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# Ribosomal protein S3 is phosphorylated by Cdk1/cdc2 during G2/M phase

*In-Soo Yoon[1,#], Ji Hyung Chung[2,#], Soo-Hyun Hahm[1], Min Ju Park[1], You Ri Lee[1], Sung Il Ko[1], Lin-Woo Kang[1], Tae-Sung Kim[3], Joon Kim[3] & Ye Sun Han[1,*]*

[1]Department of Advanced Technology Fusion, Konkuk University, Seoul 143-701, [2]Yonsei Integrative Research Institute for Cerebral & Cardiovascular Diseases, Yonsei University Health System, Seoul 120-752, [3]Laboratory of Biochemistry, School of Life Sciences and Biotechnology, and BioInstitute, Korea University, Seoul 136-713, Korea

**Ribosomal protein S3 (rpS3) is a multifunctional protein involved in translation, DNA repair, and apoptosis. The relationship between rpS3 and cyclin-dependent kinases (Cdks) involved in cell cycle regulation is not yet known. Here, we show that rpS3 is phosphorylated by Cdk1 in G2/M phase. Co-immunoprecipitation and GST pull-down assays revealed that Cdk1 interacted with rpS3. An *in vitro* kinase assay showed that Cdk1 phosphorylated rpS3 protein. Phosphorylation of rpS3 increased in nocodazole-arrested mitotic cells; however, treatment with Cdk1 inhibitor or Cdk1 siRNA significantly attenuated this phosphorylation event. The phosphorylation of a mutant form of rpS3, T221A, was significantly reduced compared with wild-type rpS3. Decreased phosphorylation and nuclear accumulation of T221A was much more pronounced in G2/M phase. These results suggest that the phosphorylation of rpS3 by Cdk1 occurs at Thr221 during G2/M phase and, moreover, that this event is important for nuclear accumulation of rpS3. [BMB reports 2011; 44(8): 529-534]**

## INTRODUCTION

Human ribosomal protein S3 (rpS3) is a component of 40S ribosomal subunit, which is important in ribosomal maturation for translational processes (1). In addition, rpS3 has been shown to have additional extraribosomal functions, such as DNA repair, gene transcription, and apoptosis (2-5). The rpS3 protein is phosphorylated by extracellular signal-regulated kinase (ERK), a MAP kinase known to play important roles in the regulation of cell growth (6). The phosphorylation event mediated by ERK1/2 is necessary for rpS3 nuclear translocation in response to DNA damage (2). Kim *et al.* have also reported that rpS3 has endonuclease activity, which is increased by protein kinase C delta (PKCδ)-dependent phosphorylation upon exposure to genotoxic stresses (3). They demonstrated that protein phosphatase 2A (PP2A) regulates the level of phosphorylated rpS3 by interacting with non-ribosomal free rpS3, but not with ribosome-associated rpS3 (7). The rpS3 protein is also known to be involved in apoptotic pathways (8). Moreover, phosphorylation of rpS3, regulated by Akt, might be a critical factor in determining either proapoptotic function or DNA repair activity in neuronal cells (4). A recent study demonstrated that phosphorylation of rpS3 promotes the DNA-binding activity of the NF-kappaB complex, thereby regulating NF-kappaB-mediated selective gene expression (5). As rpS3 is phosphorylated in response to various stimuli in cells, it seems plausible that phosphorylation status is critical for regulating the functions of rpS3 under different physiological conditions.

Herein, we focused on rpS3 phosphorylation during the cell cycle using bioinformatics analysis tools (NetPhos2.0, www.cbs.dtu.dk/services/NetPhos and KinasePhos, kinasephos.mbc.nctu.edu.tw). We identified several cyclin-dependent kinase 1 (Cdk1) putative phosphorylation sites in the amino acid sequence of rpS3. Cdk1, also known as cdc2, is a member of the serine/threonine protein kinase family, which controls cell entry into mitosis. The catalytic activity of Cdk1 requires binding of a regulatory subunit cyclin B1, which is synthesized and degraded during each cell cycle (9). Cdk1/cyclin B1 activity appears only in the G2-M border and is turned off by cyclin B1 destruction as cells enter anaphase of mitosis. When Cdk1/cyclin B1 activity is maximal during G2-M phase, this complex phosphorylates a number of proteins that regulate cellular events such as centrosome separation, chromosome condensation, and nuclear envelope breakdown (10). Moreover, a recent study by global analysis using quantitative mass spectrometry provided insights into the existence of numerous phosphorylation sites on Cdk1 substrates (11). Although increasing evidence suggests that rpS3 plays important roles in the regulation of DNA damage repair and apoptosis, in addition to its role in protein synthesis, little is known about the phosphorylation of rpS3 during the cell cycle. The present study revealed that rpS3 is a substrate of Cdk1 and is phosphorylated

