

ORIGINAL ARTICLE

Involvement of protein phosphatase 2A nuclear accumulation and subsequent inactivation of activator protein-1 in leptomycin B-inhibited cyclin D1 expression

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Leptomycin B (LMB) is a *Streptomyces* metabolite that causes the specific inhibition of the nuclear export of proteins containing a nuclear export signal (NES). LMB was reported to inhibit cell cycle progression in fission yeast and mammalian cells, however, the mechanism underlying LMB-induced cell cycle arrest is still obscure. In this study, we found that in serum-starved NIH3T3 cells, LMB inhibited serum-induced cyclin D1 expression at the level of transcription. However, this inhibition was reversed by inhibitors of protein phosphatase 2A (PP2A). Furthermore, we found that PP2A accumulated in the nucleus upon treatment with LMB. The finding prompted us to identify the functional NES in PP2A catalytic subunit α . These results indicated that LMB inhibited the chromosomal region maintenance 1 (CRM1)-dependent nuclear export of PP2A, resulting in sustained dephosphorylation in the nucleus. Although phosphorylation of c-Jun at Ser-63 is required for activator protein 1 (AP-1)-dependent expression of cyclin D1, it decreased in LMB-treated cells compared to untreated cells. Moreover, the inhibitors of PP2A restored the levels of c-Jun phosphorylated at Ser-63. We propose that inhibition of cyclin D1 expression by LMB is mediated by the LMB-induced nuclear accumulation of PP2A, leading to sustained dephosphorylation of c-Jun at Ser-63, which leads to inactivation of the transcription of the AP-1-responsive cyclin D1 gene.

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Introduction

The subcellular redistribution and compartmental sequestration of proteins have emerged as important mechanisms in the regulation of cellular response. The

transport of proteins between the nucleus and cytoplasm is an essential cellular process in all eukaryotes. The nuclear localization of a protein is usually controlled by two opposing signals: the nuclear localization signal (NLS) and the nuclear export signal (NES). The first NLS to be identified, referred to as the classic NLS, falls into two categories: a simple sequence of 3–5 basic amino-acid residues (monopartite), and a bipartite signal consisting of a basic dipeptide upstream from a simple basic sequence. Other types of NLS have been identified in heterogeneous nuclear ribonucleoproteins, ribosomal proteins and U small nuclear ribonucleoprotein (snRNPs) (Schaap *et al.*, 1991; Fischer *et al.*, 1993; Weighardt *et al.*, 1995). Conversely, a NES is a short sequence that is necessary and sufficient for nuclear export of its host protein. The NES was first identified in the viral human immunodeficiency virus-1 Rev protein (Fischer *et al.*, 1995) and in the cellular protein kinase inhibitor (PKI) (Wen *et al.*, 1995). Both sequences contain a stretch of four orderly spaced leucines. Numerous studies have contributed to the definition of the leucine-rich NES consensus sequence as Φ -X₂₋₃- Φ -X₂₋₃- Φ -X- Φ (Φ : L, I, F, V, M; X: any amino acid) (Bogerd *et al.*, 1996; Zhang and Dayton, 1998; Henderson and Eleftheriou, 2000). Mutations of leucines in the NES disrupt the ability of the protein to localize in the cytoplasm. At least 75 proteins containing a NES have been identified to date (la Cour *et al.*, 2003). These include cellular proteins, many of which are involved in transcription, cell signaling cascades, oncogenic transformation and regulation of the cell cycle. Examples include PKI, mitogen-activated protein kinase kinase (MAPKK), TFIIIA, Mdm2, p53, I κ B α , NMD3, cyclin B1, c-Abl and 14-3-3 σ (Gama-Carvalho and Carmo-Fonseca, 2001; Kutay and Guttinger, 2005). The activities of these proteins are tightly regulated by their NESs. Chromosomal region maintenance 1 (CRM1), which belongs to the family of importin-related nuclear transport receptors, directly and specifically associates with the NES and mediates the nuclear export of protein containing NESs (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997; Neville *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997; Haasen *et al.*, 1999).

Leptomycin B (LMB), a *Streptomyces* metabolite, has, until recently, been the only known small-molecule inhibitor of nuclear export (Hamamoto *et al.*, 1983a, b,

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1985). LMB was shown to interact directly with CRM1 and disrupt the interaction between CRM1 and NES, resulting in inhibition of the effects of CRM1 (Nishi *et al.*, 1994; Fornerod *et al.*, 1997; Kudo *et al.*, 1998, 1999). Since its discovery, LMB has become an important 'tool compound' for studying the regulation of nucleocytoplasmic shuttling proteins. LMB is highly specific to CRM1, and highly potent, as exemplified by its ability to block CRM1-NES binding at nanomolar concentrations *in vitro*. Using LMB to alter the cellular localization of a protein can reveal much about its cell biology.

Previous studies have demonstrated that the proliferation of rat 3Y1 fibroblasts was reversibly blocked by LMB in both the G1 and G2 phases (Yoshida and Beppu, 1988; Yoshida *et al.*, 1990). These results suggest that the molecular target of LMB, whose CRM1-dependent nuclear export is inhibited causing it to accumulate in the nucleus, is one of the components necessary for progression to both G1 and G2 in the eukaryotic cell cycle. However, the molecular mechanisms by which LMB induces cell cycle arrest remain poorly understood.

In the present study, we demonstrated that treatment with LMB induced G1 arrest in NIH3T3 cells, and suggested that the cell cycle arrest induced by LMB is related with the nuclear accumulation of protein phosphatase 2A (PP2A) α directed by its NES-dependent transport mechanism. Moreover, we have identified a functional NES in the amino-acid sequence of PP2A α , and found that disruption of this NES induced the nuclear localization of this protein.

