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Biochemical and Biophysical Research Communications 340 (2006) 302–308
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Cytoplasmic sequestration of cyclin D1 associated with cell cycle withdrawal of neuroblastoma cells

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Received 25 November 2005
Available online 12 December 2005

Abstract

The regulation of D-type cyclin-dependent kinase activity is critical for neuronal differentiation and apoptosis. We recently showed that cyclin D1 is sequestered in the cytoplasm and that its nuclear localization induces apoptosis in postmitotic primary neurons. Here, we further investigated the role of the subcellular localization of cyclin D1 in cell cycle withdrawal during the differentiation of N1E-115 neuroblastoma cells. We show that cyclin D1 became predominantly cytoplasmic after differentiation. Targeting cyclin D1 expression to the nucleus induced phosphorylation of Rb and cdk2 kinase activity. Furthermore, cyclin D1 nuclear localization promoted differentiated N1E-115 cells to reenter the cell cycle, a process that was inhibited by $p16^{INK4a}$, a specific inhibitor of D-type cyclin activity. These results indicate that cytoplasmic sequestration of cyclin D1 plays a role in neuronal cell cycle withdrawal, and suggests that the abrogation of machinery involved in monitoring aberrant nuclear cyclin D1 activity contributes to neuronal tumorigenesis.

Keywords: Cyclin D1; Neuroblastoma; Cell cycle; Cyclin-dependent kinase; Subcellular localization

Progression of the mammalian cell cycle is regulated by a family of cyclins and cyclin-dependent kinases (cdks). During the G1 phase, cyclin D1 accumulates in response to mitogenic stimulation and assembles with its catalytic partners, cdk4 and cdk6. The cyclin D1-cdk4/cdk6 complex promotes G1-to-S phase progression by phosphorylating retinoblastoma protein (Rb) together with its related p107 and p130 proteins, which bind to and suppress the transcription factor E2F, which regulates a number of genes required for DNA replication and cell cycle progression. The phosphorylation of Rb releases E2F from Rb-mediated inhibition, thereby allowing E2F to activate its target genes (reviewed by [1,2]). The activity of cyclin D1-cdk4/cdk6 is specifically inhibited by the $p16^{INK4}$ family of cdk inhibitors, whereas the $p21^{Cip1}$/$p27^{Kip1}$ family inhibits the activity of both cyclin D1-cdk4/cdk6 and cyclin A/cyclin E-cdk2 complexes. In addition to Rb phosphorylation, the cyclin D1-cdk complex sequesters the $p21^{Cip1}$/$p27^{Kip1}$ family of cdk inhibitors and thereby facilitates the activation of cyclin E/cyclin A-cdk2 kinase activities, leading to the entry into and progression of S phase.

The subcellular localization of cyclin D1 plays a role in the regulation of the activity of the cyclin D-cdk complex during the cell cycle. Cyclin D1 accumulates in the nucleus during G1 phase and redistributes to the cytoplasm when cells enter into S phase. Cyclin D1 nuclear export is regulated by the phosphorylation of cyclin D1 via GSK-3β [3,4]. We recently showed that proliferating embryonic progenitors of neurons and cardiomyocytes lose the ability to import cyclin D1 during differentiation, and that nuclear accumulation of the cyclin D1-cdk4 complex is tightly inhibited in such terminally differentiated cells [5,6]. Because a distinctive property of terminally differentiated cells is permanent withdrawal from the cell cycle, the prevention of cyclin D1 nuclear import plays a critical role as a physical barrier to prevent cell proliferation. Indeed, targeting the expression of cyclin D1 to the nucleus efficiently promotes reentry of postmitotic cardiomyocytes

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doi:10.1016/j.bbrc.2005.11.181

into the cell cycle. Nevertheless, targeting cyclin D1 expression to the nucleus induces apoptosis in postmitotic neurons. Moreover, endogenous cyclin D1 enters the nucleus under apoptotic conditions. Thus, the nuclear translocation of cyclin D1 following neuronal insult appears to trigger cell death in postmitotic neurons via inappropriate activation of the Rb pathway. Although accumulating evidence has indicated that cyclin D1 activity is critical for neuronal apoptosis, little is known about the role of cytoplasmic sequestration of cyclin D1 in cell cycle withdrawal during neuronal differentiation.