*Corresponding author. Tel: 82-2-2049-6050; Fax: 82-2-452-3410; E-mail: yshan@konkuk.ac.kr
#These authors contributed equally to this work.
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on Thr221 during mitosis depending on Cdk1 kinase activity.

## RESULTS AND DISCUSSION

### Cdk1 interacts with rpS3

To demonstrate the interaction of Cdk1 with rpS3, we carried out a co-immunoprecipitation assay with Myc-Cdk1 and FLAG-rpS3 transfection. HEK293T cells were cotransfected with Myc-tagged Cdk1 and FLAG-tagged rpS3 and then immunoprecipitated with anti-Myc or anti-FLAG antibody. As shown in Fig. 1A, immunoprecipitation with anti-Myc antibody revealed an association between Cdk1 and rpS3 (Fig. 1A, left panel). Reciprocal immunoprecipitation using anti-FLAG antibody also confirmed an interaction between rpS3 and Cdk1 (Fig. 1A, right panel). The physiological interaction of Cdk1-rpS3 in cells was confirmed by binding between endogenous Cdk1 and rpS3 in HEK293T cells (Fig. 1B). To demonstrate the direct interaction between Cdk1 and rpS3, we performed pull-down assay using recombinant GST-fusion Cdk1

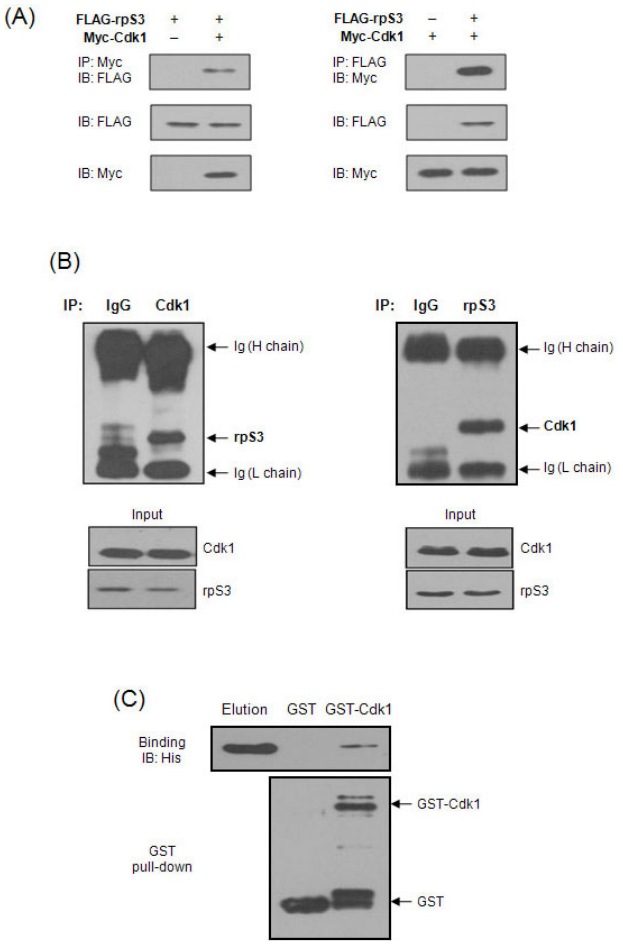


**Fig. 1.** Cdk1 interacts with rpS3. (A) HEK293T cells were transiently transfected with different sets of Myc-Cdk1 or FLAG-rpS3 expression vector. Cell lysates were subjected to immunoprecipitation (IP) with anti-Myc or anti-FLAG antibody. The immunoprecipitates were immunoblotted (IB) with anti-Myc or anti-FLAG antibody. (B) HEK293T cell lysates were subjected to immunoprecipitation with anti-Cdk1 (left panel) or anti-rpS3 (right panel) antibody, after which the immunoprecipitates were analyzed by immunoblotting with anti-Cdk1 or anti-rpS3 antibody. Immunoprecipitations using mouse IgG was used as a control for binding specificity. (C) Purified $His_6$-rpS3 was incubated with immobilized GST or GST-Cdk1. Bound proteins were detected by SDS-PAGE and immunoblot analysis.