Results

Inhibition of serum-induced G1 progression and Cdk2 activation by LMB in NIH3T3 cells

The effects of LMB on G0/G1 progression in serum-starved NIH3T3 cells are shown in Figure 1a. Before serum stimulation, more than 90% of cells arrested in G0/G1. Twenty hours after the addition of 10% serum,

approximately 60% of the cells had exited G0/G1 and initiated DNA synthesis. On the other hand, addition of LMB together with serum inhibited S-phase entry in a dose-dependent manner, with complete inhibition observed at 1 ng/mL. As the activated cyclin E-cyclin-dependent kinase (Cdk)2 complex that displays

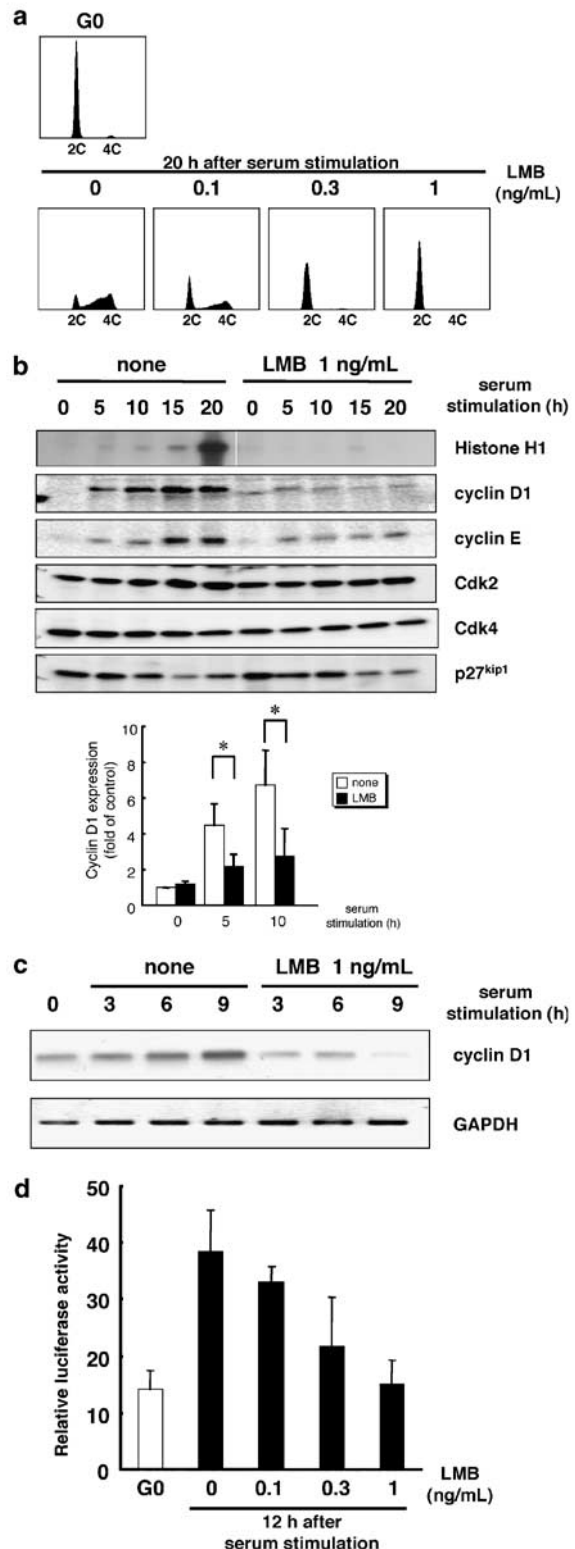


Figure 1 Inhibition of serum-induced G1 progression and G1 cyclin expression by LMB in NIH3T3 cells. Serum-starved NIH3T3 cells were restimulated with 10% serum in the presence or absence of the indicated concentrations of LMB. **(a)** The cells were collected at 20 h and DNA contents were analysed by flow cytometer. **(b)** Total cell lysates were prepared at the indicated times. Equal amounts of lysate (50 μ g) were immunoprecipitated with Cdk2 antibody, and Cdk2 immunoprecipitates were assayed *in vitro* for histone H1 kinase activity. And total protein was isolated and analysed by Western blot with antibodies recognizing cyclin D1, cyclin E, Cdk2, Cdk4 and p27^{kip1}. The cyclin D1 band intensities of three independent experiments were quantified densitometrically and represented as a fold of control. * $P < 0.05$ (Student's *t* test). **(c)** The cells were collected at the indicated times and equal amounts of total RNA were subject to Northern blot analysis for cyclin D1 expression. **(d)** The -1745D1LUC reporter was transfected into NIH3T3 cells. Serum-starved transfected cells were restimulated with 10% serum in the presence or absence of the indicated concentrations of LMB. Following 12 h incubation, cells were lysed and luciferase activity was measured. Data represent mean and s.d. of a representative experiment performed in triplicate.

in vitro histone H1 kinase activity is thought to be necessary for cells to enter the S phase, we examined the effect of LMB treatment on Cdk2 activity following restimulation of the cells (Figure 1b). Histone H1 kinase activity in cyclin E immunoprecipitates from NIH3T3 cells increased at 15 h and reached a high level at 20 h. However, only very weak histone H1 kinase activity from the cells treated with LMB was detected. This observation indicated that the inhibition of cyclin E-associated kinase activation was implicated in the inhibition of G1 progression following LMB treatment.

Inhibition of G1 cyclin expression by LMB in NIH3T3 cells

We examined the levels of expression of G1 progression-related proteins in NIH3T3 cells treated or not treated with LMB at various time points up to 20 h following serum stimulation (Figure 1b). Expression of cyclin D1 protein, which was induced at 5 h by serum restimulation, was strongly inhibited by LMB. Furthermore, LMB completely inhibited the serum-induced expression of cyclin E at 15 h. The p27^{kip1} protein was expressed under serum-starved conditions, and this expression level began to decline after 15 h. The expression profile of p27^{kip1} was not affected by LMB. In addition, LMB did not affect the expression profiles of Cdk2 and Cdk4. These results indicated that LMB inhibited the expression of cyclin D1, before cyclin E expression. Consequently, Cdk2 could not form a complex with cyclin E, resulting in the inhibition of Cdk2 activation and induction of G1 arrest in NIH3T3 cells.

LMB inhibits cyclin D1 expression at the transcriptional level

Expression of cyclin D1 is reported to be controlled at both the messenger RNA (mRNA) and protein level. To define how LMB causes the observed decrease in cyclin D1, we examined mRNA levels for serum-induced cyclin D1 at different time points upon treatment with LMB. As shown in Figure 1c, expression of cyclin D1 mRNA, which was induced at 3 h after serum restimulation, was strongly inhibited by LMB. To confirm this, we generated a human cyclin D1 promoter luciferase construct containing a cyclin D1 promoter with the 5' end residing at position -1745 and the 3' end at position +140 relative to the transcriptional initiation site and inserted it into a luciferase reporter construct (designated -1745D1LUC). The consequence of LMB treatment for promoter transactivation by serum was analysed. As illustrated in Figure 1d, treatment of NIH3T3 cells with LMB inhibited the serum-induced activation of the cyclin D1 promoter in a dose-dependent manner.

