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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 23, pp. 15601–15610, June 6, 2008


# Cdk5-mediated Phosphorylation of c-Myc on Ser-62 Is Essential in Transcriptional Activation of Cyclin B1 by Cyclin G1*[S]

Received for publication, February 6, 2008, and in revised form, April 8, 2008 Published, JBC Papers in Press, April 11, 2008, DOI 10.1074/jbc.M800987200

**Haeng Ran Seo[‡§], Joon Kim[§], Sangwoo Bae[‡], Jae-Won Soh[¶1], and Yun-Sil Lee[‡1,2]**

*From the [‡]Division of Radiation Effect, Korea Institute of Radiological and Medical Sciences, Seoul 139-706, Korea, [§]School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea, and [¶]Laboratory of Signal Transduction, Department of Chemistry, Inha University, Incheon 402-751, Korea*

**It has been reported previously that cyclin G1 enables cells to overcome radiation-induced $G_2$ arrest and increased cell death and that these effects are mediated by transcriptional activation of cyclin B1. In this study, we further investigated the mechanism by which cyclin G1 transcriptionally activates cyclin B1. Deletion or point mutations within the cyclin B1 promoter region revealed that the c-Myc binding site (E-box) is necessary for cyclin G1-mediated transcriptional activation of cyclin B1 to occur. In addition, the kinase activity of Cdk5 was increased by cyclin G1 overexpression, and Cdk5 directly phosphorylated c-Myc on Ser-62. Furthermore, cyclin G1 mediated increased radiosensitivity, and radiation-induced M phase arrest was attenuated when RNA interference of Cdk5 was treated. Taken together, the results of this study indicate that Cdk5 activation in cells that overexpress cyclin G1 leads to c-Myc phosphorylation on Ser-62, which is responsible for cyclin G1-mediated transcriptional activation of cyclin B1.**

Cell cycle arrest in response to DNA damage occurs primarily at the $G_1$/S and $G_2$/M boundaries (1, 2), with $G_1$/S arrest blocking entry of damaged DNA into the S phase and $G_2$/M arrest prohibiting entry of damaged DNA into mitosis. Therefore, both the $G_1$/S and $G_2$/M checkpoints are important for maintaining genomic stability, as well as for maintaining the fidelity of transmission of intact genomes to cellular progeny (3, 4). The eventual target of the $G_2$/M entry checkpoint is the mitotic kinase Cdc2-cyclin B1, which governs most of the processes involved in mitotic initiation and progression (5). Cyclin protein levels oscillate during the cell cycle, with B-type cyclins appearing during the S phase and accumulating in $G_2$ and mitosis before disappearing during the transition from metaphase to anaphase. Synthesis of cyclin B1 during the cell cycle is regulated primarily at the transcriptional level (6) by a cell cycle-dependent element (CDE)[3] and cell cycle gene homology region (CHR) tandem element (7). Transcription of Cdc25C, cyclin A, and the Cdc2 gene causes CDE/CHR elements to repress transcription during the early phases of the cell cycle; however, relief from this repression later in S and $G_2$ leads to their expression (8). Furthermore, although degradation by ubiquitin-mediated proteolysis leads to a large drop in cyclin B1 protein levels (6, 9, 10), it has been reported that an E-box is involved in the transcriptional regulation of cyclin B1 (11, 12). Moreover, a recent report has provided evidence that the cyclin B1 promoter is also subject to activation by c-Myc, as demonstrated by the binding of c-Myc to the activating E-box (CACGTG) of cyclin B1 (13, 14). However, the exact upstream mechanisms that cause c-Myc to bind to the E-box of the cyclin B1 promoter region during cell cycle regulation have not yet been defined.

An enhancer E-box can be defined as a transcriptional homograph, which is a short DNA element with the ability to dispense different transcriptional outputs depending upon the features of the sequence and cellular environment. For example, by recruiting the proto-oncogene c-Myc, an E-box can drive cells to become growth factor-independent, to speed through $G_1$ of the cell cycle, to avoid differentiation, or to undergo apoptotic death (15, 16). The perplexing diversity of E-box-dependent processes has fueled an intense search for trans-acting factors regulating a given E-box. Predictably, such efforts have resulted in an ever expanding list of transcription factors that can recognize E-box binding factors, including c-Myc, Arnt, Max, and MyoD (17–19).

Temporal regulation of c-Myc protein accumulation is essential for normal cell proliferation to occur, and deregulation of the c-*myc* oncogene is often observed in human cancer (20). In addition, c-Myc protein overexpression can immortalize cells, reduce their growth factor requirements, promote cell cycle progression, and inhibit differentiation (21–23). c-Myc protein is stabilized after activation of several signal transduc-

* This work was supported by the Korea Science and Engineering Foundation and the Ministry of Education, Science, and Technology through the National Nuclear Technology Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–6.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed: Division of Radiation Damage Control, Korea Inst. of Radiological and Medical Sciences, 215-4 Gongneung-Dong, Nowon-Ku, Seoul 139-706, Korea. Fax: 82-2-970-2402; Tel.: 82-2-970-1325; E-mail: yslee@kcch.re.kr.

[3] The abbreviations used are: CDE, cell cycle-dependent element; CHR, cell cycle gene homology region; GSK, glycogen synthase kinase; Cdk5, cyclin-dependent kinase 5; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; siRNA, small interfering RNA; Si-, transfected with siRNA; ChIP, chromatin immunoprecipitation; Gy, gray (unit of radiation); GST, glutathione *S*-transferase.

tion pathways that inhibit glycogen synthase kinase-3$\beta$ (GSK-3$\beta$), such as the Raf-MEK-ERK kinase cascade or the PI3K (phosphatidylinositol-3-OH kinase)-Akt pathway. The ERK and GSK-3$\beta$ kinases phosphorylate two sites, Ser-62 and Thr-58, respectively, which are near the amino terminus of c-Myc and are highly conserved in all mammalian c-Myc isofoms (24–27). These phosphorylation sites exert opposing control of c-Myc degradation through the ubiquitin-proteasome pathway (27). In response to a growth-stimulatory signal, transcription of the c-*myc* gene is increased and newly synthesized c-Myc protein is phosphorylated on the Ser-62 residue, which results in its stabilization. Phosphorylation at Ser-62 is also required for the subsequent phosphorylation of c-Myc at Thr-58 by GSK-3$\beta$, which is associated with c-Myc degradation (24, 26–28). Thr-58 phosphorylation contributes to degradation of c-Myc through the ubiquitin pathway, whereas mutation of Thr-58 to alanine results in a stable and more oncogenic c-Myc protein (24, 26, 27).

Cyclin G1, one of the target genes of p53, has been studied to determine its role as it relates to p53 activities (29, 30). However, recent observations suggest that cyclin G1 might be involved in various activities that are not associated with p53. Cyclin G1 is associated with cyclin-dependent kinase 5 (Cdk5) and non-Cdk-serine/threonine kinase, which is also known as cyclin G-associated kinase (GAK) (31, 32). In addition, because of its overexpression in human tumor cells, it has been suggested that cyclin G1 acts as an oncogenic protein (33, 34). Conversely, cyclin G1 is also involved in $G_2$/M phase arrest in response to DNA damage (35) and in the facilitation of tumor necrosis factor-induced apoptosis (36), indicating that it has a tumor-suppressing function. Despite these findings, there is limited understanding of the biochemical function of cyclin G1 when it is complexed with associated partners or of the molecular function of its associated partners.

In a previous study, we found that cyclin G1 was overexpressed in lung cancer tissues but that it regulated cyclin B1 at the transcriptional level, independently of p53. In addition, in that study, cyclin G1 was found to overcome radiation-induced $G_2$ arrest, resulting in increased radiation sensitivity (37). In this study, we further describe the mechanisms of cyclin G1-mediated transcriptional activation of cylcin B1. Cyclin G1 interacted with Cdk5, which in turn induced kinase activation of Cdk5. Active Cdk5 then phosphorylated c-Myc on Ser-62, and the phosphorylated c-Myc then bound directly to the E-box promoter region of cyclin B1, resulting in increased cyclin B1 promoter activity.