The present study examined the role of cyclin D1 subcellular localization in neuronal cell cycle withdrawal using the differentiation system of N1E-115 neuroblastoma cells. We found that cyclin D1 localized predominantly in the cytoplasm after differentiation, and that the nuclear localization of cyclin D1 is sufficient to induce differentiated neuroblastoma cells to reenter the cell cycle. These findings support the notion that the cytoplasmic sequestration of cyclin D1 plays a role in neuronal cell cycle withdrawal.

Materials and methods

Cell culture and adenoviruses. Recombinant adenovirus encoding cyclin D1 with nuclear localization signal (Ad-D1NLS), Ad-p16, Ad-p21, and Adx1W1 control viruses were prepared as described [6]. Ad-CyclinD1 (D1-WT) [7] was a gift from J.H. Albrecht. Mouse neuroblastoma cells (N1E-115) were a gift from Y. Shirasaki and maintained in DMEM (Sigma) containing 10% fetal bovine serum (FBS) and 50 μg/ml gentamicin. The media were changed to 2% FBS with 1% DMSO to induce differentiation of N1E-115 [8]. Cells were infected in 2 ml of media (per 10 cm plate) and incubated for 1 h with gentle shaking every 15 min. After infection, 8 ml of media was added and the incubation was continued for 48 h before harvesting the cells.

Cell cycle analysis. Cells were collected in suspension and fixed with 70% ethanol. After washing with phosphate-buffered saline (PBS), cells were incubated with propidium iodide (50 μg/ml) and RNase (100 μg/ml) for 30 min at 37 °C. Cell cycle profiles were analyzed using a flow cytometer (FACSCalibur; Becton Dickinson) or a laser scanning cytometer (LSC101; Olympus) as described [5].

Western blotting. Whole-cell, cytoplasmic, and nuclear extracts were prepared as described [9]. Samples were separated by 5–15% SDS–polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (Millipore). The blots were blocked in TBS (20 mM Tris–HCl, pH 7.6, 137 mM NaCl) containing 5% non-fat dried milk (Difco) and 0.1% Tween 20 (Pharmacia) at room temperature for 1 h and then incubated for 16–20 h at 4 °C with primary antibodies (Santa Cruz Biotechnology) diluted as follows: cyclin D1 (72-13G), 1:3000; cyclin A (C-19), 1:3000; cdk4 (C-22), 1:3000; cdk2 (M2-SC163), 1:3000; cdk5 (C-8), 1:3000; E2F1 (C-20), 1:3000; Rb (M-153), 1:6000; p130 (C-20), 1:3000; c-myc (9E10), 1:6000; p16 (C-20), 1:3000; p21 (F-5), 1:6000. Bands were visualized using secondary antibodies conjugated with horseradish peroxidase and the ECL chemiluminescence detection system (Amersham). Membranes were reprobed after stripping in 2% SDS, 62.5 mM Tris–HCl (pH 6.7), and 100 mM 2-mercaptoethanol at 50 °C for 30 min.

Immunofluorescent staining. Cells grown on coverslips were fixed with 3.7% formaldehyde (Polyscience) for 10 min at room temperature, and then with methanol:acetone (1:1) for 1 min, after which cells were incubated at 90 °C for 5 min in 10 mM citrate buffer. The cells were stained with either the rabbit polyclonal anti-cyclin D1 (H-295) antibody, or a mixture of the mouse monoclonal anti-cyclin D1 (72-13G) and rabbit polyclonal neuron-specific type III β-tubulin isotype antibodies (a gift from Y. Arimatsu), followed by Alexa488- and Alexa555-conjugated secondary antibodies (Molecular Probes). Nuclei were stained with TO-PRO-3 (1 μM; Molecular Probes). Images were acquired using a confocal microscope (LSM510, Carl Zeiss).