Inhibition of PP2A restored the LMB-inhibited cyclin D1 expression

We examined the mechanism underlying the LMB-induced suppression of cyclin D1 transcription using a chemical biological approach. To explore the

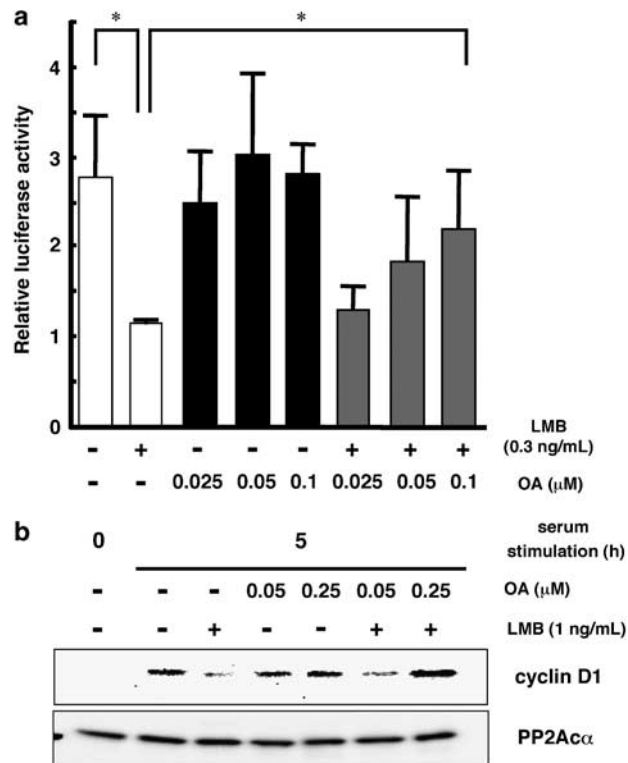


Figure 2 Evidence that PP2Acz suppresses expression of cyclin D1. (a) -1745D1LUC promoter were transfected into HEK293T cells. The transfected cells were treated with 0.3 ng/mL of LMB and/or OA (0.025, 0.05 and 0.1 μM) for 24 h and luciferase activity was measured. Data represent mean and s.d. of a representative experiment performed in triplicate. **P*<0.05 (Student's *t* test). (b) Serum-starved NIH3T3 cells were treated with 1 ng/mL of LMB and/or OA (0.05, 0.25 μM) and restimulated with 10% serum for 5 h. Total protein was isolated and analysed by Western blot with antibodies recognizing cyclin D1 and PP2Acz.

signal-transduction system involved in the inhibition of cyclin D1 expression following LMB treatment, over 90 inhibitors of signal-transduction system in the 'SCADS inhibitor kit I' (see experimental procedures) were assessed for their ability to reverse the suppressive effect of LMB on cyclin D1 promoter activity in NIH3T3 cells. After the screening of the library, we found that PP2A inhibitors, such as okadaic acid (OA), cantharidin and cytosstatin, restored the promoter activity, which was inhibited by LMB (Figure 2a and data not shown). We next examined whether PP2A inhibitors could prevent LMB-inhibited cyclin D1 expression by Western blot analysis. Treatment of serum-starved NIH3T3 cells with OA (0.25 μM) alone did not affect the expression levels of cyclin D1 at 5 h following serum restimulation. Furthermore, in the presence of OA, the expression level of cyclin D1 in LMB-treated NIH3T3 cells at 5 h was comparable to that in control cells (Figure 2b). These results suggested that PP2A is involved in the inhibitory effect of LMB on cyclin D1 gene expression.

LMB causes PP2A to accumulate in the nucleus

Because LMB is a specific inhibitor of the nuclear export of NES-bearing proteins, we hypothesized that the

inhibition of PP2A's nuclear export by LMB might be involved in the LMB-inhibited cyclin D1 expression. PP2A is a holoenzyme composed of three subunits, the catalytic subunit (PP2A/C), the structural A subunit (also known as PR65), and the regulatory B subunit (Janssens and Goris, 2001; Janssens *et al.*, 2005). To examine the possibility that LMB inhibited the nuclear export of PP2A, the subcellular distribution of PP2A catalytic subunit alpha (PP2Ac α) in control or LMB-treated NIH3T3 cells was analysed by immunostaining using anti-PP2Ac α antibody. In serum-starved NIH3T3 cells, PP2Ac α was found in both cytoplasmic and nuclear compartments (Figure 3A). Stimulation with serum did not affect its subcellular distribution. On the other hand, addition of LMB with serum for 5 h caused a progressive accumulation of PP2Ac α in the nucleus, as shown in Figure 3A. These results indicated that LMB strongly blocked the nuclear export of endogenous PP2Ac α , and suggested that the nuclear accumulation of PP2Ac α seen in the presence of LMB is mediated by the CRM1-dependent active transport mechanism. Because CRM1 interacts directly with a leucine-rich NES motif,

we next examined the primary amino-acid sequences of PP2Ac α to determine whether it contains an NES. We found that sequence between 149 and 158 conforms to this motif, as indicated by its similarity to other known NESs such as MAPKK, PKI- α , I κ B, TFIIIA, hDM2, p53 and p73 (Gama-Carvalho and Carmo-Fonseca, 2001; Kutay and Guttinger, 2005). On the other hand, we did not find any NES-like sequence in other PP2A subunits. Therefore, we generated a myc-tagged mutant PP2Ac α (PP2Ac α -mtNES) consisting of leucine to alanine conversions at residues 149, 153, 155 and 158 in the putative PP2Ac α NES (Figure 3B), as analogous mutations in other NES-containing proteins have been reported to prevent nuclear export (Bogerd *et al.*, 1996), and examined the effect to confirm the role of NES in nuclear export of PP2Ac α . The HeLa cells were transfected with pcDNA-myc-PP2Ac α or pcDNA-myc-PP2Ac α -mtNES and subcellular distribution of wild-type or mutant PP2Ac α probed with an anti-myc antibody. As shown in Figure 3C, more than 95% of HeLa cells expressing myc-tagged wt PP2Ac α showed fluorescence in both the nucleus and the cytoplasm. On