## EXPERIMENTAL PROCEDURES

*Reagents*—Nocodazole was purchased from Sigma. Monoclonal antibody for cyclin G1, cyclin B1, c-Myc, p53, ERK, p-ERK, and Cdk5 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). phospho-c-Myc (Ser-62) and phospho-c-Myc (Thr-58) were purchased from Abcam (Cambridge, MA). Phospho-GSK-3$\beta$ and GSK-3$\beta$ were purchased from Upstate (Charlottesville, VA). c-Myc and Cdk5 recombinant protein were purchased from Abnova (Taiwan).

*Plasmids and Constructs*—For overexpression of cyclin G1, human cyclin G1 cDNA was cloned into murine leukemia retrovirus MFG vector (37, 38). The cyclin B1 promoter-firefly luciferase reporter constructs, hB1-Luci, hB1-CDE mut-Luci, and hB1-CHR-Luci-mut, were kindly provided by Kurt Engeland (Universitat Leipzig, Germany). hB1-NY-F2, hB1-NY-F3, and hB1-E-box-Luci mutants were created by PCR-based targeted mutagenesis on the basis of hB1-Luci. Full-length c-Myc was then cloned into the pcDNA4-V5 plasmid after which internal deletion mutants were constructed in c-Myc-containing pcDNA3-HA vector in the c-Myc coding sequence using deletion primers. This was followed by restriction and ligation steps.

*Cell Culture*—NCI-H460 or NCI-H1299 human non-small cell carcinoma and HCT116 cells were cultured in RPMI 1640 (Invitrogen) supplemented with heat-inactivated 10% fetal bovine serum (Invitrogen) and antibiotics at 37 °C in a humidified incubator under 5% $CO_2$.

*Irradiation*—Cells were plated in 60-mm dishes and then incubated at 37 °C under humidified conditions with 5% $CO_2$ until 70–80% confluent. Cells were then exposed to $\gamma$-rays from a $^{137}$Cs $\gamma$-ray source (Atomic Energy of Canada Ltd.) at a dose rate of 3.81 Gy/min.

*Flow Cytometric Analysis*—Cells were cultured, harvested at the indicated times, and then stained with propidium iodide according to the manufacturer's protocol. The cells were then analyzed using a FACScan flow cytometer (BD Biosciences).

*Cell Transfection*—Predesigned siRNA for human cyclin G1, cyclin B1, cdk5, c-Myc, and negative control siRNA was purchased from Ambion (Austin, TX). Cells were then transfected with these siRNAs for 48 h using Lipofectamine 2000 (Invitrogen). All cells were transiently transfected using Lipofectamine or Lipofectamine Plus reagent (Invitrogen).

*Cell Cycle Analysis*—For cell cycle analysis, cells were fixed in 80% ethanol at 4 °C for at least 18 h. Next, the fixed cells were washed once with phosphate-buffered saline-EDTA and then resuspended in 1 ml of phosphate-buffered saline. After the addition of 10 $\mu$l each of propidium iodide (5 mg/ml) and RNase (10 mg/ml), the samples were incubated for 30 min at 37 °C and then analyzed using a FACScan flow cytometer.

*In Vitro Kinase Assay*—Cell lysates were incubated with cyclin G1 and Cdk5 antibody, after which the immunocomplexes were collected on protein A-Sepharose beads and resuspended in kinase assay mixture containing [$\gamma$-$^{32}$P]ATP and c-Myc as substrates. GST-cyclin G1 and GST-Cdk5 were also mixed in the kinase assay mixture. Proteins were then separated on SDS-polyacrylamide gels, after which the bands were detected by autoradiography.

*Reporter Assay*—Cells were transfected with 200 ng of $\beta$-galactosidase expression vector pSV110 and 200 ng of reporter gene, along with mutant vectors of NY-F2, NY-F3, CDE, CHR, and E-box and wild-type cyclin B1 expression vector. The cells were harvested 48 h later, and luciferase activity was then assayed, with the results normalized to the $\beta$-galactosidase expression (Promega, Madison, WI). All results represent the mean of three independent experiments.

*Electrophoretic Mobility Shift Assay*—The E-box oligonucleotide was labeled with [$\gamma$-$^{32}$P]ATP using T4 polynucleotide kinase, and 5 $\mu$g of nuclear extract was then incubated with the [$\gamma$-$^{32}$P]E-box oligonucleotide at room temperature for 30 min. The c-Myc-E-box complex was then sequentially separated

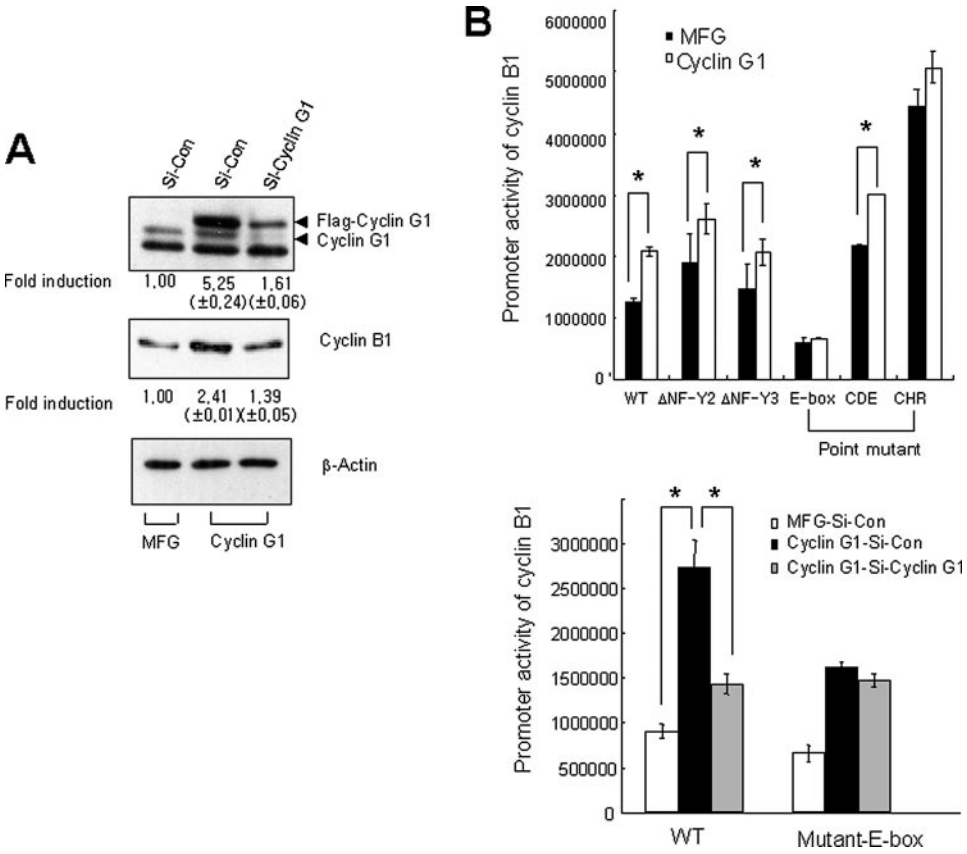



FIGURE 1. **The c-Myc binding domain is necessary for transcriptional activation of cyclin B1 by cyclin G1.** *A*, siRNA of scrambled control or cyclin G1 was transfected to MFG control and cyclin G1-overexpressing NCI-H460 cells, and Western blot analysis was performed. The relative band intensity was then calculated from densitometric scans of the immunoblots, with control values set to 1. The results represent one of three independent experiments. *B*, wild-type (*WT*) or various point or deletion (Δ) mutants of cyclin B1-Luc vectors were transfected to MFG control and cyclin G1-overexpressing cells, and a luciferase assay was then performed (*top*). Scrambled control (*Si-Con*) or siRNAs were transfected into cyclin G1-overexpressing cells (*Si-Cyclin G1*), and a cyclin B1 promoter assay was then performed using a luciferase assay (*bottom*). The results represent the means ± S.D. of three independent experiments. *, denotes statistical significance of $p < 0.05$.

from the unbound E-box on a 4% Tris borate-EDTA gel. The gel was then dried, and an autoradiogram was taken.