Histone H1 kinase assay. Lysates (100 μg) from nuclear extracts were precleared by using unconjugated Dynabeads (Dynal) for 1 h at 4 °C and then immunoprecipitated with a polyclonal antibody specific to cdk2 (M2-sc163) followed by histone H1 kinase assays as described [10]. Reaction products were separated by SDS–polyacrylamide gel electrophoresis. The gels were dried and bands were visualized by autoradiography.

Results

CyclinD1 localized in the cytoplasm in differentiated N1E-115 cells

To examine the role of cyclin D1 subcellular localization in cell cycle withdrawal during neuronal differentiation, we used the in vitro neuronal model, differentiating murine neuroblastoma N1E-115 cells, which have been widely used to study the processes of neuronal differentiation in vitro and which have been shown to differentiate in response to dimethylsulfoxide (DMSO) [8,11]. FACS analysis showed that N1E-115 cells were arrested in G0/G1 phase 4 days after differentiation (Fig. 1A). We examined the expression of cyclin D1 and the phosphorylation of Rb during N1E-115 cell differentiation by Western blotting (Fig. 1B). Rb is highly phosphorylated in undifferentiated N1E-115 cells. Consistent with previous observations

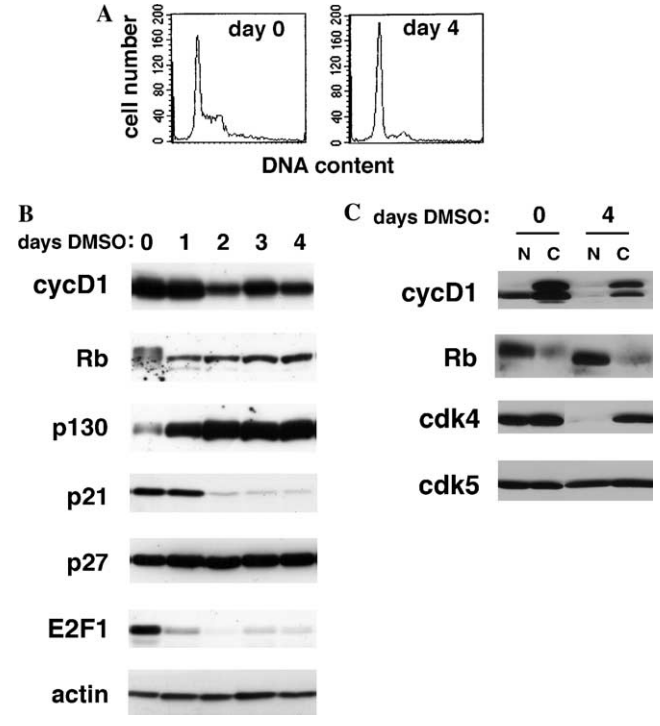

Fig. 1. Expression and subcellular localization of cell cycle regulators during differentiation of N1E-115 cells. N1E-115 cells were induced to differentiate with 2% FBS and 1% DMSO, and harvested at the indicated days thereafter. (A) Cell cycle profiles of undifferentiated and differentiated N1E-115 cells analyzed by FACS. Horizontal and vertical axes represent DNA content and cell number, respectively. (B) Western blotting of whole cell extracts using antibodies against indicated proteins. (C) Nuclear (N) and cytoplasmic (C) extracts were analyzed as in (B).

[8,11], upon differentiation induction with DMSO, Rb became hypo-phosphorylated within 24 h, which was accompanied by down-regulation of E2F1. In addition, levels of the Rb-related p130, which is up-regulated after differentiation [9,11], were also increased 24 h after differentiation. Despite the obvious changes in the Rb family of proteins and E2F1, cyclin D1 levels remained relatively constant over 24 h, then decreased, although significant amounts were still detectable 4 days after differentiation. Furthermore, an analysis of cdk inhibitors revealed that p27 somewhat increased and p21 was down-regulated following differentiation induction.