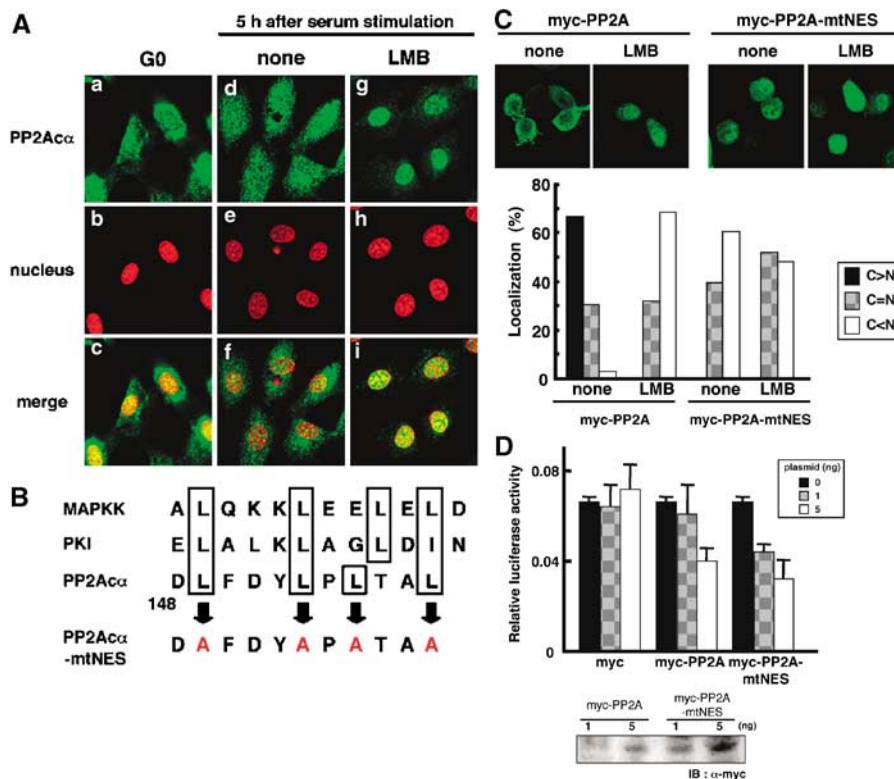


Figure 3 LMB causes PP2Ac α to accumulate in the nucleus. (A) Serum-starved NIH3T3 cells were restimulated with 10% serum in the presence or absence of 1 ng/mL of LMB. Following 5 h of incubation, cells were processed for indirect immunofluorescence microscopy with a primary antibody against PP2Ac α (a, d, g). The same cells were stained with the DNA intercalating dye, TOPRO-3 (b, e, h). (B) Schematic representation of the primary structure of PP2Ac α (human) showing a putative NES sequence. The NES sequences of MAPKK and PKI are aligned in the upper panel. Important hydrophobic residues in the sequences are boxed. (C) A myc-tagged wild-type PP2Ac α plasmid (myc-PP2A) or a mutant PP2Ac α plasmid (Leu149, 153, 155 and 158 replaced by Ala) (myc-PP2A-mtNES) was transfected into HeLa cells. Following 3 h of incubation, cells were treated with 3 ng/mL of LMB and incubated for an additional 10 h. Then cells were fixed and stained with anti-myc antibody. The distribution of myc-PP2Ac α was examined. Data represent at least 50 myc-PP2Ac α -overexpressing cells in each transfection. (D) The -1745D1LUC promoter and a myc-PP2A plasmid or a myc-PP2A-mtNES plasmid were transfected into HEK293T cells. Following 24 h of incubation, cells were lysed and luciferase activity was measured. And total protein was isolated and analysed by Western blot with an antibody recognizing myc. Data represent mean and s.d. of a representative experiment performed in triplicate.

the other hand, approximately 60% of HeLa cells expressing mutant PP2Ac α -mtNES had fluorescence exclusively in the nucleus, and the remaining 40% had in both nuclear and cytoplasm, indicating the absence of nuclear export potential of PP2Ac α -mtNES. Moreover, only weak effect of LMB on nuclear localization of PP2Ac α -mtNES was observed. In this study, we demonstrated, for the first time, that PP2Ac α undergoes active nuclear export and that this export is LMB-sensitive and mediated by a functional NES located in the PP2Ac α C terminus.

LMB might exerts its function through the AP-1 recognition sequence

In view of our findings that LMB induced nuclear accumulation of PP2Ac α and inhibited cyclin D1 expression, we hypothesized that the nuclear accumulation of PP2Ac α by LMB might induce sustained dephosphorylation and inactivation of nuclear proteins involved in the regulatory mechanism of cyclin D1 expression. One such candidate nuclear protein is transcription factor, because certain transcription factors are reported to be activated by phosphorylation. A diagram of the human cyclin D1 gene, showing several potential transcription factor binding sites, is shown in Figure 4a. To map the region of cyclin D1 promoter that is responsible for cyclin D1 expression, we generated a series of truncations in the cyclin D1 promoter-luciferase reporter construct as shown in Figure 4a, and these truncated constructs were transfected into human embryonic kidney (HEK)293T cells to determine the effect of each deletion on their promoter activities (Figure 4a, open bars). In this study, the luciferase activity obtained with the full-length -1745D1LUC construct was defined as 1.0. We found that high reporter activity was retained with the -973D1LUC construct, but significantly lower activity was observed with the longer truncated -742, -123 and -66D1LUC constructs, which lack the activator protein 1 (AP-1) response element (Figure 4a). The site-directed mutation of the AP-1 site, previously shown to abolish binding of AP-1 proteins in electrophoretic mobility-shift assays (Albanese *et al.*, 1995), in the context of the 1745-bp promoter fragment (-1745AP1mtD1LUC), reduced transactivation by 40%. We also examined the effect of LMB on each truncated construct. Deletion down to -742 did not result in significant loss of repression of LMB (Figure 4a, filled bars), indicating that the sequence between -973 and -742 in the cyclin D1 promoter, which comprises an AP-1 site, is responsible for the inhibition of the cyclin D1 promoter by LMB. Taken together, LMB induced the inhibition of cyclin D1 expression possibly through the AP-1 enhancer element.