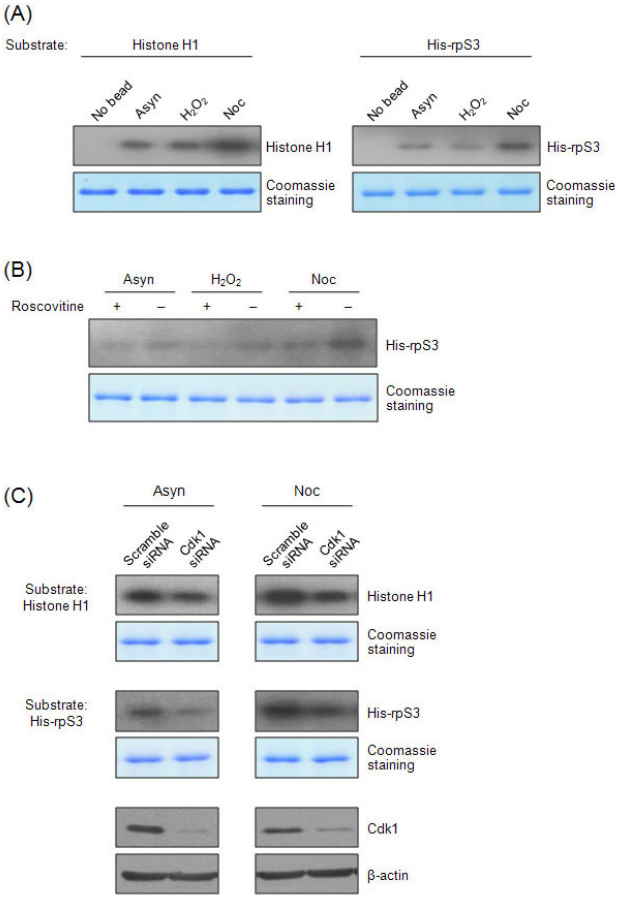


**Fig. 2.** Cdk1 phosphorylates rpS3. (A) HEK293T cells were treated with nocodazole (0.1 μg/ml, 15 h) or $H_2O_2$ (100 μM, 30 min). Cell lysates were prepared, and Cdk1 was immunoprecipitated with anti-Cdk1 antibody. Precipitated samples were subjected to in vitro kinase assay with histone H1 or His-rpS3 as a substrate. (B) Cell lysates were subjected to immunoprecipitation with anti- Cdk1 antibody, and precipitated samples were treated with 100 μM roscovitine or DMSO. Cdk1 kinase assay was performed using His-rpS3 as a substrate. (C) Following 24 h of transfection with Cdk1 siRNAs, cells were treated with 0.1 μg/ml of nocodazole for 15 h. Cell lysates were prepared, and precipitated samples with anti-Cdk1 antibody were subjected to in vitro kinase assay with histone H1 or His-rpS3. Asyn, asynchronized cells; Noc, nocodazole-treated (G2/M phase) cells.

and $His_6$-tagged rpS3 proteins. $His_6$-rpS3 interacted with GST-Cdk1, whereas GST alone did not exhibit any pull-down activity with $His_6$-rpS3 protein (Fig. 1C). These results suggest that Cdk1 directly interacts with rpS3 in cells.

### Cdk1 phosphorylates rpS3

In mammalian cells, rpS3 has been shown to be a substrate of several kinases (2-4). It has also been reported that Cdks phosphorylate several proteins involved in cell cycle checkpoints, such as p53, p21Cip1, and MDM2 (12, 13). Cdk1, a ubiquitously expressed serine/threonine protein kinase, is involved in the regulation of events during eukaryotic cell division (9, 10). To examine whether or not Cdk1 phosphorylates rpS3, we performed *in vitro* kinase assays using Cdk1 immunoprecipitates in HEK293T cells. Cdk1 phosphorylated rpS3 protein as well as histone H1, a typical substrate of Cdk1 (Fig. 2A). Interestingly, the level of phosphorylation increased in nocodazole-arrested mitotic cells compared with asynchronously growing cells or DNA-damaging agent ($H_2O_2$)-treated cells (Fig. 2). However, phosphorylation of rpS3 was significantly attenuated in the presence of roscovitine, a specific inhibitor of Cdk1 (Fig. 2B), confirming that rpS3 phosphorylation was caused by Cdk1-mediated phosphorylation. After treatment with Cdk1 siRNA, we observed a significant reduction in the phosphorylation of both substrates, histone H1 and rpS3 (Fig. 2C).

