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Biological Effects of G1 Phase Arrest Compound, Sesquicillin, in Human Breast Cancer Cell Lines

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Abstract—Sesquicillin, isolated from fungal fermentation broth, strongly induced G1 phase arrest in human breast cancer cells. During G1 phase arrest, the expression level of cyclin D1, cyclin A, and cyclin E was decreased, and the expression of CDK (cyclin-dependent-kinase) inhibitor, protein $p21^{Waf1/Cip1}$, was increased in a time-dependent manner in a breast cancer cell MCF-7. Interestingly, the G1 phase arrest induced by sesquicillin also occurred independently of the tumor suppressor protein, p53. Sesquicillin inhibits the proliferation of MCF-7 via G1 phase arrest in association with the induction of CDK inhibitor protein, $p21^{Waf1/Cip1}$, and the reduction of G1 phase related-cyclin proteins.

Introduction

Cell-cycle dysregulation has important consequences in relation to human disease and inappropriate cell cycle progression is a critical feature in tumor cell. Especially, it is the hallmark of transformed cells that they lack appropriate checkpoint control. G1 phase of cell cycle is an important period where various complex signals interact to determine whether to divide or to remain in a state of quiescence (known as G0).[1] For a cell to pass the G0 state, cyclin-dependent-kinases (CDKs) have to be activated and phosphorylates the tumor suppressor protein, retinoblastoma (Rb) protein, that normally is found in a hypophosphorylated form bound to the transcription factor, E2F. Phosphorylation of Rb leads to the release of transcriptionally active E2F and the S phase progression can occur. It was reported that the CDK-Rb pathway at G1/S transition phase is heavily mutated in human tumor cells. The cyclin-dependent-kinase inhibitors (CDKIs) have different patterns of expression in diseases. The CDK inhibitors Kip/Cip inactivate the cyclin/CDK complexes in the G1 phase leading to cell-cycle arrest and the mutations or deletions of INK4 (inhibitor of cyclin-dependent kinase 4) have been described in human malignancies.[2] This observation has raised a level of interest in the G1 phase regulatory molecules such as CDK, cyclins, Rb, CDKIs, and p53 as potential therapeutic targets in diseases where control of inappropriate cellular proliferation would be a therapeutic benefit.[3]

In the course of screening for G1 phase arrest compound, sesquicillin was isolated from a microbial organism. Sesquicillin has previously been reported as having bronchos pasmolytic, antihypertensive, anti-inflammatory, and laxatico activities.[4] Recently, it has also been reported to be an inhibitor of glucocorticoid-mediated signal transduction.[5]

In the present study, the effects of sesquicillin on the cell cycle and proliferation were investigated in the human breast cancer cell lines. To investigate the potential mechanisms of the G1 phase arrest activity of sesquicillin, the expression of cell-cycle-related proteins (cyclins, CDKs, and p53), the activity of CDKs, and phosphorylation states of Rb protein in cancer cells were also determined. In addition, the expression levels of CDKIs, such as $p21^{Waf1/Cip1}$, $p27^{Kip1}$, and $p16^{INK4}$, were examined in MCF-7 cells.[6]

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Results and Discussion

Isolation

The ethylacetate extract of the fermentation broth of fungal strain F60063 was fractionated by silica gel flash chromatography and HPLC. Finally, compound **1** was obtained as a colorless powder. The structure of this compound was elucidated by spectroscopic method (NMR, MS, IR, and UV). Its melting point (165–167 °C) and other spectral data were identical to those of sesquicillin whose structure has previously been reported (Fig. 1).[5]

Proliferation activity of several cancer cell lines treated with sesquicillin

An effect of sesquicillin on the cell proliferation of the five cell lines MCF-7, T47D, MDA-MB-468 (human breast cancer cell), SW620 (human colon cancer cell), and SNU354 (human gastric cancer cell) was examined using a cell proliferation assay kit. Sesquicillin exhibited a dose dependent inhibition of cell growth in a broad range of concentrations and the GI_{50} value of sesquicillin for in vitro growth inhibition was 10 μg/mL for MCF-7, 1.2 μg/mL for MDA-MB-468, 12 μg/mL for SW620, and 10 μg/mL for SNU354, respectively.