We have shown that cyclin D1 becomes predominantly cytoplasmic during the differentiation of neuronal progenitor cells [5]. We next examined cyclin D1 subcellular localization during N1E-115 cell differentiation. Western blots of nuclear and cytoplasmic extracts revealed that, similar to postmitotic neurons, cyclin D1 and its partner cdk4 were localized both in the nucleus and cytoplasm of proliferating N1E-115 cells, but predominantly redistributed to the cytoplasm after differentiation, whereas their critical substrate, Rb, became hypo-phosphorylated and localized in the nucleus (Fig. 1C). The redistribution of cdk4 was supported by the fact that the cytoplasmic cyclin D1 forms a complex with cdk4 in differentiated primary neurons and in N1E-115 cells ([5] and data not shown). In contrast, cdk5, a neuronal cell-specific cdk, was detected both in the nucleus and cytoplasm but its expression and subcellular localization did not change after differentiation.

The subcellular localization of cyclin D1 during differentiation was further examined by immunofluorescent staining with the polyclonal anti-cyclin D1 antibody. Fig. 2A shows that cyclin D1 was localized in both the nucleus and cytoplasm in undifferentiated cells (day 0), but became predominantly cytoplasmic after differentiation (day 4). To confirm these results, cells were stained with the monoclonal anti-cyclin D1 and neuron-specific type III β-tubulin antibodies (Fig. 2B). Again, cyclin D1 was predominantly in the cytoplasm of the type III β-tubulin positive cells 4 days after differentiation, whereas cyclin D1 localized in the nucleus in undifferentiated cells.

Ectopic expression of cyclin D1-NLS in the nucleus can induce Rb phosphorylation, E2F target gene products, and cdk2 kinase activity in differentiated N1E-115 cells

To examine the role of the subcellular localization of cyclin D1 in cell cycle withdrawal of N1E-115 cells, we

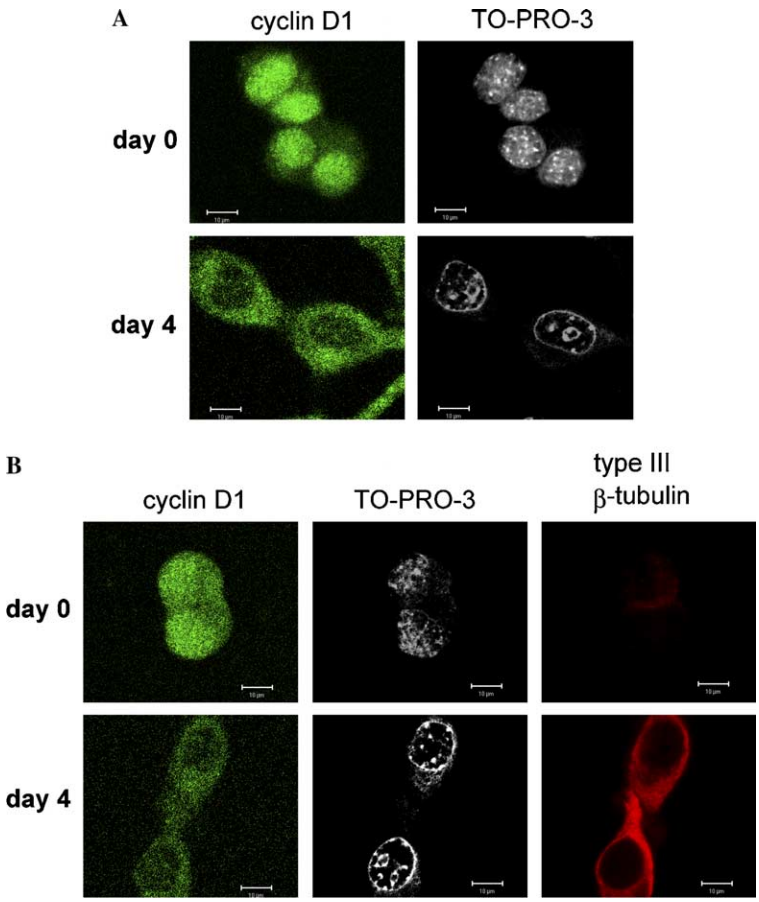

Fig. 2. Cytoplasmic localization of D-type cyclins in differentiated N1E-115 cells. N1E-115 cells were induced to differentiate for 4 days. (A) Undifferentiated (day 0) and differentiated (day 4) cells were stained with anti-cyclin D1 antibody (H-295). (B) Same as (A), except that cells were double-stained with anti-cyclin D1 (72-13G) and anti-neuron-specific type III β-tubulin antibodies. Nuclei were visualized with TO-PRO-3. Bar, 10 μm.