*Protein Synthesis*—For protein synthesis assays, MFG control and cyclin G1-overexpressing NCI-H460 cells were resuspended in Met/Cys-free Dulbecco's modified Eagle's medium plus 2% dialyzed fetal bovine serum and then preincubated for 30 min at 37 °C for recovery. The cells were then labeled with [$^{35}$S]methionine (5 Ci, 3000 Ci/mmol) for the times indicated in Fig. 3, after which 500 μl of cell suspension were lysed in SDS sample buffer and then incubated with c-Myc antibody. Next, the immunocomplexes were collected on protein A-Sepharose beads and then separated on SDS-polyacrylamide gels, after which the bands were detected by autoradiography.

*Chromatin Immunoprecipitation Assay*—NCI-H460 cells were cross-linked with 1% formaldehyde in serum-free RPMI at room temperature for 5–10 min. The chromation was then sheared by sonication on ice, which yielded chromatin fragments of 100–600 bp. Chromatin complexes were then immunoprecipitated with no antibody, anti-c-Myc, anti-phospho-c-Myc Ser-62, or anti-phospho-c-Myc Thr-58 antibodies for 12–18 h. The binding of c-Myc and phospho-c-Myc to the cyclin B1 promoter was then detected by quantitative real-time PCR using a DNA Engine 2 Opticon thermal cycler (MJ Research) and the LightCycler-FastStart DNA Master SYBR Green I mix (Roche Applied Science). The real-time PCR cycling conditions were as follows: 94 °C for 5 min followed by 32 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C followed by fluorescence measurement. The polymerization temperature was set to 68 °C to allow accurate fluorescence measurements. Following PCR, a thermal melt profile was performed to identify the amplicons. Ct was determined by manually setting the threshold level of fluorescence during the early phase of the PCR amplification.

*In Vitro Translation and GST Pulldown Assay*—*In vitro* translation of the c-Myc protein was performed using the TNT transcription-translation system (Promega). GST pulldown assays were performed by incubating GST protein alone or by incubating GST-cyclin fusion proteins immobilized on GST beads with *in vitro* translated $^{35}$S-labeled protein. Next, bound proteins were isolated by incubating the mixture for 2 h at 4 °C and then washing the mixture with Nonidet P-40 lysis buffer. The proteins were then eluted in SDS buffer and separated by SDS-PAGE. Finally, the gels were developed using autoradiography.

## RESULTS

*E-box-dependent Transcriptional Activation of Cyclin B1 by Cyclin G1*—Transfection of the retroviral vector containing cyclin G1 to NCI-H460 lung cancer cells increased cyclin B1 protein levels by more than 2-fold. However, siRNA treatment of cyclin G1 reversed this phenomenon (Fig. 1*A*, *Si-Cyclin G1*). Because a previous study had indicated that overexpression of cyclin G1 induces cyclin B1 at the transcriptional level (37), the pathway by which cyclin G1 regulates the transcription activity of cyclin B1 was evaluated. Deletion or point mutations within the cyclin B1 promoter region revealed that increased cyclin B1 promoter activity in cells that overexpressed cyclin G1 was diminished by transfection of additional genes containing mutation of the E-box site in the cyclin B1 promoter region, which is the c-Myc binding site (Fig. 1*B*, *top*). In addition, the level of promoter activity was found to be similar to that of Si-cyclin G1-transfected cells (Fig. 1*B*, *bottom*). However, the transfection of point mutants containing CDE or CHR sites, which are the cell cycle-dependent transcriptional elements in the cyclin B1 promoter, did not affect the transcriptional activ-

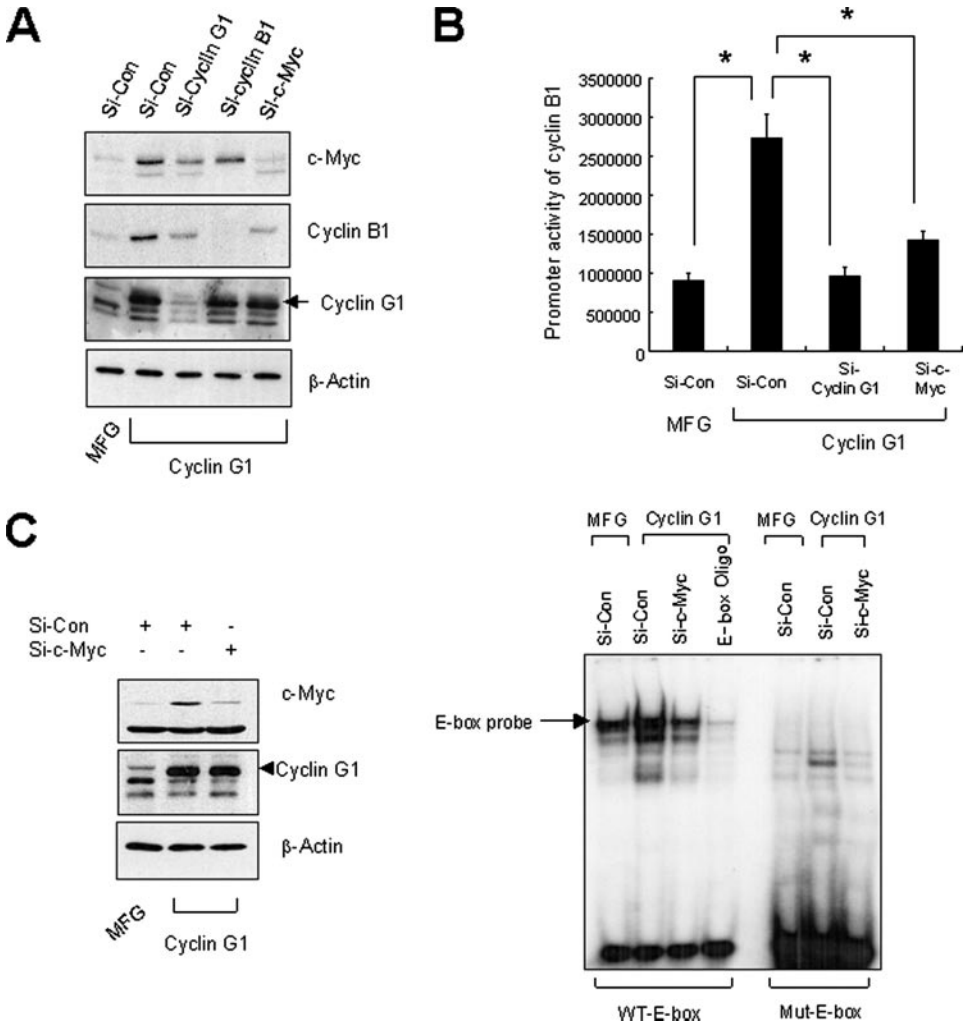



FIGURE 2. **Involvement of c-Myc in cyclin G1-mediated transcriptional activation of cyclin B1.** siRNAs of scrambled control (*Si-Con*), cyclin G1 (*Si-Cyclin G1*), cyclin B1 (*Si-Cyclin B1*), or c-Myc (*Si-c-Myc*) were transfected to MFG control and cyclin G1-overexpressing cells, and Western blotting (*A*) and luciferase assay (*B*) were then performed. The results represent the means ± S.D. of three independent experiments. *, denotes statistical significance of $p < 0.05$ *C*, siRNA of scrambled control or c-Myc was transfected to MFG control and cyclin G1-overexpressing NCI-H460 cells, and Western blot analysis was then performed (*left*). A gel mobility shift analysis was done using a wild-type E-box (*WT-E-box*) or mutant E-box (*Mut-E-box*) probe on the extracts of the MFG control or cyclin G1-transfected NCI-H460 cells with or without transfection siRNA of c-Myc. Nuclear extracts were prepared, and the c-Myc DNA binding activity was then measured using $^{32}$P-labeled E-box fragments (*right*). Representative results from three independent experiments are shown.

ity of cyclin B1 in cells that overexpressed cyclin G1. Furthermore, in cells that overexpressed cyclin G1, the transcription activity of cyclin B1 was unaffected by transfection of deletion mutants containing NF-Y (ΔNF-Y2 and ΔNF-Y3), which are the dominant activating elements in the cyclin B1 promoter (Fig. 1*B*, *top*). Taken together, these results indicate that cyclin G1 overexpression increases the binding activity of c-Myc on the E-box of the cyclin B1 promoter site.