Induction of G1 arrest in the sesquicillin treated human breast cancer cell lines

To assess effects of sesquicillin on the cell cycle, a time course FACS analysis was performed in drug treated cells. 3×10^5 cells (30% confluence in flask) were seeded into a T25 flask with RPMI 1640 complete medium, and incubated overnight. The medium was replenished either with sesquicillin (20 μg/mL) or with vehicle solvent 0.1% DMSO containing the medium. Figure 2 and Table 1 show that, when sesquicillin (20 μg/mL) was treated in the MCF-7 cells, the cell cycle was arrested in the G1 phase. After 48 h sesquicillin treatment, more than 90% of the MCF-7 cells were in the G1 phase. The cell viability was consistently greater than 80% in cells treated for 72 h, and then there was a slight loss of viability.

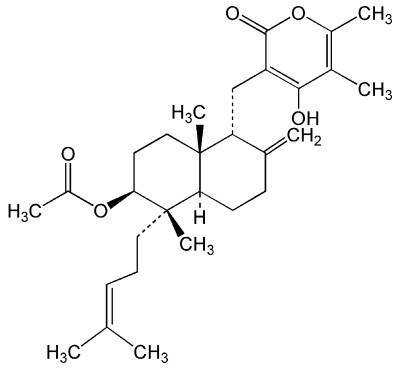

Figure 1. Structure of sesquicillin.

Effects of sesquicillin on the protein level of cyclins and CDKs in MCF-7 cells

To understand the molecular events involved in the sesquicillin activity regarding G1 phase arrest, the effect on the expression level of cyclins and CDKs was investigated in sesquicillin treated MCF-7 cells. Because the G1/S transition is regulated by D-type cyclins that bind to and activate CDK4, and cyclin E which activates CDK2. The level of G1 related proteins, such as CDK4, CDK2, cyclin D1, E, and A, when sequicilin was treated, showed a quiet different pattern as demonstrated in Figure 3. There was a little detectable decrease in the level of CDK1, CDK2, and CDK4, even after 48 h exposure to sesquicillin. However, unlike CDKs, the expression levels of cyclins were dramatically decreased in sesquicillin-treated MCF-7 cells. In particular, cyclin D1 level almost completely disappeared after 12–48 h treatment. The cyclin A and cyclin E level was also markedly decreased after 24 h.

In vitro kinase activity, and regulation of Rb phosphorylation by sesquicillin

CDK2/cyclin E is activated later at the G1/S boundary and throughput the S phase, and CDK4/cyclin D1 is activated in G1 only. Functional activation of the two kinases is certainly regulated from the G1 to S transition. As sesquicillin induced G1 phase arrest in MCF-7 cells, we tested whether sesquicillin has in vitro inhibitory activity of G1 phase-related kinases (CDK4/cyclin D1 and CDK2/cyclin E). In vitro kinase assay was performed in various concentration of sesquicillin (from 1 up to 100 μg/mL) with purified enzyme CDK4/cyclin D1 and CDK2/cyclin E. Sesquicillin did not exhibit any in vitro inhibition activity at effective concentrations shown to inhibit proliferation or induce G1 phase arrest (data not shown).

The tumor suppressor protein Rb has a central role in G1 cell cycle progression.[7–10] The highly phosphorylated form of Rb is increased significantly at the G1–S boundary. As the hypophosphorylated form of Rb

Table 1. Effects of sesquicillin on cell cycle progression of human breast cells

Time		% of cells		
		G1	S	G2/M
12 h	DMSO	56.58[a]	34.03	8.89
		57.82[b]	30.20	11.98
	Sesquicillin	68.47	20.43	11.09
		78.56	12.41	9.03
24 h	DMSO	58.08	31.06	10.86
		63.97	23.25	12.78
	Sesquicillin	87.41	6.46	6.13
		83.65	5.90	10.45
48 h	DMSO	55.62	34.63	9.74
		66.01	20.68	13.32
	Sesquicillin	92.94	4.34	2.72
		84.33	4.24	11.24

[a]MCF-7 cell.
[b]T47D cell.