examined whether nuclear targeting of cyclin D1 could induce the phosphorylation of endogenous Rb, thereby activating downstream events that are regulated by Rb in differentiated N1E-115 cells. We efficiently targeted cyclin D1 to the nucleus using the recombinant adenovirus encoding cyclin D1 linked to nuclear localization signals (Ad-D1NLS), which has been shown to localize in the nucleus and induces Rb phosphorylation in terminally differentiated cells [5,6]. We infected N1E-115 cells with Ad-D1NLS 48 h after differentiation and then prepared nuclear and cytoplasmic extracts 48 h later. Western blots revealed that cyclin D1-NLS protein, which migrates more slowly than endogenous cyclin D1 [6], dose-dependently entered the nucleus of differentiated N1E-115 cells (Fig. 3A). This process was accompanied by the nuclear localization of endogenous cdk4 (Fig. 3A, lanes 4 and 5), as well as the induction and nuclear localization of cdk2 (lanes 4, 5, 9, and 10). Consistent with these results, the expression of D1NLS converted the majority of the Rb and p130 proteins into their slowly migrating, hyperphosphorylated forms (pRb and pp130, respectively) (Fig. 3B) and induced expression of cyclin A and E2F-1 proteins (Fig. 3C). The induction of endogenous cdk2 and cyclin A proteins by Ad-D1NLS suggested that the expression of cyclin D1-NLS could induce cdk2 kinase activity, which is activated following Rb phosphorylation during the cell cycle. To test this notion, nuclear extracts were immunoprecipitated with cdk2 and histone H1 kinase was assayed. Fig. 3D shows that, like undifferentiated N1E-115 cells, cdk2 immunoprecipitates had kinase activity in differentiated cells infected with Ad-D1NLS, but not in cells infected with the control virus.

Overexpression of cyclin D1-NLS induced cell cycle reentry in differentiated N1E-115 cells

The above results indicated that the nuclear import of ectopic cyclin D1 in differentiated N1E-115 cells activates the Rb regulatory pathway that is required for cell cycle progression. Therefore, we questioned whether Ad-D1NLS infection could induce the reentry of differentiated N1E-115 cells into the cell cycle. FACS analysis revealed that Ad-D1NLS dose-dependently increased the fractions of cells in the S and G_2 phase (Fig. 4A). Our previous studies have shown that overexpressed wild-type cyclin D1 proteins are retained in the cytoplasm of postmitotic neurons and cardiomyocytes. Thus, the nuclear localization of cyclin D1 is tightly inhibited in terminally differentiated cells. The present study, however, found that the ectopic expression of cyclin D1-NLS resulted in the nuclear localization of endogenous cyclin D1 (Fig. 3A; lanes 4 and 5), suggesting the possibility that the overexpression of wild-type cyclin D1 overcomes the inhibition of cyclin D1 nuclear import in differentiated N1E-115 cells and thus induces their entry into the cell cycle. In agreement with this, Fig. 4B shows that infection with an adenovirus encoding wild-type cyclin D1 increased the fraction of cells in S and G2 phase.