*Involvement of c-Myc in Cyclin G1-mediated Transcriptional Activation of Cyclin B1*—Cyclin G1 overexpression increased the expression of c-Myc, which is one of the target proteins that bind to the E-box site. Therefore, we evaluated c-Myc expression to determine whether it was related to the cyclin G1-mediated transcriptional activation of cyclin B1. Treatment of cells that overexpressed cyclin G1 with Si-c-Myc inhibited cyclin B1 protein levels as well as its promoter activity, suggesting that cyclin G1-mediated c-Myc expression was involved in the promoter activation of cyclin B1 by cyclin G1 (Fig. 2, *A* and *B*). Furthermore, the increased DNA binding activity that was observed when an E-box probe was added to cyclin G1-transfected cell lysates was reduced by treatment with Si-c-Myc, and when cells that had been treated with a mutant E-box probe were used, the DNA binding activity disappeared completely (Fig. 2*C*).

*Increased Protein Stability of c-Myc by Cyclin G1*—The mRNA level of c-Myc in cells that overexpress cyclin G1 did not change (supplemental Fig. 1). Therefore, we examined whether *de novo* protein synthesis or the protein stability of c-Myc was affected by cyclin G1. The half-life of c-Myc following treatment with cycloheximide was increased by cyclin G1 overexpression when compared with that of control MFG cells (Fig. 3*A*). However, when we examined c-Myc protein synthesis using [$^{35}$S]methionine, no differences were observed between the control vector and cyclin G1-transfected cells (Fig. 3*B*), which suggests that cyclin G1 affects c-Myc protein stability.

*Increased Phosphorylation of c-Myc on Ser-62 by Cyclin G1*—Because c-Myc stability is regulated by the phosphorylation status of Thr-58 and Ser-62 (24, 26–28), the phosphorylation of c-Myc were examined. Increased phosphorylation of c-Myc on both Ser-62 and Thr-58 was observed in cells that overexpressed cyclin G1 (Fig. 4*A*). When the phosphorylation levels of c-Myc in the same amount of immunoprecipitated c-Myc protein were examined, expression of phospho-c-Myc on Ser-62 was found to be increased by cyclin G1 overexpression; however, no difference was observed in the expression of phospho-c-Myc on Thr-58 when control and cyclin G1-overexpressing cells were evaluated (Fig. 4*B*). This finding suggests that cyclin G1 overexpression stabilizes the c-Myc protein via phosphorylation on Ser-62. The DNA binding activity observed when we used the E-box probe also suggests that increased DNA binding activity to the E-box probe in cells that overexpressed cyclin G1 was inhibited by the addition of antibody to phospho-c-Myc on Ser-62; however, this did not occur when antibody to phospho-c-Myc on Thr-58 was added (supplemental Fig. 2). This finding suggests that phospho-c-Myc on Ser-62 was responsible for the increased DNA binding activity to the

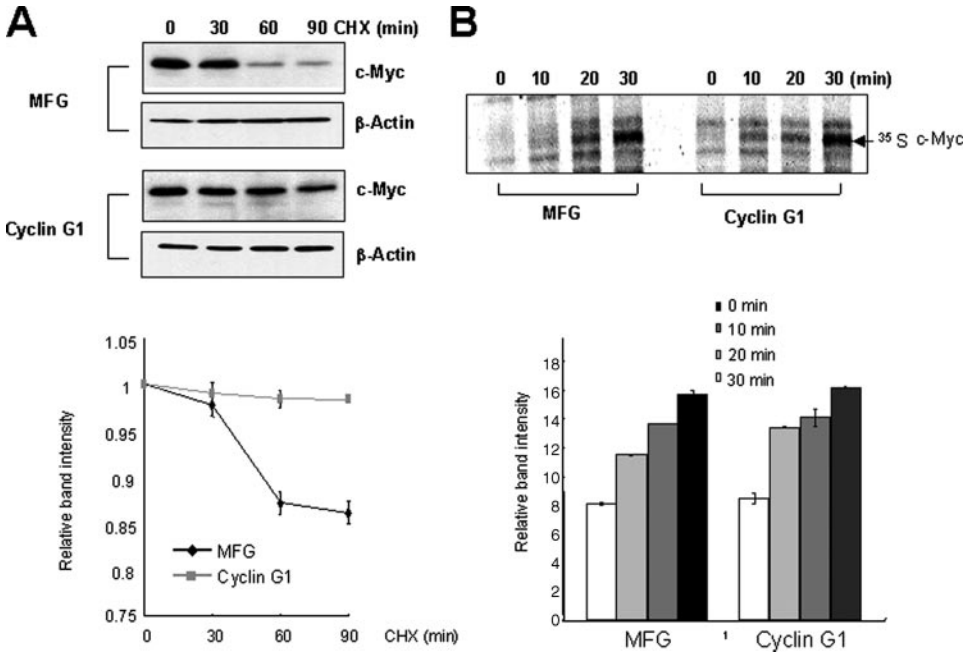
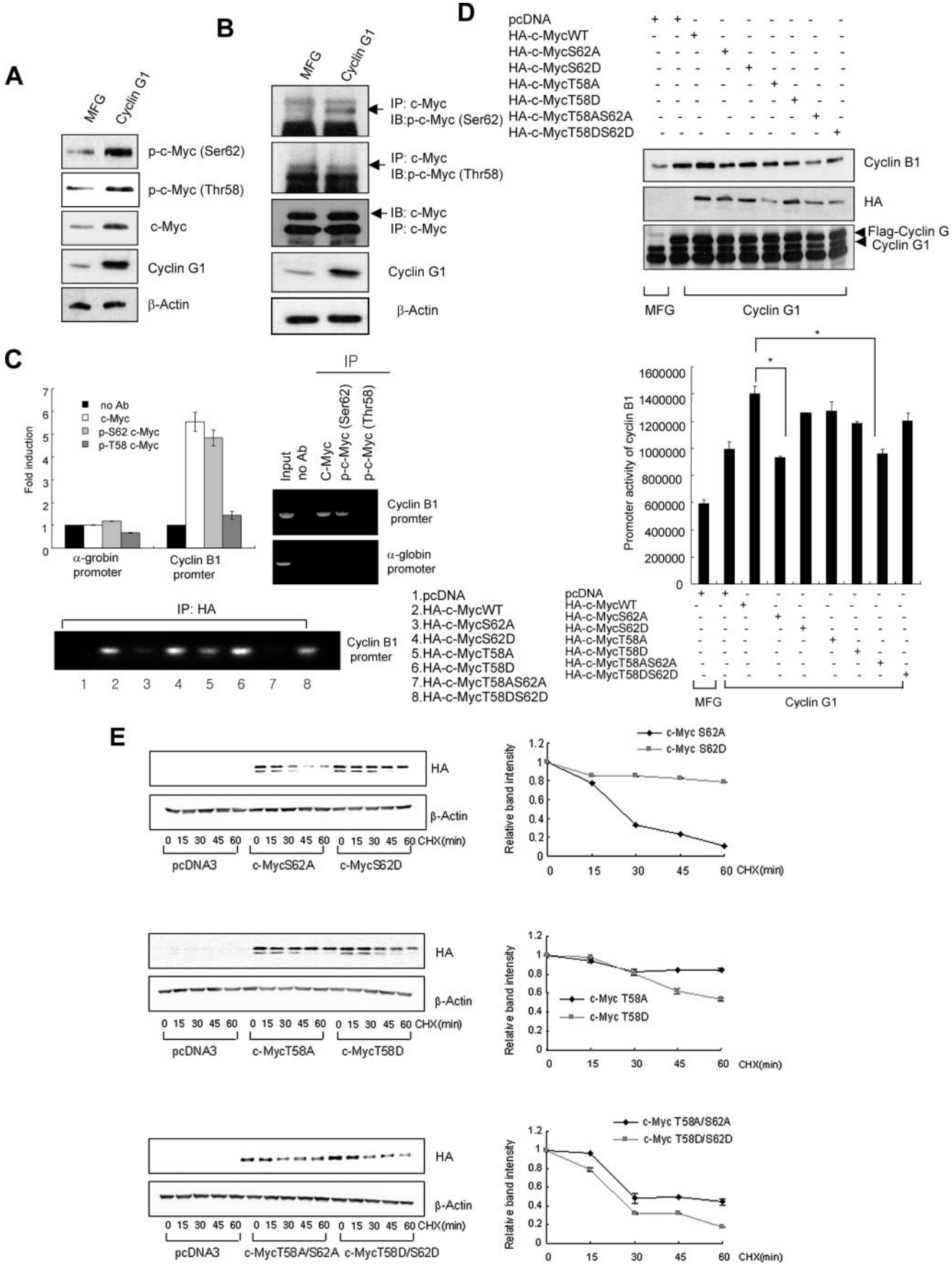