### Rps3 is phosphorylated mainly in G2/M phase

To further confirm whether or not rpS3 phosphorylation by Cdk1 occurs at a specific stage of the cell cycle, *in vitro* kinase assays were carried out with Cdk1 immunoprecipitates prepared from asynchronized cells, nocodazole-treated (G2/M phase) cells, or excess thymidine-induced S phase cells. In all cases, cell cycle positions were confirmed using flow cytometry (Fig. 3A). Phosphorylation of rpS3 was much stronger with Cdk1 from G/M phase cells compared to that from asynchronized or S phase cells (Fig. 3B). The same results were obtained with another reaction using histone H1, a typical substrate of Cdk1 (Fig. 3B). Fig. 3C shows that equal amounts of protein were loaded from asynchronized cells, S phase, or G2/M phase cells. These results suggest that Cdk1 regulates rpS3 phosphorylation during G2/M phase. In previous studies, several ribosomal proteins were reported to regulate the cell cycle by inhibiting MDM2-mediated feedback regulation of p53 (14-16). Interestingly, the ribosomal protein RPL6 has been shown to promote cell cycle progression through upregulation of cyclin E expression (17). In contrast, another ribosomal protein, rpS36, leads to delay of cell cycle progression and inhibition of cell proliferation (18). Furthermore, the regulatory functions of many ribosomal proteins have been shown to be dependent on phosphorylation status (19-21). Thus, rpS3 targeted by Cdk1 during mitosis seems to play a role in regulating the cell cycle, although further study will be required to elucidate the biological significance of rpS3 phosphorylation in cells.

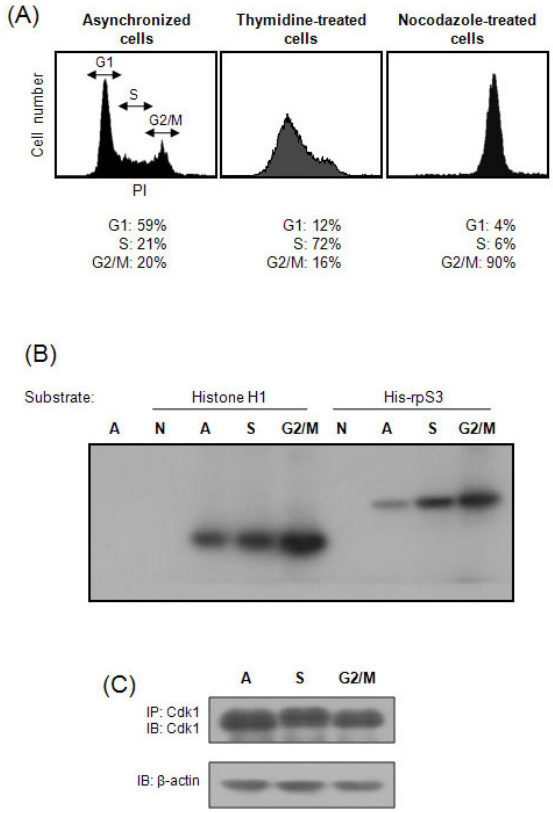


**Fig. 3.** RpS3 is phosphorylated mainly in G2/M phase. (A) To arrest the cell cycle in S phase, HEK293T cells were incubated with 3 mM thymidine for 18 h. Then, cells were washed with medium and incubated in fresh medium for 2 h. G2/M phase cells were collected by treatment with 0.1μg/ml of nocodazole for 12 h. An aliquot of cells was stained with propidium iodide (PI) and analyzed by FACS. (B) Cell lysates from HEK293T cells in each cell cycle stage were prepared, and Cdk1 was immunoprecipitated with anti-Cdk1 antibody. Precipitated samples were subjected to *in vitro* kinase assay with histone H1 or His-rpS3 as a substrate. (C) Cell lysates were subjected to immunoprecipitation with anti-Cdk1 antibody, and precipitated samples were analyzed by immunoblotting with anti-Cdk1 antibody. The β-actin levels in the total cell lysates were detected by immunoblotting with anti-β-actin antibody. N, no beads; A, asynchronized cells; S, S phase cells; G2/M, G2/M phase cells.