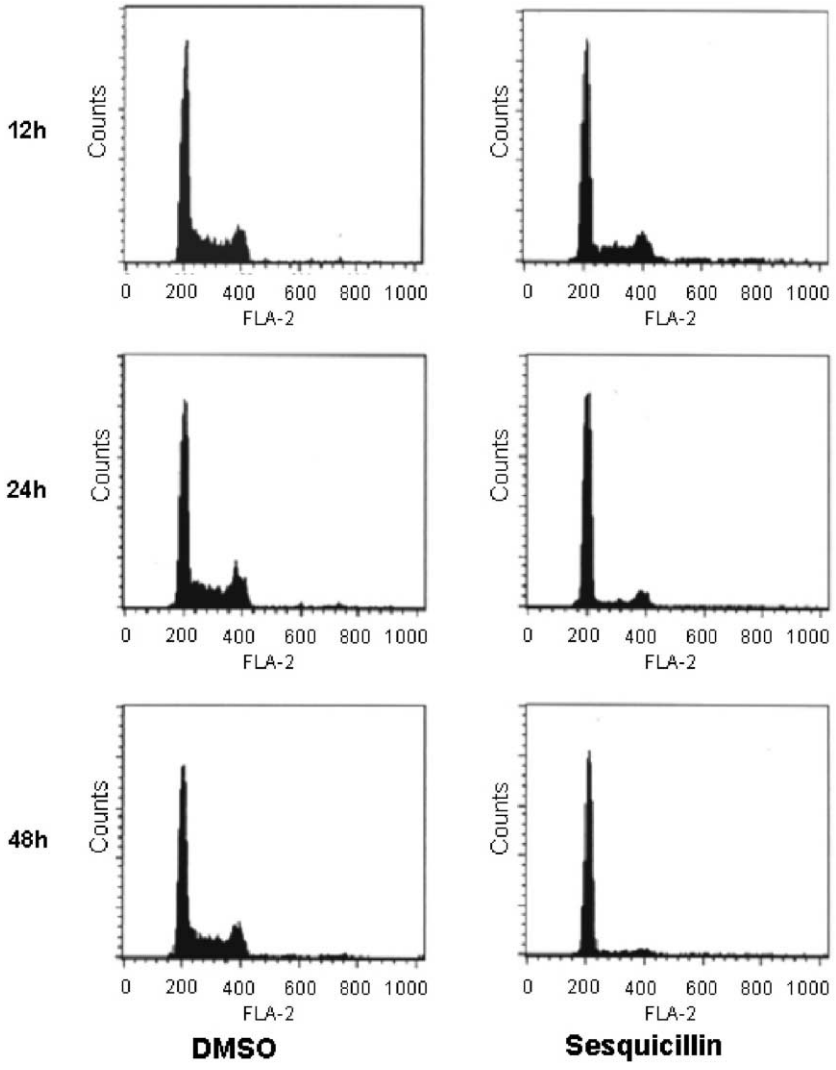

Figure 2. Cell-cycle profiles of MCF-7 cell in the presence of sesquicillin. Human breast cancer cell line, MCF-7 was treated with sesquicillin (20 μg/mL) or with vehicle solvent (0.1% DMSO) for the indicated times. At 12, 24, and 48 h after sesquicillin treatment, cells were harvested and fixed with 70% EtOH. Fixed cells were incubated with RNase A and stained with propidium iodide. 20,000 fixed cells were subjected FACScalibur analysis to determine the distribution of cells throughout the G1, S, and G2/M phase.

migrates faster than the hyperphosphorylated form on a SDS-PAGE, two types of Rb proteins are, thus, easily discernable by western blot analysis. To determine CDK4 activity in sesquicillin treated cells, the degree of phosphorylation of the Rb protein was analyzed by western blot analysis with monoclonal anti-Rb antibody. As shown in Figure 4, sesquicillin caused a significant decrease in the amount of hyperphosphorylated Rb (ppRb), and a large amount of hypophosphorylated Rb (pRb) was also detected at 12 h. After 36 and 48 h exposure, ppRb protein could be not detected in sesquicillin treated cells and a trace amount of pRb was observed. However, Rb protein was hyperphosphorylated in untreated control cells, which means that the cell was not arrested in G1 phase.

Effects of sesquicillin on the CDK-inhibitor protein (CDKI), $p21^{Waf1/Cip1}$ and $p27^{Kip1}$

Cells express at least two families of low molecular weight proteins that serve as natural inhibitors of CDKs/cyclin, the $p21^{Waf1/Cip1}$ and the $p16^{INK4}$ gene family.[6] The INK4 families directly interact with the catalytic subunits of CDK4 and CDK6, and inhibit the formation of complexes with cyclins.[11] On the other hand, members of the Cip/Kip are able to interact with both the cyclin and CDK subunits.[12,13] Especially, they affect the activities of cyclinD-, E-, and A-dependent kinases.