FIGURE 3. **Increased protein stability of c-Myc by cyclin G1.** *A*, protein extracts were prepared at the indicated time points following cycloheximide treatment (20 $\mu$g/ml) of MFG control and cyclin G1-overexpressing NCI-H460 cells. Western blotting was performed using anti-c-Myc (*top*), and the relative band intensity was then calculated by comparing densitometric scans of the sample immunoblots with the values of control samples, which were set to 1 (*bottom*). The results represent one of three independent experiments. *B*, protein synthesis was conducted in MFG control and cyclin G1-overexpressing NCI-H460 cells with [$^{35}$S]methionine for 30 min. Samples were collected every 10 min and analyzed to determine the rate of protein synthesis (*top*). Relative band intensity was calculated by comparing densitometric scans of the sample blots with those of control samples (0 time point), which were set to 1 (*bottom*). Means ± S.D. is shown.

E-box of the cyclin B1 promoter in cells that overexpressed cyclin G1. To directly test the *in vivo* binding activity of c-Myc to the cyclin B1 promoter, we performed chromatin immunoprecipitation (ChIP) assays. Quantitative PCR and PCR data carrying the E-box DNA fragment in ChIPs of cyclin G1-overexpressing cells yielded signals for c-Myc and phospho-c-Myc (Ser-62) antibodies. However, the antibody of phospho-c-Myc (Thr-58) did not bind to the cyclin B1 promoter, and when we performed a ChIP assay of the $\alpha$-globin promoter, no signals were detected (Fig. 4*C*, *top*). Moreover, a ChIP assay conducted using the lysates of cells transfected with various point mutants revealed that transfection of plasmids encoding wild-type or phospho-c-Myc on Ser-62 resulted in direct binding to the cyclin B1 promoter region but that transfection of plasmid encoding phospho-c-Myc on Thr-58 did not (Fig. 4*C*, *bottom*). Further, transfection with the point mutant containing c-Myc on Ser-62 (c-MycS62A, a phosphorylation-defective mutant) did not induce cyclin B1 protein expression or cyclin B1 promoter activity, even in the cyclin G1-overexpressing cells. However, the phospho-mimetic form still induced cyclin B1 protein expression and promoter activity in the case of c-MycS62D. Moreover, a mutation of c-Myc on Thr-58 (c-MycT58A) did not affect cyclin G1-mediated cyclin B1 protein expression or promoter activity. Additionally, transfection of c-MycT58A to cyclin G1-overexpressing cells increased cyclin B1 protein expression and its promoter activity; however, this increase was not found in the transfection of MycT58AS62A. This finding suggests that c-Myc phosphorylation on Ser-62 is important for increased cyclin B1 promoter activity by cyclin G1 (Fig. 4*D*). Protein stability was increased following cycloheximide treatment of c-MycS62D- or c-MycT58A-transfected cells, even though these cells showed a shortened half-life (Fig. 4*E*), which indicates that phosphorylation of c-Myc on Ser-62 by cyclin G1 overexpression increased the protein stability of c-Myc, as well as the binding activity of the E-box region of the cyclin B1 promoter. However, the phospho-defective form of c-Myc increased the protein stability of Thr-58.

*Activation of Cdk5 by Cyclin G1 Phosphorylates c-Myc on Ser-62*—The activity of Cdk5, a binding partner of cyclin G1, was increased in cyclin G1-overexpressing cells (supplemental Fig. 3). In addition, treatment of the cells with Si-Cdk5 or Si-cyclin G1 inhibited this kinase activity when c-Myc protein was used as a substrate. Increased phosphorylation of c-Myc on Ser-62 and cyclin B1 protein expression in cyclin G1-overexpressing cells were also inhibited by treatment with Si-Cdk5 or Si-cyclin G1. This suggests that Cdk5 is involved in cyclin G1-mediated c-Myc phosphorylation and cyclin B1 expression, as well as in cyclin B1 transcriptional activity (Fig. 5, *A* and *B*). To determine whether cyclin G1 or Cdk5 directly regulated c-Myc phosphorylation, an *in vitro* kinase assay was performed. Treatment with GST-cyclin G1 protein alone did not induce $^{32}$P incorporation when c-Myc was used as a substrate. However, treatment with GST-Cdk5 protein induced c-Myc phosphorylation, and co-treatment with GST-cyclin G1 and GST-Cdk5 protein enhanced $^{32}$P incorporation, which suggests that Cdk5 is responsible for c-Myc phosphorylation and co-treatment of cyclin G1 and that Cdk5 potentiates this phenomenon (Fig. 5*C*). An *in vitro* translational assay using a TnT kit also indicated that Cdk5 directly bound to c-Myc and that cyclin G1 potentiated this binding (Fig. 5*D*). Furthermore, addition of GST-cyclin G1 protein to the cell lysates of NCI-H596 resulted in a lower expression of cyclin G1, which in turn increased the level of c-Myc phosphorylation on Ser-62 via activation of Cdk5 by cyclin G1. Additionally, cell lysates that were treated with GST-Cdk5 protein showed greater c-Myc phosphorylation than those treated with only GST-cyclin G1 protein, and co-treatment with GST-cyclin G1 and GST-Cdk5 proteins dramatically increased these phenomena (Fig. 5*E*). Taken together, these results indicate that cyclin G1-mediated c-Myc phosphorylation on Ser-62 and cyclin B1 expression resulted from Cdk5 activation by cyclin G1.

*Binding Site of c-Myc in the Interaction with Cdk5*—The binding of c-Myc with Cdk5 was detected in the immunoprecipitates of NCI-H460 cells following transfection with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MFG
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p-c-Myc (Thr58)
c-Myc
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B
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IB:p-c-Myc (Ser62)
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Cyclin B1
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Flag-Cyclin G
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p-S62 c-Myc
p-T58 c-Myc
Fold induction
α-grobin promoter
Cyclin B1 promter
Input
no Ab
C-Myc
p-c-Myc (Ser62)
p-c-Myc (Thr58)
Cyclin B1 promter
α-globin promoter
IP: HA
Cyclin B1 promter
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4.HA-c-MycS62D
5.HA-c-MycT58A
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7.HA-c-MycT58AS62A
8.HA-c-MycT58DS62D
Promoter activity of cyclin B1
pcDNA
HA-c-MycWT
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HA-c-MycS62D
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MFG
Cyclin G1
E
HA
β-Actin
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pcDNA3
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c-MycS62D
Relative band intensity
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c-Myc S62D
CHX(min)
HA
β-Actin
pcDNA3
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c-MycT58D
c-Myc T58A
c-Myc T58D
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c-Myc T58D/S62D