Reduction of c-Jun N-terminal phosphorylation correlates with the induction of PP2A activity following LMB treatment

The major components of the AP-1 complex have been studied to be c-Jun and c-Fos proteins. Previous studies

have shown that c-Jun is transiently phosphorylated in its N-terminal activation domain on serine residues 63 and 73 (Ser-63 and Ser-73), modification that is expected to increase c-Jun transactivation potential (Minden *et al.*, 1994; Karin *et al.*, 1997; Bakiri *et al.*, 2000). To examine whether the phosphorylation of c-Jun at N-terminal activation domain is related to the expression of cyclin D1, we overexpressed wild-type c-Jun or mutant c-Jun in which Ser-63 and Ser-73 were replaced by alanine, and examined the effect of these proteins on cyclin D1 promoter activity. As shown in Figure 4b, wild-type c-Jun strongly activated the cyclin D1 reporter in a dose-dependent manner. In contrast, replacing Ser-63 with alanine decreased its transcriptional activation of the cyclin D1 promoter. Furthermore, the ability of c-Jun to activate cyclin D1 transcription was seriously diminished by the replacing of both Ser-63 and Ser-73 with alanines. Therefore, we thought c-Jun as the possible candidate nuclear protein that is functionally involved in the mechanism of the inhibition of cyclin D1 expression induced by LMB. If this was the case, phosphorylation levels of c-Jun would decrease by the treatment with LMB. Hence, we examined the phosphorylation status of c-Jun in NIH3T3 cells by Western blot analysis using phospho-specific antibody against Ser-63 of c-Jun. When serum-starved NIH3T3 cells were restimulated by serum, c-Jun phosphorylation was strongly induced reaching a maximum at 30 min and returning to the basal level at 4 h after stimulation, whereas a potent inhibitory effect of LMB was readily detectable (Figure 4c). Treatment with LMB had no detectable effect on the total protein levels of c-Jun. The experiment suggested that the repression of the AP-1 activity observed in NIH3T3 cells may be due at least in part to a reduction in N-terminal phosphorylation of c-Jun. Furthermore, to elucidate whether decreased phosphorylation level of c-Jun in LMB-treated cells was caused by PP2A accumulated in nucleus, LMB-treated cells were incubated with OA and the phosphorylation level of c-Jun was examined. As shown in Figure 4d, the inhibition of PP2A activity by the treatment with 0.25 μ M OA in the presence of LMB restored the protein level of phosphorylated c-Jun to those of control cells. To confirm that dephosphorylation of c-Jun Ser-63/Ser-73 by PP2A is involved in LMB-induced inhibition of cyclin D1 expression, we constructed constitutive active mutant c-Jun in which Ser-63 and Ser-73 were both replaced by aspartic acid (S63/73D) (Bakiri *et al.*, 2000), and examined whether the ability of this mutant c-Jun (S63/73D) to activate cyclin D1 promoter is inhibited by LMB. As shown in Figure 4e, LMB inhibited the cyclin D1 promoter activity induced with wild-type c-Jun expression, whereas it failed to inhibit the cyclin D1 promoter activity induced with c-Jun (S63/73D). Taken together, we proposed the molecular mechanism for LMB-inhibited cyclin D1 expression as follows: LMB induced nuclear accumulation of PP2Ac α by inhibiting its CRM1-dependent nuclear export, leading to enhancement of dephosphorylation and inactivation of c-Jun in the nucleus, thereby failing to stimulate cyclin D1