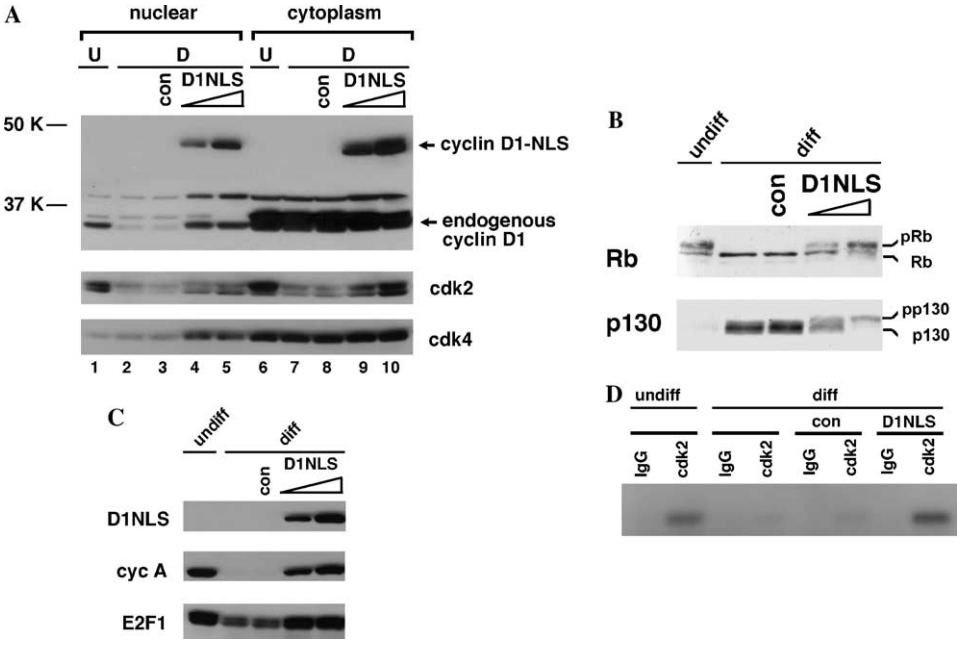

Fig. 3. Nuclear expression of cyclin D1 induced Rb phosphorylation, E2F target gene products and cdk2 kinase activity in differentiated N1E-115 cells. (A) N1E-115 cells were induced to differentiate for 2 days and then infected with Ad-con (m.o.i. = 100) or Ad-D1NLS (m.o.i. = 50 and 250). Cells were further incubated in differentiated media for 48 h and then Western blotted. Positions of endogenous cyclin D1 and myc-tagged cyclin D1-NLS are indicated. U, undifferentiated cells; D, differentiated cells. (B and C) Nuclear extracts were assessed by Western blotting using antibodies against Rb and p130 proteins (B), or myc-tag, cyclin A and E2F1(C). (D) Nuclear extracts were immunoprecipitated with cdk2 antibody or control rabbit IgG and then cdk2 kinase activities were determined by using histone H1 as a substrate.

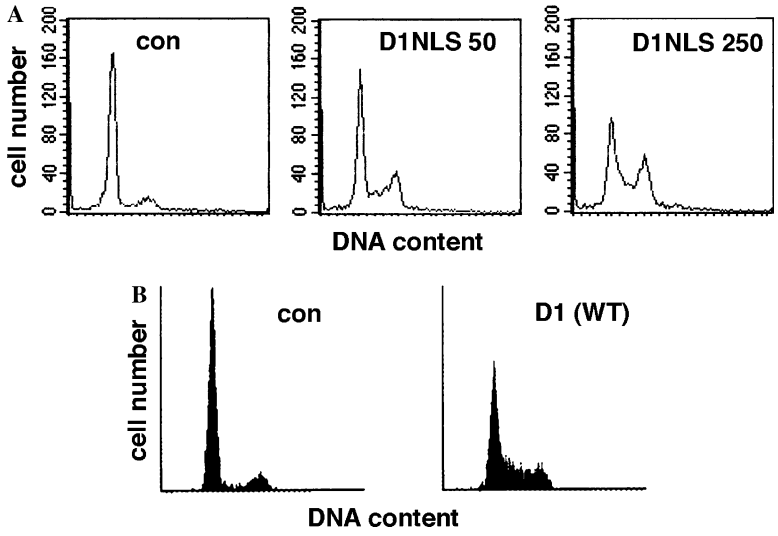

Fig. 4. Overexpression of cyclin D1 induced cell cycle progression, but not apoptosis in differentiated N1E-115 cells. (A) Cells treated as described in Fig. 3 were processed for FACS analysis. (B) Differentiated N1E-115 cells were infected with Ad-D1 (WT) and then analyzed by LSC. Horizontal and vertical axes represent DNA content and cell number, respectively.

