS[□]SPYKFPS[□]SLRIPGGNIYI
 SPLK[□]SPYKISEGLPT[□]PTKMT[□]TPRSRI
 LVSIGESFGTSEKFQKINQMVCNSD
 RVLKRS[□]AE[□]GSNPPKPLKKLRFDIEG
 SDEADGSKHLPGESKFQQLAEMT
 STRTRMQKQKMND[□]SDMTSNKEEK

B

C

		Relative P^{32} radioactivity	
		CDK2/cyclin A	CDK4/cyclin D1
P-Ser	P-Ser	18622 (65.2%)	25038 (82.0%)
	P-Thr	9729 (34.0%)	5152 (16.9%)
P-Tyr	P-Tyr	232 (0.8%)	351 (1.1%)
	Total	28583 (100%)	30541 (100%)

Figure 5. Phospho-amino acid analysis of human RB C-terminal domain after phosphorylation reaction by CDK2/cyclin A or CDK4/cyclin D1. (A) Amino acid sequence of the RB C-terminal (a.a. 780 to 928) are shown and the possible phosphorylation sites of serine and threonine residues are marked with rectangular boxes. (B) After the phosphorylation reaction using P^{32} -ATP, the RB C-terminal protein was separated in 15% SDS-PAGE gel and transferred to PVDF membrane. The radioactive RB band was excised and hydrolyzed. The mixture of hydrolyzed amino acids were separated on TLC plate along with cold phosphoserine, phosphothreonine and phosphotyrosine as references. The radioactivity on each assigned phosphoamino acid was autoradiographed. (C) The radioactivity on each spot was quantitated using BAS P^{32} phospho-imager (Kodak) and summarized along with % value in parenthesis compared to the sum of radioactivity from all three spots.

are seven possible sites that can be phosphorylated by CDKs within this peptide (Figure 5A). Among these residues, five are serines and two are threonines as depicted by letters within rectangle box in Figure 5A. In the phosphorylation by CDK2, the ratio of p^{32} radioactivity distribution in serine spot: threonine spot: tyrosine spot is 65.1:34.0:0.9% respectively. Whereas, in case of CDK4, its distribution in serine spot: threonine spot: tyrosine spot was 81.9:16.9:1.1% respectively (Figure 5B and C). These data indicate that CDK2/cyclin A tends to phosphorylate two threonine residues with about 2 fold higher preference than CDK4/cyclin D1. In addition, CDK4/cyclin D1 prefers serine residues for its phosphorylation site more than CDK2/cyclin A does. This differential preference for phosphorylation site between two CDKs will result in differently phosphorylated RB C-terminal proteins. At this time it is not clear the functional significance of this differential phosphorylation between two CDKs. However, we interpret that differential phosphorylation of RB C-terminal by CDK2 and CDK4 would be necessary for cell cycle stage dependent association and dissociation of RB protein with its specific binding proteins. This is based on the fact that more than 10 proteins are known to bind RB protein (Weinberg, 1995) and most of these bindings are shown to be affected by serine/threonine phosphorylation in RB C-terminal. The sum of p^{32} radioactivity in the spots of all three amino acids after the phospho-amino acid analysis reaction was almost identical between CDK2/cyclin A and CDK4/cyclin D1 (Figure 5C). This indicates two enzymes share a similar reaction rate when ATP is provided sufficiently, confirming the similar turn over rates between them as shown above.

p16 inhibition of CDK4/cyclin D1 activity is potent and may not be interfered by the complex formation between CDK4 and cyclin D1

p16 is known to bind tightly to CDK4 protein and inhibits CDK4/cyclin D1 enzymatic activity, probably by preventing CDK4 from forming an active structural conformation exerted by binding of cyclin D1 (Pavletich, 1999; Russo *et al.*, 1998). When we measured the potency of this inhibition using the standard assay, it gives about 3 nM of IC_{50} value (Figure 6). Whereas Flavopiridol, a compound under the clinical trial (Bible, 1996; Knockaert *et al.*, 2002) as CDK4 inhibitor showed 60 nM of IC_{50} in a parallel measurement similar to the reported value of 70 nM. As reported previously, p16 did not inhibit CDK2/cyclin A (data not shown). This confirmed that p16 is a very potent and selective inhibitor against CDK4/cyclin D1. Next we performed this inhibition assay with a preincubation of p16 and CDK4/cyclin D1 for an hour before subject to inhibition assay on a purpose to give

Figure 6. Inhibition of CDK4/cyclin D1 activity by its inhibitors. In the absence or presence of various amounts of purified p16 protein with or without preincubation, inhibition assay was performed 3-5 times under the standard assay condition. In a same way, inhibition by Flavopiridol was performed as well and compared to that by p16. Percent of relative CDK4/cyclin D1 activity compared to the activity without inhibitor at various concentrations of inhibitors was plotted with standard deviations. IC_{50} value was assigned as the concentration of inhibitor that gives 50% inhibition of CDK4/cyclin D1 activity. filled circle; inhibition curve by p16 without preincubation, filled triangle; inhibition curve by p16 with preincubation, open circle; inhibition curve by Flavopiridol.

an enough time to interfere CDK4/cyclin D1 complex by p16. Interestingly this preincubation did not change the IC_{50} value (Figure 6). This suggests that the degree of complex formation between CDK4 and cyclin D1 does not affect the inhibition by p16 at all. In other words, p16 could inhibit CDK4 whether it is alone or complexed with cyclin D1 with a same efficiency.