### Rps3 Thr221 phosphorylated by Cdk1 and its nuclear accumulation

RpS3 contains two putative Cdk1 phosphorylation consensus sites (T42 and T221) (22). In addition, a serine residue (S6) of rpS3 was previously shown to be a target of protein kinase (3). We generated His6-fusion proteins of rpS3 wild-type (WT) and mutants in which the candidate phosphorylation residues (T42, T221, and S6) were converted to alanine. We then performed *in vitro* kinase assays using WT and mutant proteins

(T42A, T221A, and S6A) of rpS3. The phosphorylation of rpS3 by the Cdk1 immunoprecipitate was significantly reduced in the T221A mutant alone. In contrast, no marked reduction in the level of rpS3 phosphorylation was observed in any of the other mutant proteins (Fig. 4A). Notably, the phosphorylation of T221A mutant protein was reduced not only in asynchronized cells, but also in G2/M phase cells, where the increase in rpS3 phosphorylation was much more pronounced (Fig. 4A). The interaction between Cdk1 and rpS3 also occurred much more strongly in G/M phase cells than in asynchronized cells (Fig. 4B). These evidences strongly suggest that Cdk1 is a possible candidate for the phosphorylation of T221 of rpS3 in G2/M phase. Previous reports show that nuclear accumulation of rpS3 is required for its intracellular function (4, 5, 7). We found that rpS3 WT was localized to the nucleus more in G2/M phase cells compared to asynchronized cells; however, nuclear accumulation of mutant T221A was significantly decreased (Fig. 4C). On the other hand, accumulation of the phosphorylation-mimicking form (T221D) of rpS3 in the nucleus was increased. These results suggest that T221 phosphorylation of rpS3 is important for its nuclear accumulation in G2/M phase.