Cdk inhibitors suppressed S phase entry induced by cyclin D1-NLS

We examined whether the reentry of differentiated N1E-115 cells into the cell cycle induced by ectopic cyclin D1 is dependent upon its kinase activity. FACS analysis revealed that co-infection with a recombinant adenovirus expressing $p21^{Cip1}$ (Ad-p21) with Ad-D1NLS inhibited the cell cycle reentry of differentiated N1E-115 cells (Fig. 5A). Specifically, a virus expressing $p16^{INK4}$, which specifically inhibits D-type cyclin kinase activity, also prevented the cell cycle reentry induced by Ad-D1NLS, indicating that cyclin D-dependent kinase activity contributes to the cell cycle reentry of differentiated N1E-115 cells. The cell cycle arrest by the cdk inhibitors was further confirmed by Western blot analysis, showing that expression of E2F-1, which was induced by Ad-D1NLS infection, was inhibited by exogenous $p16^{INK4}$ and $p21^{Cip1}$ in differentiated N1E-115 cells (Fig. 5B). Taken together, these results indicate that cyclin D1 activity in the nucleus is sufficient to induce the reentry of differentiated N1E-115 cells into the cell cycle, and support the notion that cyclin D1 cytoplasmic sequestration plays a critical role in neuronal cell cycle withdrawal.

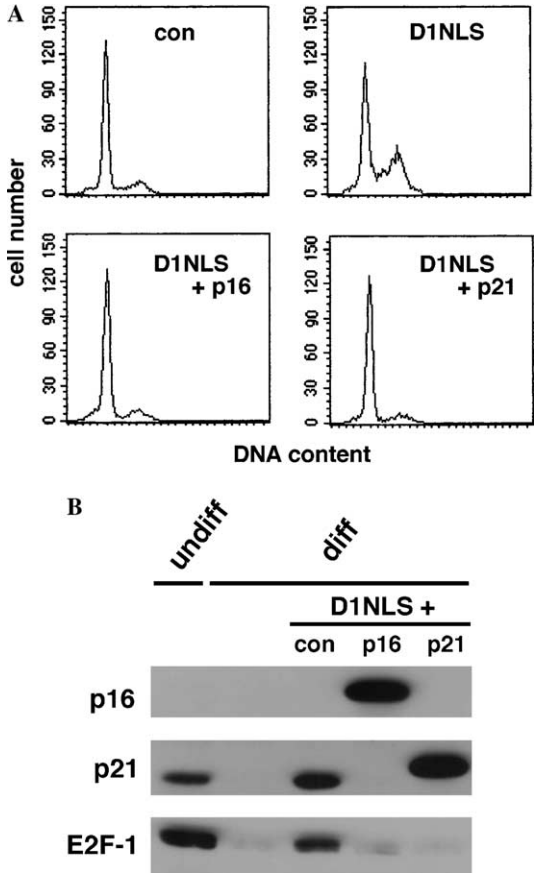

Fig. 5. Cdk inhibitors suppressed cell cycle progression induced by cyclinD1-NLS (A) Differentiated N1E-115 cells were infected with Ad-con alone or with Ad-D1NLS (m.o.i. = 50), together with either Ad-p16 (m.o.i. = 50) or Ad-p21 (m.o.i. = 50), and then processed for FACS analysis. (B) Western blots of cells treated as in (A) with indicated antibodies.

Discussion

The regulation of D-type cyclin-dependent kinase activity is critical for neuronal differentiation and apoptosis. We have shown that proliferating neuronal progenitor cells lose the ability to import cyclin D1 during differentiation and that cyclin D1-cdk4 complex is sequestered in postmitotic cortical neurons [5]. Consistently, a recent study has reported that the nuclear/cytoplasmic ratio is low in neurons of adult brain, whereas it is very high in those of embryonic brain [12]. Although the subcellular localization of cyclin D1 plays a role in the regulation of cyclin D-cdk complex activity during the cell cycle, the role of the cytoplasmic sequestration of cyclin D1 in the cell cycle withdrawal during neuronal differentiation remains to be