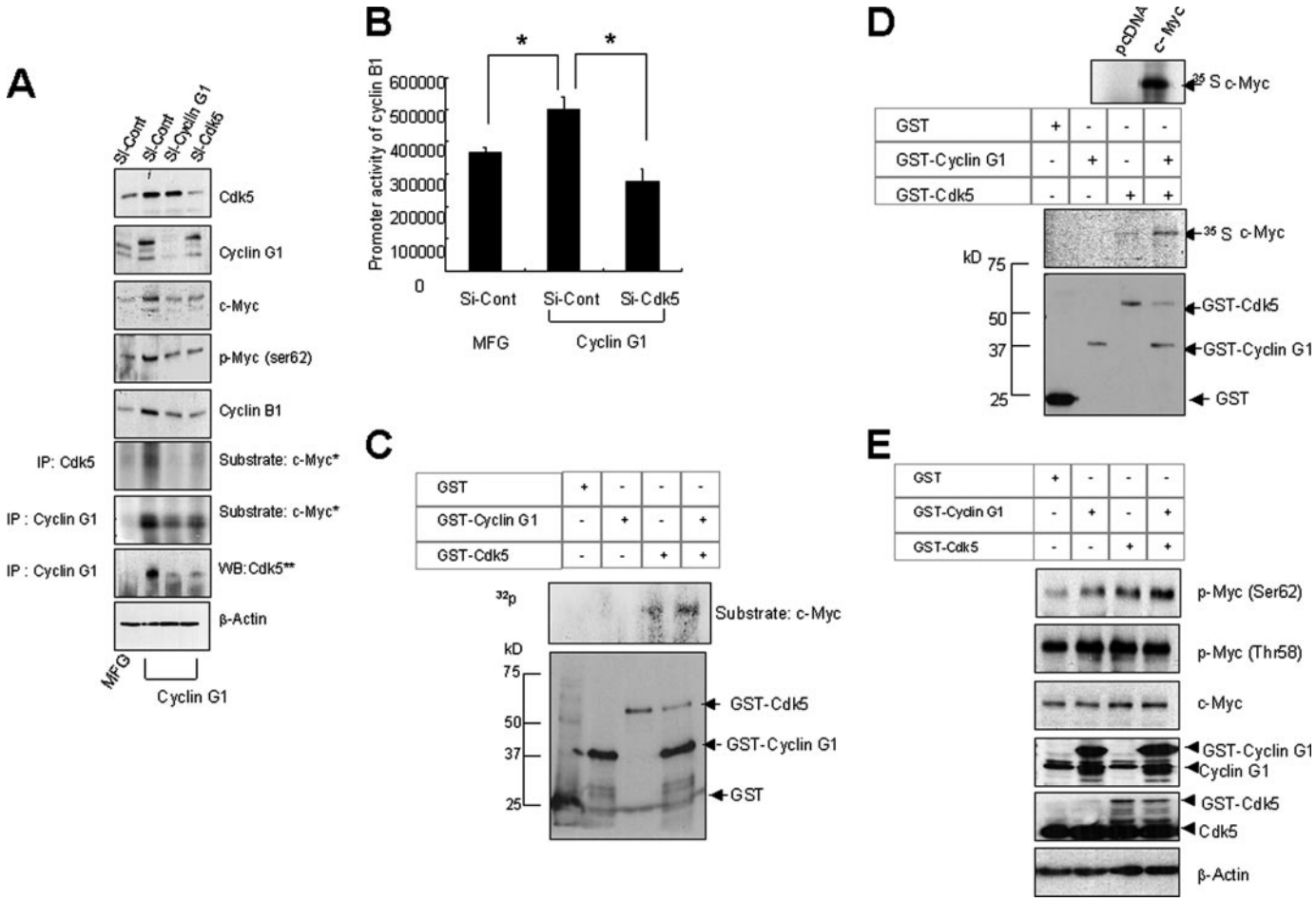


FIGURE 5. **Cdk5 activation by cyclin G1 phosphorylates c-Myc on Ser-62.** *A*, siRNA of scrambled control (*Si-Cont*), cyclin G1 (*Si-Cyclin G1*), cyclin B1 (*Si-Cyclin B1*), or Cdk5 (*Si-Cdk5*) were transfected to MFG control and cyclin G1-overexpressing cells. Western blot (*WB*), immunoblotting, or kinase assays were performed following immunoprecipitation (*IP*). *, denotes kinase assay; **, denotes immunoprecipitation assay. *B*, siRNA of scrambled control or Cdk5 was transfected to MFG control and cyclin G1-overexpressing NCI-H460 cells, and a cyclin B1 promoter assay was performed by luciferase assay. The results represent the means ± S.D. of three independent experiments. *, denotes statistical significance of $p < 0.05$ *C*, an *in vitro* kinase assay was conducted using cyclin G1, Cdk5, and c-Myc recombinant proteins. *D*, an *in vitro* translation and a GST pulldown assay were conducted using cyclin G1, Cdk5, and c-Myc recombinant proteins. *E*, Western blotting was performed using NCI-H596 cell lysates with or without GST-cyclin G1 or GST-Cdk5 recombinant proteins.

c-Myc (Fig. 6*A*); therefore, we evaluated the binding activity of several deletion constructs of c-Myc. The F4 fragment was found to bind to Cdk5; however, no interactions were observed between Cdk5 and fragments F1, F2, and F3 (Fig. 6*B*). These findings indicate that amino acid sequence 1–100 of the amino terminus of c-Myc is the binding site that interacts with Cdk5.

*Involvement of Increased Activity of Cdk5 in Cyclin G1-overexpressing Cells in Radiation-induced M Phase Arrest and Cell Death*—It has been reported that cyclin G1 overexpression results in increased radiation-induced M phase arrest and cell death. In this study, activation of Cdk5 by cyclin G1 phosphorylated c-Myc on Ser-62, which was in turn responsible for increased cyclin B1 promoter activity. Therefore, we evaluated Cdk5 to determine whether it was involved in M phase arrest and cell death. Phosphorylated histone H3, a mitosis marker, was induced by overnight nocodazole block and release for a greater length of time in cells that overexpressed cyclin G1 than in MFG control cells. However, when cells that overexpressed cyclin G1 were treated with Si-Cdk5, this phenomenon was blocked (Fig. 7*A*, *top*). Increased $G_2$/M phase arrest by nocodazole treat-

FIGURE 4. **Increased phosphorylation of c-Myc on Ser-62 by cyclin G1.** *A*, Western blot analysis of the phosphorylated forms of c-Myc (Ser-62 and Thr-58) in control and cyclin G1-overexpressing cells *B*, immunoblotting (*IB*) analysis of the phosphorylated forms of c-Myc (Ser-62 and Thr-58) following immunoprecipitation (*IP: c-Myc*) was performed in MFG control and cyclin G1-overexpressing cells *C*, ChIP-quantitative PCR analysis was performed to determine the *in vivo* binding of c-Myc to the cyclin B1 promoter. The α-globin promoter was used as a negative control in the ChIP-quantitative PCR (*left*). A ChIP assay was conducted in NCI-H460 cells or in cells transfected with various point mutant plasmids of c-Myc as reported under "Experimental Procedures." Amplification was performed using oligonucleotides specific to the cyclin B1 (region −329/−3) gene. *D*, NCI-H460 cells were transiently transfected with control or mutant c-Myc plasmids. Western blot analysis and luciferase assays were performed, and the results are presented as the means ± S.D. of three independent experiments. *E*, protein extracts were prepared at the indicated time points following cycloheximide treatment (20 μg/ml) of NCI-H460 cells transfected with pcDNA3 and various point mutant plasmids of c-Myc that were hemagglutinin (*HA*)-tagged, and Western blot analysis was then performed using anti-hemagglutinin antibody. The relative band intensity was calculated from densitometric scans of immunoblots, with the control value set to 1. The results represent one of three independent experiments.