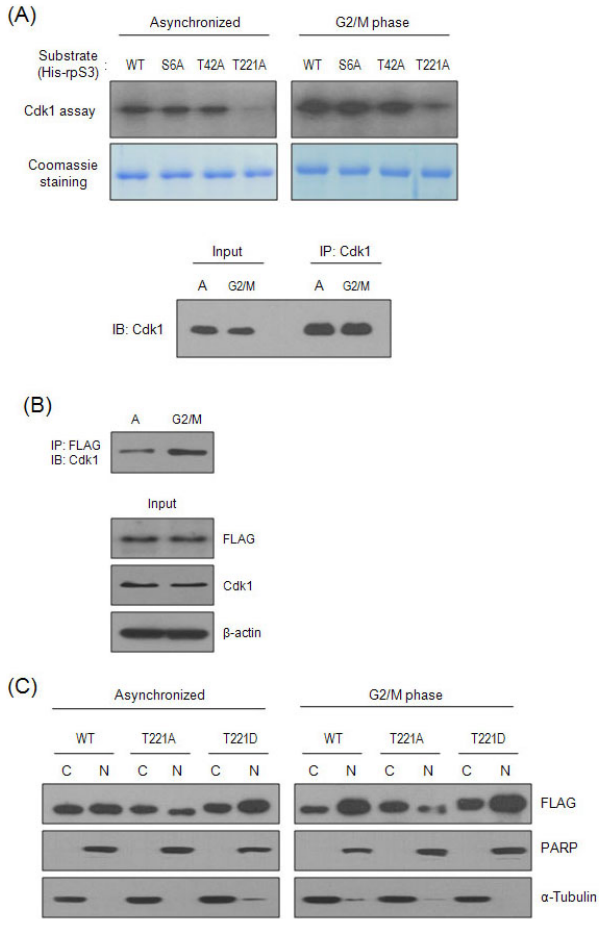


**Fig. 4.** Rps3 Thr221 phosphorylation and its nuclear accumulation. (A) Asynchronized and G2/M phase cell lysates were subjected to immunoprecipitation with anti-Cdk1 antibody. Cdk1 kinase assay was performed using His-rpS3 wild-type (WT) and mutants (S6A, T42A, T221A) as substrates. Equal amounts of substrates were monitored by Coomassie blue staining. To confirm that equivalent amounts of Ckd1 were present in the kinase assays, input samples and immunoprecipitates were assayed for Cdk1 levels by immunoblot analysis (lower panel). A, asynchronized cells; G2/M, G2/M phase cells. (B) HEK293T cells were transiently transfected with FLAG-rpS3 (WT) expression vector. Asynchronized or G2/M phase cell lysates were immunoprecipitated with anti-FLAG antibody and analyzed by immunoblotting with anti-Cdk1 antibody. (C) HEK293T cells were transiently transfected with FLAG-rpS3 expression vectors harboring rpS3 wild-type (WT) or mutants (T221A or T221D). Equal amounts of cytosolic and nuclear protein were separated by SDS-PAGE, and nuclear accumulation of rpS3 was detected by immunoblot analysis. C, cytosolic fraction; N, nuclear fraction.

In summary, we demonstrated here that Cdk1 phosphorylates rpS3 at T221 and that this event is essential for nuclear accumulation of rpS3, especially during G2/M phase. Our data provided the first proposal concerning the relationship between Cdk1 and rpS3 in the cell cycle, though further investigation is required to determine the contribution of Cdk1 to phosphorylation of rpS3 at T221 in the cell cycle.

## MATERIALS AND METHODS

### Cell lines

Human kidney embryonic HEK293T cells were grown in Dulbecco's modified Eagle's (DMEM, Invitrogen, Carlsbad, CA) medium containing 10% FBS (Invitrogen) and 1% penicillin-streptomycin solution (Sigma, St. Louis, MO) at 37°C in a 5% $CO_2$ incubator. HEK293 cells were seeded onto 6-well plates at a density of $1 \times 10^6$ cells per well and incubated for 24 h before the experiment.

### Plasmid construction

Gene fragments corresponding to cDNA coding regions of human rpS3 and Cdk1 (Accession no. NM_001005; rpS3, NM_001786; Cdk1) were amplified using PCR. A Cdk1 fragment was inserted into *Eco*RI and *Sal*I sites of pCMV Tag3A (Stratagene, La Jolla, CA), and rpS3 fragments were inserted into *Bam*HI and *Xho*I sites of pCMV Tag2C (Stratagene). All constructs were confirmed by both restriction enzyme mapping and DNA sequence analyses.

### Transfection

Recombinant plasmid DNAs were transiently transfected into 80-90% confluent HEK293T cells using Lipofectamine™ 2,000 reagent (Invitrogen) according to the manufacturer's protocol. After 24 h of incubation, cells were collected and lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA, 1 mM NaF, 1 mM $Na_3VO_4$, 1% Nonidet P-40, 10μg/ml of PMSF, and protease inhibitor cocktail) for 30 min at 4°C. After centrifugation, supernatants were

collected and used in immunoblot analysis or immunoprecipitation experiment.

**Cell fractionation**

To prepare the nuclear and cytosolic protein extracts, cells were seeded and cultured to 90% confluency on 60-mm dishes. Following washing, cells were harvested and collected by centrifugation. Cells were suspended into buffer A (10 mM HEPES, pH 7.9, 50 mM NaCl, 1 mM DTT, 0.1 mM EDTA, and protease inhibitor cocktail) for 20 min on ice. An equal volume of buffer B (0.1% NP-40 in buffer A) was then added, and the suspension was allowed to sit for 20 min on ice. Following centrifugation, the supernatant (cytosol fraction) was collected and subjected to centrifugation at 5,000 × g for 2 min to remove cellular debris. The nuclear pellet was washed two times with buffer A and resuspended using buffer C (10 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM DTT, and 1 mM EDTA). The nuclear fractions were cleared of debris by centrifugation at 13,000 rpm for 15 min at 4°C. Protein concentrations were measured using DC protein assay (Bio-Rad, Hercules, CA).

**GST pull-down assay**

We used the GST gene fusion system (Amersham Biosciences, Uppsala, Sweden) to generate GST-Cdk1 fusion protein. For GST pull-down assays, fusion proteins were adsorbed to glutathione-Sepharose 4B beads. $His_6$-purified rpS3 protein was then incubated with GST or GST fusion protein in binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.3 mM DTT, 0.1% NP-40, and protease inhibitor cocktail). The binding reaction was incubated for 3 h at 4°C, after which the beads were washed four times with the same buffer. The bound proteins were then subjected to 10% SDS-PAGE analysis. Proteins were detected by immunoblot analysis.