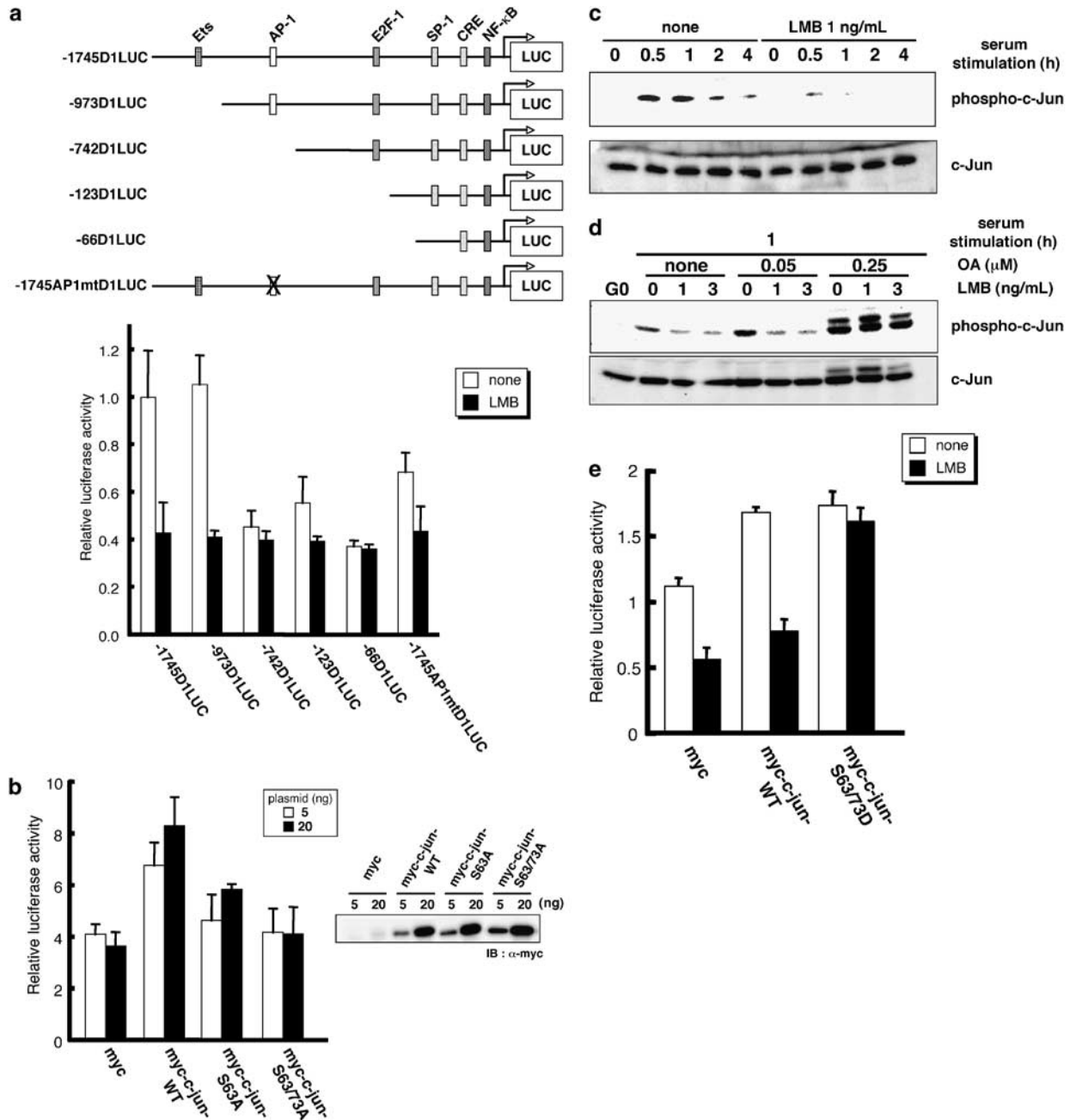


Figure 4 Reduction in c-Jun N-terminal phosphorylation correlates with the induction of PP2A activity following LMB treatment. (a) Schematic presentation of the promoter elements in the cyclin D1 promoter. A series of promoters with 5'-deletions were transfected into HEK293T cells. The transfected cells were treated with 0.3 ng/mL of LMB for 24 h and luciferase activity was measured. Data represent mean and s.d. of a representative experiment performed in triplicate. (b) The -1745D1LUC promoter and a myc-tagged wild-type c-jun plasmid (myc-c-jun-WT) or a mutant c-jun plasmid (Ser63 or/and 73 replaced by Ala) (myc-c-jun-S63A and S63/73A) were transfected into HEK293T cells. Following 24 h of incubation, cells were lysed and luciferase activity was measured. Data represent mean and s.d. of a representative experiment performed in triplicate. And total protein was isolated and analysed by Western blot with an antibody recognizing myc. (c) Serum-starved NIH3T3 cells were restimulated with 10% serum in the presence or absence of 1 ng/mL of LMB for the periods indicated. Total protein was isolated and subjected to Western blot with antibodies recognizing c-Jun phosphorylated at Ser-63 (phospho-c-Jun) and total c-Jun (c-Jun). (d) Serum-starved NIH3T3 cells were treated with LMB (1, 3 ng/mL) and/or OA (0.05, 0.25 μ M) and restimulated with 10% serum for 1 h. Total protein was isolated and analysed by Western blot with antibodies recognizing phospho-c-Jun and c-Jun. (e) The -1745D1LUC promoter and a myc-tagged wild-type c-jun plasmid (myc-c-jun-WT) or a mutant c-jun plasmid (Ser63 and 73 replaced by Asp) (myc-c-jun-S63/73D) were transfected into NIH3T3 cells. The transfected cells were treated with 0.1 ng/mL of LMB for 24 h and luciferase activity was measured. Data represent mean and s.d. of a representative experiment performed in triplicate.

expression. However, OA failed to reverse LMB-induced cell cycle arrest (data not shown).

Discussion

LMB is a specific inhibitor of CRM1-dependent nuclear export of NES-containing proteins, and it is known to induce cell cycle arrest in mammalian fibroblasts. However, the inhibitory mechanism of LMB-induced cell cycle arrest has yet to be clarified. In this study, we found that LMB inhibited serum-induced expression of cyclin D1, thereby inhibiting downstream events such as cyclin E expression and Cdk2 activation required for G1/S transition (Figure 1a and 1b). Furthermore, LMB was found to cause a significant reduction in promoter activity of cyclin D1, which was reflected in the overall decrease in cyclin D1 mRNA detected by northern blot analysis (Figure 1c and d). Thus, we proposed that the inhibition of cyclin D1 expression at the level of transcription by LMB is, at least in part, responsible for LMB-induced cell cycle arrest at G1.

We also found that PP2A inhibitors, such as OA, cantharidin or cytosstatin, restored the LMB-inhibited cyclin D1 expression (Figure 2). Moreover, we found that LMB induced the nuclear accumulation of PP2A in NIH3T3 cells (Figure 3). These findings suggested that the sustained dephosphorylation activity of PP2A accumulated in the nucleus is involved in the inhibition by LMB of cyclin D1 expression.

Next we sought to identify the signaling molecule, whose sustained dephosphorylation by nuclear-accumulated PP2A is responsible for LMB-inhibited cyclin D1 expression. The most likely candidate molecule was c-Jun, an important component of transcription factor AP-1. This idea had come from following reported facts: (1) The activation of c-Jun depends on the phosphorylation of Ser-63 and Ser-73, which enables c-Jun to bind CBP (CREB binding protein) and form a larger complex to activate the transcription (Bannister *et al.*, 1995). (2) PP2A represses AP-1 activity by dephosphorylation of c-Jun on Ser-63 (Alberts *et al.*, 1993; Al-Murrani *et al.*, 1999; Ramirez *et al.*, 2005). (3) c-Jun enhances cell proliferation through induction of cyclin D1 transcription, shown by several reports (Watanabe *et al.*, 1996; Shiozawa *et al.*, 2004; Whang *et al.*, 2005). The possibility that the involvement of c-Jun in the mechanism of LMB-induced reduction of cyclin D1 expression was further supported by our findings: (1) serum-induced cyclin D1 expression is required for AP-1 site in the promoter region of cyclin D1 gene, and the residual promoter activity of AP-1 site-deleted or point-mutated cyclin D1 promoter construct is no longer inhibited by LMB treatment. (2) overexpression of c-Jun induced an increase in cyclin D1 promoter activity, indicating that c-Jun is involved in cyclin D1 expression. Consistent with this result, fibroblasts derived from c-Jun^{-/-} embryo display reduced expression of cyclin D1 (Wisdom *et al.*, 1999). (3) the phosphorylation of c-Jun at Ser-63 and Ser-73 is required for its transcriptional