To determine whether G1 phase arrest of MCF-7 cells by sesquicillin was related to the expression of the CDKIs, we investigated the effects of sesquicillin on $p16^{INK4}$, $p21^{Waf1/Cip1}$, and $p27^{Kip1}$ by western blot analysis. When the cells were treated with sesquicillin (20 μg/mL), we could not detect the $p16^{INK4}$ protein (data not shown) and the protein level of $p27^{Kip1}$ was not changed for 48 h in treated and control cells (Fig. 5a). However, $p21^{Waf1/Cip1}$ was induced in sesquicillin treated MCF-7 cells. An increase in $p21^{Waf1/Cip1}$ level due to sesquicillin was detected within 24 h after the compound treatment and reached a maximum after 48 h. The induction of $p21^{Waf1/Cip1}$ by sesquicillin was accom-

panied by an increase in the steady-state levels of $p21^{Waf1/Cip1}$ mRNA (Fig. 5b), and the kinetics of induction was also consistent with the time course for the inhibition of Rb phosphorylation. As p53 regulates $p21^{Waf1/Cip1}$ expression at the transcriptional level in response to DNA damage and stress signal, we performed immunoblotting for p53 level.[14] The expression level of p53 was measured by western blot analysis after compound treatment from 12 to 48 h. Like $p21^{Waf1/Cip1}$, p53 protein was also increased after 24 h of treatment and the kinetics of induction were consistent with the time course of p21 induction.

Effects of sesquicillin on the tumor suppressor protein, p53

Both p53-dependent and p53-independent pathways have been reported to contribute to the regulation of $p21^{Waf1/Cip1}$ expression.[15,16]

To address whether the growth-inhibitory response to sesquicillin is dependent on the p53 status of MCF-7 cell expressing HPV16-E6 protein was used. The E6 protein of Human papilloma virus 16 (HPV16) specifically targets p53 for degradation via the ubiquitination-dependent pathway.[17–20] Thereby, HPV16-E6 expressing cells are rendered p53-deficient cells. Cell-cycle profiles were examined following sesquicillin treatment for HPV16-E6-MCF7, and it was observed that G1-phase cells were accumulated after sesquicillin treatment in p53-deficient MCF-7 cells (after 48 h, 83% of G1 cells). These findings demonstrate that p53 is not absolutely required. It is probably not related to DNA damage for the G1 arrest in MCF-7 cell by sesquicillin, because DNA fragmentation by the compound in the cell was not detected on the gel electrophoresis (data not shown).

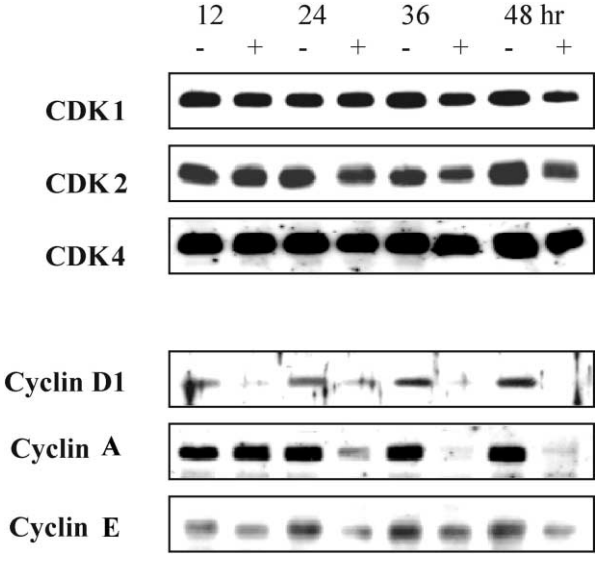

Figure 3. Effects of sesquicillin on the cell cycle related proteins in MCF-7 cell. MCF-7 cells were treated with sesquicillin (20 μg/mL) or with the vehicle control (0.1% DMSO) for the indicated times. The protein production of the cell cycle components was determined by western blot analysis using specific antibodies. The same cell extracts were utilized for the analysis of each cell-cycle protein and equal sample loading was confirmed by Ponceau S staining of the western blot membranes.