**Coimmunoprecipitation**

Total cell lysates were collected from cells transiently transfected with different sets of plasmid DNAs and incubated for 3 h with 2μg of anti-c-Myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C with gentle rotation. Antibody and bound proteins were incubated with 30μl of protein A/G-Sepharose beads at 4°C for 12 h. Samples were precipitated with centrifugation at 3,800 rpm for 5 min, washed five times with washing buffer (1 : 1 mixture of lysis buffer and PBS), and mixed with gel loading buffer. Immunoprecipitated samples were resolved on 8% SDS-polyacrylamide gel and subjected to immunoblot analysis.

**Immunoblot analysis**

Proteins resolved on 8-12% SDS-polyacrylamide gel were transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Bioscience). The membranes were blocked overnight at 4°C with 5% non-fat dried milk in TBS-T (TBS with 0.05% Tween 20) and then incubated with primary antibodies, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. Protein bands were detected using ECL Western blotting detection reagents (Pierce, Rockford, IL).

**Cell cycle synchronization**

Cells were plated at $5 \times 10^5$ cells/60-$mm^2$ dish. To arrest cell cycle in S phase, after 24 h of incubation, the cells were treated with 3 mM thymidine for 18 h. Then, the cells were washed with medium and incubated in fresh medium for 2 h. To collect G2/M phase cells, the transfected cells were treated with 0.1μg/ml of nocodazole for 12 h. An aliquot of cells was stained with propidium iodide (PI) and analyzed by FACS.

**Fluorescence-activated cell sorting (FACS) analysis**

Cells were trypsinized and centrifuged at 1,000 rpm for 10 min. The cells were fixed using 1 ml of 70% ethanol for 30 min, followed by centrifugation at 3,500 rpm for 3 min. After washing with ice-cold PBS, the cell pellets were resuspended in 0.5 ml of PBS containing 50 μg/ml of propidium iodide (Sigma) and 100 μg/ml of RNase (Sigma). After 30 min of incubation in the dark, cells were analyzed using a flow cytometer. Fluorescence emitted from the PI-DNA complex was estimated using a minimum of 10,000 cells per sample and analyzed using Cell Quest Alias software (BD Biosciences, Rockville, MD).

***In vitro* Cdk1 kinase assay**

After 3 h of incubation of cell lysates with Cdk1-specific monoclonal antibody (5μg), samples were incubated with 50μl of protein A/G-Sepharose beads at 4°C for 12 h. Immune complexes were washed five times with washing buffer (1 : 1 mixture of lysis buffer and PBS) and kinase reaction buffer (20 mM Tris-HCl, pH 7.4, 15 mM $MgCl_2$, and 1 mM DTT). After washing, samples were added with 1μg of substrate (histone H1 or rpS3), 200 μM of ATP, and 5 μCi of [$^{32}$P-γ]ATP in 30 μl of reaction buffer. Reactions were run for 25 min at 30°C and terminated by addition of SDS-PAGE sample buffer. Samples were subjected to 12% SDS-PAGE, and phosphorylation was detected by autoradiography.

**Cdk1 siRNA**

Cdk1 siRNA oligonucleotides targeting the sequences 5'-GGGUUCCUAGUACUGCAATT-3' and 5'-UUGCAGUACUAGGAACCCCTT-3' were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were transfected with Cdk1 siRNAs (5 nM each) using Lipofectamine 2,000 (Invitrogen). Following 24 h of transfection, the level of Cdk1 was analyzed with anti-Cdk1 antibody (Santa Cruz Biotechnology). A scrambled siRNA (GFP siRNA, 5'-GGGCACAAGCUGGAGUACAACUACA-3') was used as the control.

**Cytosolic and nuclear fractions**

Cells were resuspended in buffer A (10 mM HEPES, pH 7.9, 50 mM NaCl, 1 mM DTT, 0.1 mM EDTA, and protease in-

hibitors) for 20 min. Samples were added with an equal volume of buffer B (0.1% NP-40 in buffer A) and incubated on ice for 20 min. Following centrifugation at 12,000 rpm for 2 min, the supernatant (cytosolic fraction) was collected. The nuclear pellet was washed three times with buffer A and resuspended in buffer C (10 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM DTT, 1 mM EDTA). The nuclear fraction was collected by centrifugation at 12,000 rpm for 15 min. PARP and $\alpha$-tubulin were used as loading controls of cytosolic and nuclear fractions, respectively.

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