activation of the cyclin D1 promoter. This result is consistent with previous studies showing that c-Jun phosphorylation, mimicked by the use of aspartic acid residues at Ser-63 and Ser-73, significantly increased the positive effect of c-Jun on cyclin D1 transcription (Bakiri *et al.*, 2000). (4) the level of c-Jun phosphorylated at Ser-63 was significantly decreased in LMB-treated NIH3T3 cells compared to untreated cells. (5) OA restored the LMB-induced repression of both the phosphorylation of c-Jun at Ser-63 and the expression of cyclin D1. (6) LMB failed to inhibit the cyclin D1 promoter activity induced with constitutive active mutant c-Jun (S63/73D). Taken together, we demonstrated that the nuclear accumulation of PP2A induced by LMB led to sustained dephosphorylation of c-Jun at Ser-63, therefore reducing the transcription of the AP-1-responsive cyclin D1 gene. The ability of c-Jun to bind DNA and thus contribute to the induction of AP-1 responsive genes is also regulated by the phosphorylation of Thr-231, Ser-243 and Ser-249, located at the C-terminal DNA binding domain, an event which inhibits DNA-binding and, consequently, the activity of AP-1. It has been demonstrated that extracellular signal-regulated kinase (ERK)1 and ERK2 are the kinases that phosphorylate these residues (Papavassiliou *et al.*, 1995). Therefore, we hypothesized that LMB might induce the repression of AP-1 activity through upregulation of ERK activity. Indeed, ERK activity was prolonged and ERK was accumulated in the nuclei following LMB treatment, although LMB did not alter the protein expression of ERK1/2 and activation by serum (Supplementary data 1). However, selective inhibition of ERK activity with U0126 did not restore the cyclin D1 expression inhibited by LMB, therefore it is not likely that LMB-induced inhibition of cyclin D1 expression is due to LMB-induced activation of ERK 1/2. AP-1 activity can be regulated by the amount of AP-1 protein components in the cells (Chiu *et al.*, 1989; Angel and Karin, 1991). AP-1 consists of dimers between members of the Fos (c-Fos, FosB, Fra-1 and Fra-2), Jun (c-Jun, JunB and JunD) or activating transcription factor families. Extensive analyses of mice and of cell lines have indicated that each family member has distinct biological functions. For example, Jun B has been reported to repress the cyclin D1 promoter (Bakiri *et al.*, 2000). Therefore, we cannot exclude the possibility that Jun B is involved in LMB-inhibited cyclin D1 promoter activity.

What had remained unclear is how LMB induced nuclear accumulation of PP2A. PP2A is a holoenzyme composed of three subunits, the catalytic subunit (PP2A/C), the structural A subunit (also known as PR65), and the regulatory B subunit (Janssens and Goris, 2001; Janssens *et al.*, 2005). We searched for leucine-rich sequence with conserved spacings and hydrophobicity that fits the criteria established for an NES in the primary amino-acid sequences of these three subunits, and we found a putative NES lie between 149 and 158 in human PP2A α . Mutations in the leucine residues within this putative NES led to a decrease in the nuclear export activity, indicating that the sequence

located between 149 and 158 in human PP2A α is a functional NES. The amino-acid sequence of human PP2A α is 99.7% identical to that of mouse PP2A α , in which only N-terminal Val-5 in human PP2A α is replaced by Leu in mouse PP2A α . Therefore, PP2A α 's NES is completely conserved in two species. Taken together, we proposed that LMB induced nuclear accumulation of PP2A through inhibiting the nuclear export of NES-containing PP2A α in human and mouse cells. This is the first report to identify the functional NES of PP2A α , although we could not find NLS-like sequences in PP2A α . Recent results have shown that a specific interaction between an importin β superfamily protein and the A subunit of PP2A (PR65) (Lubert and Sarge, 2003). Furthermore, although there are more than a dozen of B subunits in three different families, reported are the presence of a cluster of basic residues near the carboxyl terminus and a consensus bipartite NLS in all three B' variants (Csontos *et al.*, 1996). Thus, ABC trimetric PP2A shuttles between the nucleus and the cytoplasm mediated by the PP2A α NES which we identified and by the NLS in A or B subunit of PP2A but nuclear export predominates, resulting in the appearance of cytoplasmic sequestration (Figure 3). Indeed, we found that B' subunit of PP2A was observed predominantly in cytoplasm, but was accumulated in nucleus following LMB treatment (supplementary data 2).

OA is also known to be a potent inhibitor of protein phosphatase 1 (PP1). PP1 consists of a catalytic subunit (PP1c) associated with various regulatory subunits, forming numerous oligomeric enzymes (Wera and Hemmings, 1995; Campos *et al.*, 1996). PP1c and PP2Ac are 67% homologous and most of the amino acids crucial for the three-dimensional structure of PP1c are conserved in PP2Ac (Goldberg *et al.*, 1995). Although OA inhibited PP1 at low concentrations (<100 nM) *in vitro*, it failed to inhibit PP1 but not PP2A up to 1 mM in cultured cells (Favre *et al.*, 1997). Therefore, it is not likely that PP1 is also responsible for the LMB-induced reduction of cyclin D1 expression. Protein phosphatase 4 (PP4), protein phosphatase 5 (PP5) and protein phosphatase 6 (PP6) as well as PP2A are members of the PPP gene family of serine/threonine protein phosphatases, and they show similar sensitivities to OA. Like PP2A, their protein phosphatase activities are regulated by the binding of each catalytic subunit to their partner proteins such as regulatory subunit and structural subunit. Therefore, although we could not find any NES-like motif in the primary amino-acid sequence in PP4, PP5 or PP6, it would be possible that they shuttle between the nucleus and cytoplasm through the NES and the NLS of their partner proteins, and that PP4, PP5 or PP6 are also, at least in part, involved in the LMB-induced reduction of cyclin D1 expression.

In summary, this study presents evidence suggesting that LMB inhibits nuclear export of PP2A, and nuclear accumulated PP2A induced sustained dephosphorylation of the c-Jun N-terminal transactivation domain, therefore suppressing the transcription of the cyclin D1 gene. However, OA failed to restore LMB-induced cell

cycle arrest at G1 phase under the condition where OA restored the repression of cyclin D1 expression induced by LMB (data not shown). This result can be explained by the other's findings that PP2A regulates diverse cellular processes, and physiological targets of PP2A include protein kinases involved in mitogenic signaling and the cell cycle, and numerous transcription factors (Cohen *et al.*, 1990; Mumby and Walter, 1993; Shenolikar, 1994; Wera and Hemmings, 1995). Furthermore, because a number of proteins affecting the cell cycle, whose subcellular localization is regulated by the LMB-sensitive transport mechanism (Graves *et al.*, 2001; Yashiroda and Yoshida, 2003; Uchida *et al.*, 2004; Sigoillot *et al.*, 2005), it is likely that other mechanism(s) than inhibition of cyclin D1 expression may also be involved in LMB-induced cell cycle arrest at G1 phase. Lecane *et al.* (2003) reported that LMB induced nuclear accumulation of p53, expression of p21^{WAF1} and growth arrest in primary cell train. The finding may explain why cell cycle arrest was not restored by the inhibition of PP2A. Further studies for a full elucidation of the inhibition mechanism of cell cycle progression by LMB are now under investigation.