In conclusion, reduction of cyclin D1, A, and E protein levels, induction of $p21^{Waf1/Cip1}$, and reduction of pRb phosphorylation are responsible for sesquicillin-induced G1 arrest in MCF-7 cells. Hypophosphorylation of Rb by both decreased expression of cyclin D1 and increased expression of $p21^{Waf1/Cip1}$ is responsible for sesquicillin-dependent cell-cycle arrest. Interestingly, sesquicillin can induce cell cycle arrest in a p53 independent manner and many tumors lost p53 function suggest that the compound will be a good candidate for the development of cell growth controlling agents.

Experimental

Sesquicillin

The producing organism, fungal strain F60063, was isolated from soil samples in Korea and the fermenta-

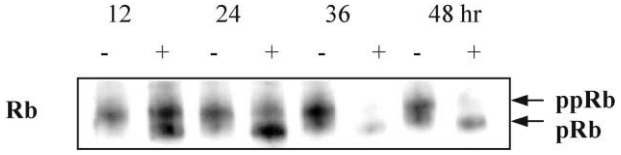

Figure 4. Effect of sesquicillin on Rb phosphorylation in MCF-7 cells. MCF-7 cell was treated with sesquicillin (20 μg/mL) or with vehicle control (0.1% DMSO) for the indicated times. The cell extracts were fractionated in 7.5% SDS-PAGE, and western blots were probed with anti-Rb antibodies (14001A, Pharmingen, USA). The level of endogenous Rb phosphorylation was determined by the characteristic migration of the hyperphosphoylated (ppRb) and hypophosphorylated (pRb) forms of Rb. Equal sample loading was confirmed by Ponceau S staining of the western blot membranes.

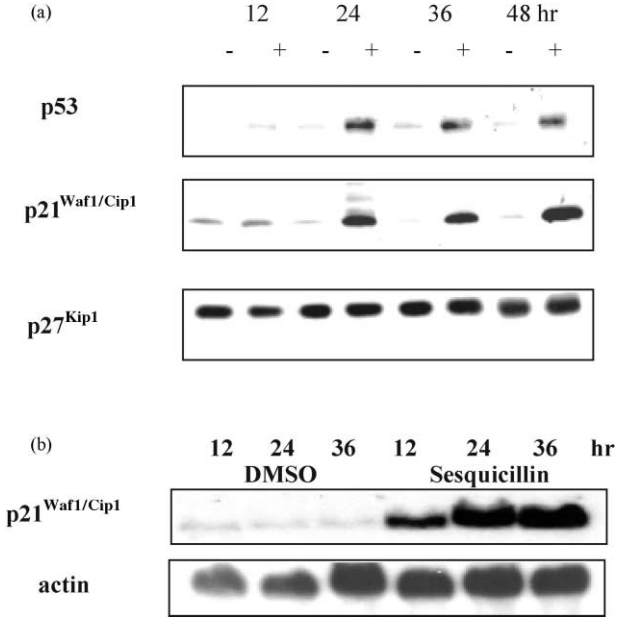

Figure 5. Effect of sesquicillin on CDKI, $p21^{Waf1/Cip1}$, p^{27Kip1}, and p53 in MCF-7 cells. MCF-7 cells were treated with sesquicillin (20 μg/mL) or with the vehicle control (0.1% DMSO) for the indicated times. In panel (a), MCF-7 lysates were subjected to western blot analysis to determine the expression levels of $p21^{Waf1/Cip1}$, p^{27Kip1}, and p53. In panel (b), total RNA was prepared, and $p21^{Waf1/Cip1}$ mRNA was detected by northern blot analysis. The same blot was probed with a cDNA to β-actin to demonstrate comparable levels of mRNA per lane.

tion was carried out at 25 °C with agitation at a rate of 150 rpm for 6 days. The culture broth was filtered to remove the mycelium and the filtrate was applied on a column of Diaion HP-20. The active elute was subjected to silica gel column chromatography and further purified by HPLC to give a white powder (Fig. 1)

Cell cultures and cell proliferation assays

Human gastric cancer cell line SNU354 was obtained from the Korean Cell Line Bank and other carcinoma cell lines were obtained originally from ATCC. The tumor cells were cultured in RPMI 1640 (Gibco/BRL, NY, USA) supplemented with 10% heat-inactivated FBS and 100 mM Hepes. MCF7-neo and MCF7-HPV16-E6 expressing cell were cultured in RPMI 1640 containing G418 (400 ug/mL). MCF10A cells were cultured in DMEM/F12 supplement with 5% horse serum.