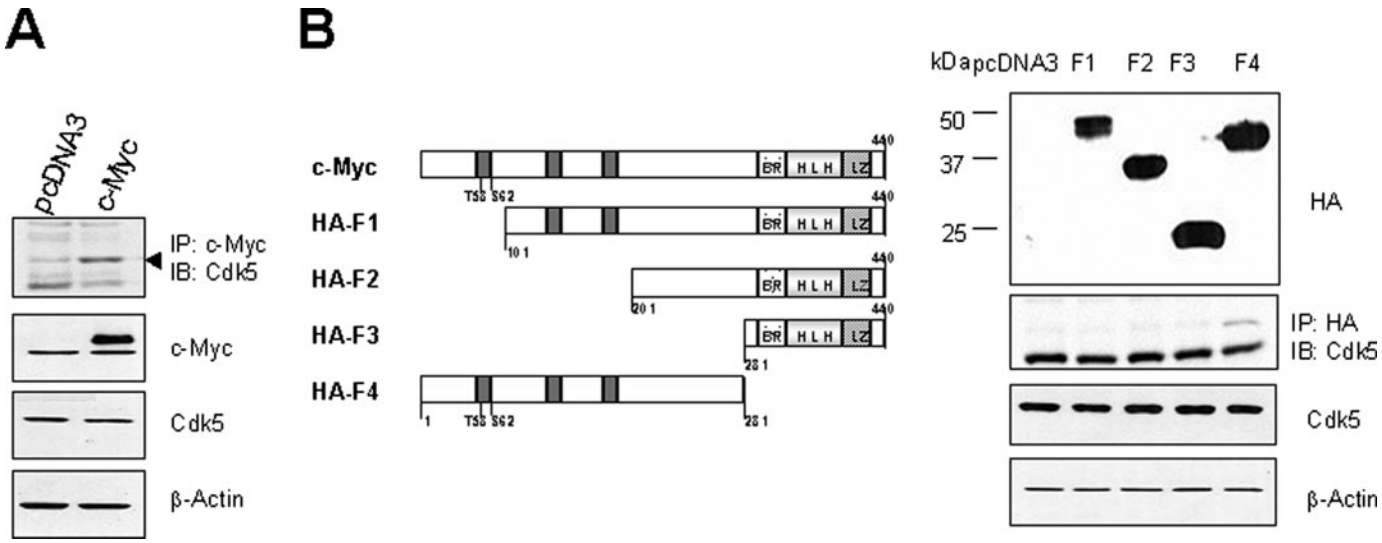


FIGURE 6. **Amino-terminal sequence of c-Myc is the binding site for interaction with Cdk5.** *A*, pcDNA3 and c-Myc were transfected to H460 cells, and Western blot or immunoblotting (*IB*) was conducted following immunoprecipitation (*IP*). *B*, various deletion constructs of hemagglutinin (*HA*)-tagged c-Myc (*left*) were transfected to H460 cells. Western blot or immunoblotting was performed following immunoprecipitation (*right*).

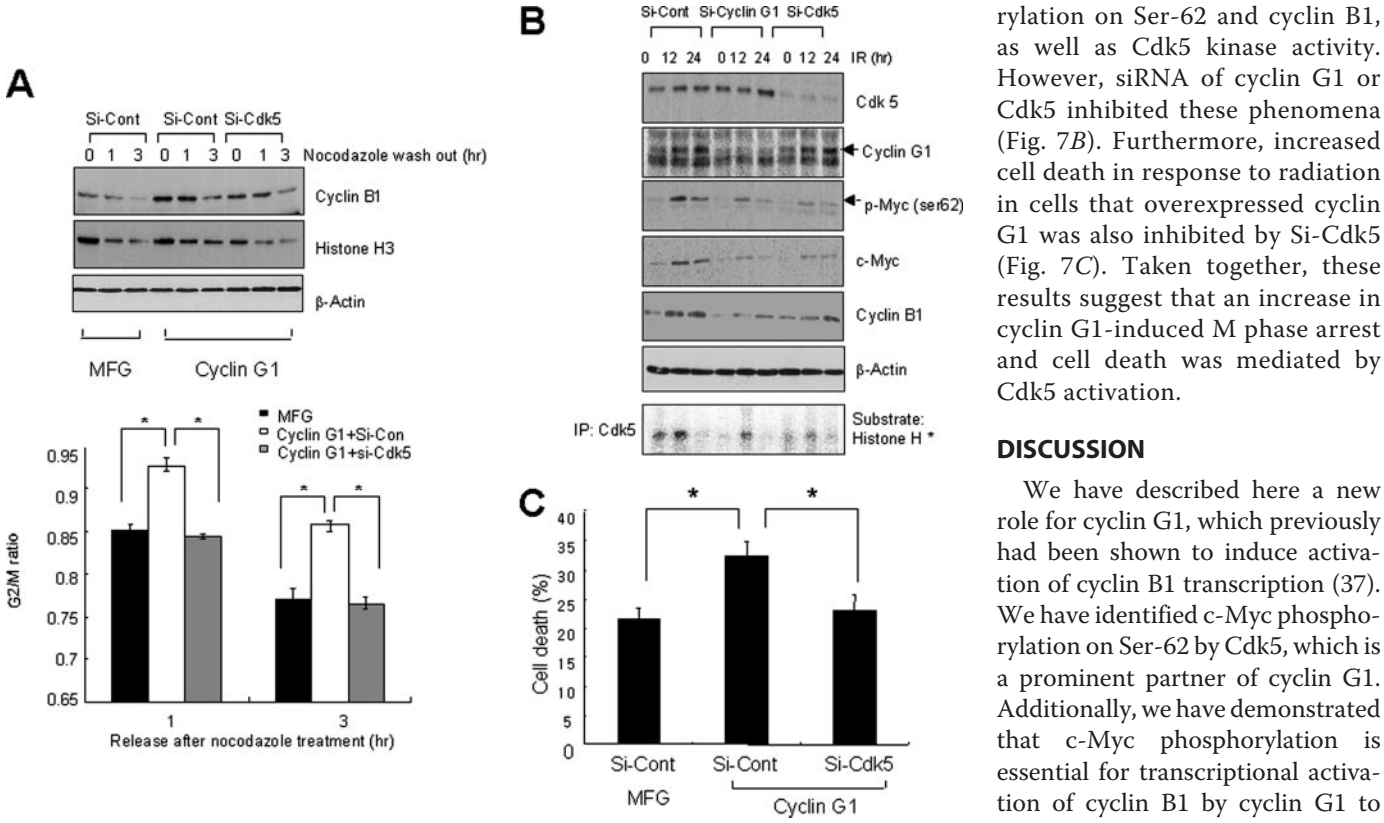


FIGURE 7. **Inhibition of cyclin G1-mediated radiation sensitivity by knockdown of Cdk5.** *A*, siRNAs from scrambled control (*Si-Con*) or Cdk5 (*Si-Cdk5*) were transfected to MFG control and cyclin G1-overexpressing NCI-H460 cells. Following overnight treatment with 200 nM nocodazole, cells were cultured with new media. Western blot analysis (*top*) and flow cytometry (*bottom*) were then performed at the indicated time points. *B*, Western blot or kinase assay of NCI-H460 cells transfected with siRNA of control, cyclin G1, or Cdk5 was conducted at the indicated time points following treatment with 5-Gy radiation. The results represent one of three independent experiments. *, denotes kinase assay. *IP*, immunoprecipitation. *C*, flow cytometric analysis after propidium iodide staining was conducted following 48 h of 5-Gy radiation using MFG control and cyclin G1-overexpressing NCI-H460 cells. The results represent the means ± S.D. of three independent experiments. *, denotes statistical significance of $p < 0.05$.

ment in cells that overexpressed cyclin G1 was also inhibited by Si-Cdk5 (Fig. 7*A*, *bottom*). Additionally radiation increased the expression of cyclin G1 and c-Myc phosphorylation on Ser-62 and cyclin B1, as well as Cdk5 kinase activity. However, siRNA of cyclin G1 or Cdk5 inhibited these phenomena (Fig. 7*B*). Furthermore, increased cell death in response to radiation in cells that overexpressed cyclin G1 was also inhibited by Si-Cdk5 (Fig. 7*C*). Taken together, these results suggest that an increase in cyclin G1-induced M phase arrest and cell death was mediated by Cdk5 activation.

## DISCUSSION

We have described here a new role for cyclin G1, which previously had been shown to induce activation of cyclin B1 transcription (37). We have identified c-Myc phosphorylation on Ser-62 by Cdk5, which is a prominent partner of cyclin G1. Additionally, we have demonstrated that c-Myc phosphorylation is essential for transcriptional activation of cyclin B1 by cyclin G1 to occur. Cyclin G1 was originally identified as a novel member of the cyclin family and is one of the target genes of transcriptional factor p53 (29, 30). However, in our system, the role that cyclin G1 plays in transcriptional activation of cyclin B1 exhibited a p53-independent pattern (supplemental Fig. 4). In addition, the cyclin G1-mediated increase of cyclin B1 expression was observed in both p53 wild-type and null cells. Moreover, there are reports that p53 can

repress the transcription of cyclin B1 (14), which suggests that p53 function is not involved in cyclin B1 transcription.