Materials and methods

Materials

LMB was generated as described previously (Hamamoto *et al.*, 1983a). The SCADS inhibitor kit I was a gift from the Screening Committee of Anticancer Drugs supported by a Grant-in-Aid for Scientific Research on Priority Area 'Cancer' from The Ministry of Education, Culture, Sports, Science and Technology, Japan.

Cell culture, synchrony and transfections

NIH3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS) and antibiotics. HEK293T and HeLa cells were cultured in DMEM containing 10% fetal bovine serum plus antibiotics. For synchronization/re-stimulation experiments, the cells were seeded in DMEM with 10% CS. After 48 h, the cells were re-fed in DMEM with 0.2% CS. Then, 48 h later, the cells were re-stimulated with medium containing 10% CS. The cells were transiently transfected with Lipofectamine Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cells were harvested 13 h after transfection and processed for immunofluorescence microscopy as described below.

Plasmid construction

The reporter constructs of the human cyclin D1 promoter were a gift from Richard G Pestell (Watanabe *et al.*, 1996; Albanese *et al.*, 1999). A 1745-bp fragment of the human cyclin D1 gene was obtained by polymerase chain reaction (PCR) and cloned into the plasmid pGL3-Basic (Promega, Madison, MA, USA) to generate -1745D1LUC. Site-directed mutagenesis was used to mutate the AP-1 site at -840 (5'-TGAGTCA-3' to 5'-TGCCGCA-3') to generate -1745AP1mtD1LUC. A series of deletion constructs were generated by PCR amplification of the desired portion of the insert and were ligated into pGL3-Basic. The nucleotide sequence of each construct was verified by DNA sequencing. To generate the C-terminally myc/His-tagged PP2A α , a PCR product was generated using primers

that amplified the complete coding region of PP2Ac α (without the stop codon), and ligated in-frame into pcDNA3.1/myc-His(-)A (Invitrogen). PCR-amplified PP2Ac α was sequenced to ensure that unintentional mutations were not introduced. The NES in PP2Ac α was mutated by substituting alanines for the leucines at positions 149, 153, 155 and 158 (numbers refer to the human PP2Ac α amino-acid sequence) by PCR mutagenesis and the mutant was subcloned into the same vectors as wild-type PP2Ac α for the expression of myc/His-tagged versions.

Flow cytometry

The cells were collected by trypsinization, combined with cells floating in the medium, and then stained with propidium iodide. The cell suspension was analysed using an EPICS Elite and EXPO32 software analysis program (Beckman Coulter, Fullerton, CA, USA).

Western blot analysis

The cells were lysed with lysis buffer (50 mM Tris-HCl; (pH 7.4), 150 mM NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% NP-40) containing protease inhibitors. Then, 50 μ g of the total cell lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a Hybond-P membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA), and probed with specific antibodies. This was followed by detection with the ECL Western blotting detection system (Perkin-Elmer Biosystems, Foster City, CA, USA) and LAS-1000 (Fuji Film, Tokyo, JAPAN). Primary antibodies included anti-cyclin D1 (556470; BD Biosciences, Franklin Lakes, NJ, USA), anti-cyclin E (#06-459; upstate, Charlottesville, VA, USA), anti-Cdk4 antibody (#06-139; upstate), anti-Cdk2 antibody (sc-163; Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA), anti-p27^{kip1} (610241; BD Biosciences), anti-c-Jun (#9162; Cell Signaling Technology, Beverly, MA, USA), anti-phospho-c-Jun (558036; BD Biosciences) and anti-PP2Ac α (610555; BD Biosciences). The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Ig)G or anti-mouse IgG (GE Healthcare Bio-Sciences Corp.).

Cdk2 assay

The cells were harvested and lysed using lysis buffer as described above. After centrifugation, the clarified supernatant material was precleared by incubation with proteinA/G-sepharose beads (GE Healthcare Bio-Sciences Corp.) for 1 h and then immunoprecipitated by incubation with Cdk2 antibody for 1 h. Immune complexes were recovered with proteinA/G-Sepharose and washed with ImmunoPrecipitant (IP) washing buffer (50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes) (pH 7.4), 150 mM NaCl, 2.5 mM ethyleneglycoltetraacetate (EGTA), 1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.2% Tween20). The immunoprecipitates were collected and suspended in reaction buffer (50 mM Hepes (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 2.5 mM EGTA, 10 mM β -glycerophosphate, 1 mM NaF and 0.1 mM sodium orthovanadate) and reacted with 1 μ g of histone H1 (Roche

Diagnostics, Basel, Switzerland) and 5 μ Ci of [γ -³²P]adenosine triphosphate, for 15 min at 30°C. The reaction was terminated by the addition of SDS-PAGE sample buffer. The samples were incubated for 5 min at 95°C and separated by SDS-PAGE, and the gel was autoradiographed.

Northern analysis

Total RNA was extracted from cultured cells by using RNeasy (Qiagen, Crawley, UK). Total RNA concentrations were determined, and equal amounts (100 μ g) of total RNA were separated by formaldehyde gel electrophoresis and transferred to a membrane (GE Healthcare Bio-Sciences Corp.). A 888 base pair fragment of the mouse cyclin D1 cDNA was amplified by PCR and randomly labeled with [γ -³²P] deoxycytidine triphosphate (GE Healthcare Bio-Sciences Corp.). Northern blots were evaluated by autoradiography and quantified by PhosphorImager analysis. Membranes were stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase as the loading control.

Reporter gene assay

NIH3T3 and HEK293T cells were transfected with 300 ng of luciferase reporter and 100 ng of a control β -galactosidase reporter using lipofectamine Reagent. At specific indicated time points after transfection, luciferase and β -galactosidase activities were determined. The result represents the average of three experiments.

Immunofluorescence microscopy

The cells were fixed with 3% paraformaldehyde, permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS) and blocked in 3% bovine serum albumin in PBS. For determining the distribution of endogenous PP2Ac α , the cells were stained with monoclonal anti-PP2Ac α antibody (1:100 dilution). For detection of ectopically expressed PP2Ac α , the cells were stained with 0.2 μ g/mL of anti-myc primary antibody (sc-40; Santa Cruz Biotechnology). They were incubated with 2 μ g/mL of anti-mouse Alexa Fluor 488 (Invitrogen). The nuclei were stained with 1 μ g/mL of TOPRO-3. The cells were observed using a laser-scanning confocal microscope system (Radiance2000; BIO-RAD, Hercules, CA, USA). A minimum of 50 cells were scored for each coverslip.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>).