determined. To investigate the role of the subcellular localization of cyclin D1 in neuronal cell cycle withdrawal, we have made use of N1E-115 neuroblastoma cells, which were derived from the embryonic neural crest cells. We show that cyclin D1 became predominantly cytoplasmic as cells underwent cell cycle withdrawal during differentiation. Moreover, targeting cyclin D1 expression to the nucleus activates the Rb regulatory pathway leading to the reentry into the cell cycle in differentiated N1E-115 cells, which was inhibited by $p16^{INK4a}$, a specific inhibitor against D-type cyclin activity. These results indicate that the cytoplasmic sequestration of cyclin D1 is characteristic of neuronal differentiation and suggest that the cytoplasmic sequestration of cyclin D1 plays a role in neuronal cell cycle withdrawal.

Growing evidence has indicated that neuronal cell death following DNA damage is a consequence of inappropriate activation of the cell cycle [13–15]. Cyclin D1-dependent kinase activity is elevated in primary cortical and sympathetic neurons following DNA damage and the inhibition of cyclin D-dependent kinase promotes the survival of primary sympathetic and cortical neurons under a variety of apoptotic conditions [16–23]. Recently, we have shown that subcellular localization of cyclin D1 plays a critical role in neuronal cell death and survival. Targeting the expression of cyclin D1 in the nucleus induces apoptosis in postmitotic cortical neurons, and that cyclin D1 redistributes from the cytoplasm to the nucleus under apoptotic conditions [5]. Therefore, the nuclear translocation of cyclin D1 after DNA damage appears to trigger cell death in postmitotic neurons via activation of the Rb pathway. Nevertheless, the results presented here indicate that cyclin D1 activity in the nucleus induced the reentry of differentiated N1E-115 cells into the cell cycle without apparent apoptotic induction. The inability of nuclear targeting of cyclin D1 to induce apoptosis implies that N1E-115 neuroblastoma cells lost the ability to induce apoptosis in response to inappropriate nuclear cyclin D1 activity. In agreement with this notion, a number of studies have suggested that the apoptotic response is impaired in human neuroblastoma [24]. It has been shown that the expression of caspase 8, a key intermediate of apoptotic cascade, is absent in a significant percentage of neuroblastoma cell lines [25–27]. Furthermore, survivin, an inhibitor of the apoptotic response, is accumulated in aggressive stages of neuroblastoma [28,29]. Therefore, inactivation of such anticancer apoptotic defenses might contribute to the resistance to apoptotic induction by nuclear cyclin D1 activity in N1E-115 neuroblastoma cells.

Our recent studies have demonstrated that ectopically expressed wild-type cyclin D1 proteins are retained in the cytoplasm of postmitotic neurons and cardiomyocytes, and that in cardiomyocytes, cyclin D1-NLS, but not wild-type cyclin D1, induces cell cycle reentry, indicating that cyclin D1 nuclear import is tightly inhibited in these types of terminally differentiated cells [5,6]. Therefore, the inhibition of cyclin D1 nuclear accumulation is likely to be a critical barrier for maintaining the postmitotic state. However, the present study showed that the overexpression of wild-type cyclin D1 overcomes the inhibition of cyclin D1 nuclear import and thereby induces the cell cycle in differentiated N1E-115 cells. In agreement with this, another study has reported that cyclin D1 overexpression can induce reentry into the cell cycle from differentiated neurons derived from P19 embryonic carcinoma cells [30]. Therefore, the tight regulation of cyclin D1 subcellular localization is apparently impaired in differentiated neuroblastoma cells. Thus, the abrogation of the machinery preventing cyclin D1 nuclear import, together with that monitoring the aberrant induction of nuclear cyclin D1 activity, might allow the survival and proliferation of damaged neurons and thereby contribute to neuronal tumorigenesis. Further studies are required to elucidate the precise mechanism involved in preventing cyclin D1 nuclear accumulation in postmitotic neurons.

Acknowledgments

We thank J.H. Albrecht, Y. Shirasaki, and Y. Arimatsu for materials. This study was supported in part by Grants-in-Aid for Scientific Research (16209054 to MI) from the Japan Society for the Promotion of Science and by Long Term Invitation Program (to PS) from the Japan Society for the Promotion of Science and the National Research Council of Thailand.

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