Cyclin G1 has been shown to play an important role in various biological phenomena (36, 39, 40) including apoptosis (34), and the molecules that it associates with have been identified (31, 41). However, the functional consequences of cyclin G1 are still the subject of debate. For instance, transcription of cyclin G1 is induced within a few hours of growth factor stimulation of quiescent cells (42). In addition, retroviral vector-mediated gene transfection of antisense cyclin G1 inhibits proliferation of human osteogenic sarcoma cells (43), which suggests that cyclin G1 plays a pivotal role in mammalian cell growth regulation. Conversely, some data suggest that cyclin G1 has growth-inhibitory activity (36, 39, 44). Furthermore, a recent study concluded that a low level of cyclin G1 results in a lack of growth-inhibitory activity and may even promote growth (37), whereas high levels of cyclin G1 suppress growth activity.

Previously, we found that cyclin G1 increases radiation-induced M phase arrest and cell death as a result of increased cyclin B1 promoter activity. Synthesis of cyclin B1 during the cell cycle is regulated primarily at the transcriptional level (37), and cell cycle-related transcription by this promoter is regulated by a CDE and CHR tandem element (7). However, the cyclin B1 promoter is also subject to activation by c-Myc.

Here, we have demonstrated that cells that overexpress cyclin G1 increased the promoter activity of cyclin B1 through activation of the E-box, which suggests that activation of the cyclin B1 promoter by cyclin G1 is dependant on c-Myc (Fig. 1). Indeed, in our system, cyclin G1 overexpression increased the c-Myc protein level (Fig. 2). However, levels of c-Myc mRNA and c-Myc protein synthesis were not altered by cyclin G1, but the protein stability of c-Myc was increased (Fig. 3). Therefore, we evaluated the phosphorylation status of c-Myc to determine whether it was affected by cyclin G1. It has been reported that phosphorylation sites Thr-58 and Ser-62 are associated with c-Myc degradation (24–27), and in this study we observed that cyclin G1 increased c-Myc phosphorylation on Ser-62. However, increased phosphorylation of c-Myc on Thr-58 in cells that overexpressed cyclin G1 was not the direct consequence of overexpression of cyclin G1 (Fig. 4). Phosphorylation of c-Myc on Ser-62 is regulated by ERK1/2 activation, and GSK-3$\beta$ is involved in c-Myc phosphorylation on Thr-58. Nevertheless, because cyclin G1 overexpression did not affect the status of ERK1/2 and GSK-3$\beta$ phosphorylation (supplemental Fig. 5), we excluded c-Myc phosphorylation by ERK1/2 or GSK-3. Although the mechanism by which phospho-c-Myc on Thr-58 increased by cyclin G1 is unclear, it is possible that increased phospho-c-Myc on Ser-62 affected the stability of phospho-c-Myc on Thr-58. Indeed, when we examined the half-life of the phospho-mimetic (c-MycT58D) or phospho-defective (c-MycT58A) forms of c-Myc on Thr-58, c-MycT58A was found to have greater stability than c-MycT58D (Fig. 4). In our system, the phospho form of c-Myc on Ser-62 was found to increase the binding activity of the cyclin B1 promoter region. It has been reported that c-Myc phosphorylation on Ser-62 and Thr-58 has no impact on the transcriptional activity of E-box-containing promoters (45–47). However, several studies have suggested that phosphorylation of c-Myc enhances the transcriptional activity of its target genes (27, 48, 49). Our data also suggest that phosphorylation of c-Myc on Ser-62, but not on Thr-58, affects its ability to bind to the E-box of cyclin B1 (Fig. 4). It is not clear why only phospho-c-Myc on Ser-62 enhanced the promoter activity of cyclin B1; however, it has been shown that c-MycS62A fails to be recruited to the E-box site of $\gamma$-glutamyl cysteine synthetase promoter (48), which is similar to the results of this study.

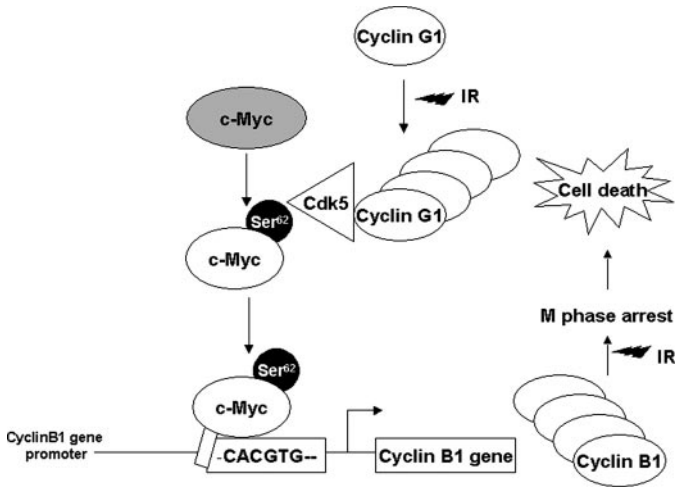


FIGURE 8. **Hypothetical scheme of Cdk5-mediated c-Myc phosphorylation on Ser-62 in cyclin G1-overexpressing cells.** Cdk5, the binding partner of cyclin G1, was responsible for c-Myc phosphorylation on Ser-62, and this stable form of phosphorylated c-Myc then bound to the E-box promoter region of cyclin B1, which resulted in increased radiation-induced M phase arrest and cell death in cyclin G1-overexpressing cells.

Cyclin G1 does not have kinase activity; therefore, we evaluated binding partners of cyclin G1, such as Cdk5, to determine whether they were involved in c-Myc phosphorylation. Cdk5 kinase activity was found to be increased by cyclin G1 overexpression, and knockdown of Cdk5 by siRNA showed inhibition of c-Myc phosphorylation on Ser-62 as well as inhibition of cyclin B1 promoter activity. In addition, an *in vitro* kinase assay revealed that Cdk5 alone induced c-Myc phosphorylation and that additional treatment with cyclin G1 potentiated this phenomenon, whereas cyclin G1 alone did not (Fig. 5). Furthermore, the results of this study revealed an interaction between c-Myc and Cdk5 (Fig. 6), which suggests that this interaction was important for the phosphorylation of c-Myc by Cdk5 to occur.

We previously reported that cyclin G1 was overexpressed in cancer tissues, including cancerous lung tissues (37). In this study, the cyclin G1 levels in several lung cancer cell lines were found to be well correlated with c-Myc phosphorylation on Ser-62, as well as with cyclin B1 expression, both of which were mediated by Cdk5 (supplemental Fig. 6). Taken together, these results suggest that the cyclin B1 expression level in cyclin G1-overexpressing cancer cells was controlled by Cdk5. It has been shown that cyclin G1 overexpression results in increased radiation sensitivity as a result of the increased transition from the $G_2$ to M phase, which occurs due to cyclin B1 promoter activation (37). In this study, inhibition of Cdk5 by siRNA treatment was found to reduce radiation-induced $G_2$/M phase arrest and cell death in cyclin G1-overexpressing cells. This suggests that Cdk5-mediated activation of c-Myc phosphorylation on

Ser-62, which is responsible for the transcriptional activation of cyclin B1, is essential for increased radiation sensitivity by cyclin G1 (Fig. 7).

In conclusion, the results of this study provide an explanation for the mechanism by which cyclin G1 regulates cyclin B1 promoter activity. We propose that Cdk5, the binding partner of cyclin G1, is responsible for c-Myc phosphorylation on Ser-62 and that this phosphorylated c-Myc has an increased protein half-life and binds to the E-box promoter region of cyclin B1. Further, we suggest that these effects result in increased cyclin B1 promoter activation in cyclin G1-overexpressing cells, which are frequently observed in cancer cells (Fig. 8).

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