Cells (5,000 cells) were seeded into 96-well plates in RPMI 1640, or DMEM containing 10% FBS. After 24 h, cells were replenished with fresh complete medium containing either sesquicillin or 0.1% DMSO. After incubation for 48 h, the cell proliferation reagent WST-1 (Roche, Mannheim, Germany) was added to each well. The amount of WST-1 formazan produced was measured at 450 nm using an ELISA Reader (Bio-Rad, Hercules, CA, USA)

FACS procedure

To analyze the DNA content by flow cytometry, cells were trypsinized from culture flask bottom. After 5 min of centrifugation at 300*g*, the supernatant was removed, the cells then were washed twice in PBS solution and fixed with 3 mL of ice cold 70% ethanol overnight. Fixed cells were harvested by centrifugation at 300 *g* for 3 min and washed twice in PBS containing 1% FBS. Collected cells were resuspended in PBS (100 μL/ 1×10^5 cells) and treated with 100 μg/mL of RNase A at 37 °C for 30 min. Propidium iodide was then added to a final concentration of 50 μg/mL. 20,000 fixed cells were analyzed with a FACScalibur (Becton Dickinson, Mountain View, CA, USA). Cell cycle distribution was analyzed using the Modifit's program (Becton Dickinson, CA, USA).

Protein analysis

Cell lysates were prepared by a reported method.[21] Briefly, cells were lysed with RIPA [50 mM Tris-Cl (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF and 10 μg/mL each of aprotinin, leupeptin and pepstatin] on ice for 1 h, and the lysates were clarified by centrifugation (12,000 rpm for 10 min at 4 °C). The protein concentration was determined using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, CA, USA) with BSA as a standard.

For western blot analysis, 10 μg protein lysates were separated by electrophoresis on SDS-polyacrylamide gel. Gels were electroblotted onto PVDF membrane and blots were blocked with TBST (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20) containing blocking reagents for 1 h. The primary antibodies, polyclonal anti-cyclin D1 (sc-753), anti- cyclin A (sc-596), cyclin E (sc-481), CDK1 (sc-403), anti-CDK2 (sc-163), and anti-CDK4 (sc-601) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal anti-Rb (14001A) was obtained from Pharmingen (San Diego, CA, USA). The secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit IgG from Santa Cruz (Sc-2004), and horseradish peroxidase conjugated goat anti-mouse IgG from Transduction Laboratories (M15345). The antibodies were used at the manufacture recommended dilutions. Blots were incubated in primary antibodies for 2 h at room temperature, then washed five times in 0.1% TBST. Proteins were visualized with chemiluminescence POD reagents (Roche, Germany) and exposed to chemiluminescence film.

Northern blot analysis

Total RNA was purified using an RNeasy mini kit (Qiagen, Valencia, CA, USA). Fifteen micrograms of total cellular RNA was size-fractionated through 1% agarose-formaldehyde gel and blotted onto a nylon membrane. After prehybridization, the membrane was hybridized with ^{32}P-labeled human cDNA probes for $p21^{Waf1/Cip1}$. β-Actin was used as a loading control to monitor the quantity and integrity of the mRNA. Hybridization was performed at 42 °C in a solution of 1 mM EDTA, 250 mM Na_2HPO_4, 1% hydrolysated casein, 7% SDS, pH 7.4, overnight. The membrane was washed in 2×SSC (1×SSC represents 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 0.5% SDS twice for 20 min, then washed once with 0.5×SSC and 0.1% SDS at 65 °C for 20 min. The membrane was subsequently dried and exposed to X-ray films at −70 °C with intensifying screen.

Kinase assay

The enzyme used in the kinase assay was purified from recombinant baculovirus infected insect cells (*Sf21*). GST-Rb/c-terminal region (792–928 amino acid regions) and histone H1 were used as enzyme substrate for CDK4/cyclin D1 and CDK2/cyclin E kinase, respectively. Each kinase assay was performed with 50 μM cold ATP and 0.5 μCi [γ-^{33}P]-ATP for 30 min in a microfilter plate (Millipore, MA, USA).

Acknowledgements

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