Discussion

Cell proliferation is the fundamental basic process for life. In cell proliferation, development and differentiation, CDK4/cyclin D1 activity plays a pivotal regulatory role (Sherr, 1994; Ekholm *et al.*, 2000). In this reason, cellular CDK4/cyclin D1 activity is tightly controlled by various positive and negative regulations. Its unbalanced regulation often results in diseases such as cancer (Sherr, 1996; Robles *et al.*, 1998; Krimpenfort, *et al.*, 2001). In this respect, the precise understanding of CDK4/cyclin D1 is important to elucidate the mechanisms of related diseases and develop their therapeutic intervention strategies. Most studies on the activities of CDK4/cyclin D1 have been done at cellular level. Therefore results presented here from the biochemical studies of purified CDK4/cyclin D1 protein compared to CDK2/cyc A provide us with another crews for the better understanding of

CDK4/cyclin D1.

One of interesting facts from this study is that the K_m of CDK4/cyclin D1 for ATP is 418 M. Most protein kinases involved in the signal transduction are known to have k_m for ATP in the range of 1-30 M. Given that cellular ATP concentration varies from 10 M to 1 mM, cellular CDK4/cyclin D1 activity should depend significantly on the variation of intracellular ATP concentration. In other words, control of intracellular ATP concentration could be one of key tools to regulate the cellular activity of CDK4/cyclin D1. Another interesting data shown here to suggest a possible tight relationship between CDK4/cyclin D1 activity and intracellular ATP concentration is that the inhibition of cellular CDK4 activity by its specific pharmacological inhibitor, Flavopiridol increases the cellular ATP content whereas the inhibition of CDK2 activity by its known inhibitor, Roscovitine does not give any significant effect on cellular amount of ATP. The reason for increase of ATP upon cellular CDK4 inhibition is not clear at this time. One of possible interpretations is that cells might resist to an abrupt cell cycle blocking by increasing cellular ATP content. The inhibition of CDK2/cyclin A by p27 is known to be dependent on cellular amount of ATP (Sheaff *et al.*, 1997). According to the paper, high concentration of ATP causes the ATP binding pocket of CDK2 filled by ATP, which lead to CDK2/cyclin A activity free from the inhibition by p27. In supporting this observation, it has been shown from the X-ray crystallographic study that the binding region of p27 in CDK2 includes ATP binding pocket (Jeffrey *et al.*, 1995). Taken together, these observations imply that cellular ATP concentration plays a role in the regulation of cell cycle process. In addition, ATP is known to have mitogenic activities in several cells (Chung *et al.*, 1997). However we need more works to decipher molecular mechanisms for the increase of cellular ATP amount upon the inhibition of CDK4/cyclin D1 activity.

RB is phosphorylated in cycling cells and becomes unphosphorylated as cells are arrested in G0/G1 (Goodrich *et al.*, 1991; Lin *et al.*, 1991). The phosphorylation of RB is known to affect its binding to other proteins such as E2F and this is one of the main mechanisms to regulate cellular RB function (Stevens, 2003). *in vitro*, all of CDK1, CDK2 and CDK4 can phosphorylate RB and the phosphorylated sites exist mostly in its C-terminal region (Endicott, 1999). However, each CDK has a distinct role in its corresponding cell cycle phase. It is possible to speculate that each CDK might phosphorylate serine and threonine residues in RB C-terminal with a different pattern from other CDKs and this might contribute to a distinct complex formation between RB and cellular proteins depending on a specific cell

cycle phase. In data presented here, it is confirmed that CDK2 and CDK4 generate differently phosphorylated RB C-terminal proteins. We postulate that this different phosphorylation pattern might be necessary for the regulation of interactions between RB and cellular proteins differently in mid G1 and S phase. However, in order to elucidate a concrete cellular meaning of this differential phosphorylation in respect of cell cycle regulatory function by RB, further studies should be necessary.

The reason why CDK4/cyclin D1 draws a lot of research interests is that its activated activity is directly linked to cancer (An *et al.*, 1999; Krimpenfort *et al.*, 2001; Sotillo *et al.*, 2001). This fact has stimulated a lot of research efforts to develop its specific inhibitors as an anticancer agent (Senderowicz *et al.*, 2000; Knockaert *et al.*, 2002). Two CDK4/cyclin D1 inhibitors have been tested extensively for therapeutic possibility of cancer. One is p16, a natural specific protein inhibitor and the other is a small molecular weight chemical, flavopiridol (Bible *et al.*, 1996; Liggett *et al.*, 1998; Harrington *et al.*, 2001). So far the exact degree of inhibition of CDK4/cyclin D1 by p16 has not been published yet. In this study, we obtained 3 nM of IC_{50} value for p16 while flavopiridol gave 70 nM of IC_{50} value. This indicates the inhibition by p16 is very potent, compared to the chemical inhibitor, flavopiridol. p16 protein is known to bind to monomeric CDK4 protein. The fact that preincubation of CDK4/cyclin D1 with p16 before starting enzyme inhibition reaction did not improve the measured IC_{50} value at all suggests that CDK4 binding to p16 is much more preferential over its association with cyclin D1. These inhibitory features could make p16 as a good molecule for targeting *in vivo* CDK4 activity in cancer cells. However its delivery technique into cancer cells within body could be a big huddle to develop it as a therapeutic purpose.

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