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Expression of Cdk5 and its activators in NT2 cells during neuronal differentiation

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Abstract

We have recently developed a rapid protocol involving NT2 cell aggregation and treatment with retinoic acid (RA) to produce terminally differentiated CNS neurons. As a first step to explore the functional roles of cell-cycle regulatory proteins in the process of neuronal differentiation, the expression profiles of cyclin-dependent kinases (Cdks) and their regulators were examined in NT2 cells following treatment with RA. One of the Cdks, Cdk5, has been demonstrated to affect the process of neuronal differentiation and suggested to play an important role in development of the nervous system. We found that the expression of Cdk5 was gradually increased, while its activators (p35 and p39) as well as Cdk5 kinase activity were induced in NT2 cells during the process of neuronal differentiation. Moreover, both p35 and p39 were localized along the axons and varicosity-like structures of differentiated NT2 neurons. Taken together, our results demonstrated that NT2 cells provide a good *in vitro* model system to examine signaling pathways involved in the regulation of Cdk5 activators and to elucidate the functional roles of Cdk5 in neuronal differentiation.

Keywords: Cdk5, cell cycle, cyclin-dependent kinase, embryonal carcinoma, neuronal differentiation, NT2.

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The process of neuronal differentiation in the CNS is well orchestrated, requiring a complex series of events such as mitotic arrest, cellular differentiation and apoptosis (reviewed by Gao and Zelenka 1997). Several neuroblastoma cell lines have been extensively used to study the molecular mechanism of neuronal differentiation. Unlike neuroblastoma cells, embryonal carcinoma cells such as PCC7-Mz1 (Heiermann *et al*. 1992; Berger *et al*. 1997), P19 (Jones-Villeneuve *et al*. 1982) and NT2 (Andrews 1984) are pluripotent and possess developmental properties that resemble the stem cells of early embryos. NT2 cells, a human embryonal carcinoma cell line, become post-mitotic CNS neurons upon treatment with retinoic acid (RA; Andrews 1984). Accumulating evidence supports the use of NT2 cells as an *in vitro* model to study the process of early neuronal differentiation in the CNS (Miller *et al*. 1990; Abraham *et al*. 1991; Moasser *et al*. 1995; Cheung *et al*. 1997, 2000, 2001). While the conventional methodology normally requires at least 2 months to obtain mature neurons, we have recently developed a rapid protocol to differentiate NT2 cells using cell aggregation and RA (Cheung *et al*. 1999).

Terminally differentiated neurons are post-mitotic and become growth arrested in the G_0 phase of cell cycle. The cell cycle is controlled by a protein family of cyclin-dependent kinases (Cdks), the activities of which are positively regulated by forming complexes with cyclins, or negatively, by binding to Cdk inhibitors (reviewed in Hengstschlager *et al*. 1999; Pavletich 1999; Sherr and Roberts 1999). The expression of Cdks is differentially regulated during neuronal differentiation and the activities of Cdks in neuronal cells are correlated with the commitment of neurons to withdraw from the cell cycle (Hayes *et al*. 1991; Kranenburg *et al*. 1995; Yan and Ziff 1995). Unlike other members of the Cdk family, Cdk5 has distinct functions that are mainly restrictive to neuronal development. The kinase activity of Cdk5 requires the association with its activators, p35 (Lew *et al*. 1994; Tsai *et al*. 1994) or p39 (Tang *et al*. 1995). Unlike Cdk5, however, the expression of p35 and p39

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Abbreviations used: Cdk, cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FBS, fetal bovine serum; RA, retinoic acid; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

is more restrictive, being detected only in post-mitotic neurons (Tsai *et al*. 1993) and muscle (Lazaro *et al*. 1997; Fu *et al*. 2001).

Recent studies have demonstrated that expression of dominant negative Cdk5 in primary rat cortical neurons inhibits neurite outgrowth (Nikolic *et al*. 1996), implicating an important functional role of Cdk5 in neuronal differentiation. More than 60% of Cdk5(–/–) mice died at the embryonic stage (Ohshima *et al*. 1996, 1999). These mutant mice showed abnormal neuronal migration with lack of proper stratification in the cortex and hippocampus, and defects in the cerebellum foliation. Although several proteins that serve a regulatory role at the growth cone have been identified to be the substrates of Cdk5, e.g. Pak1 (Nikolic *et al*. 1998), the precise mechanism underlying the functions of Cdk5 in neuronal development remains to be elucidated. Recent studies have shed light on some of the factors that regulate the expression and activity of Cdk5 and its activators. For example, the expression of p35 mRNA and protein as well as p35-associated Cdk5 kinase activity can be induced by laminin (Paglini *et al*. 1998; Li *et al*. 2000), neurotrophic factors (Tokuoka *et al*. 2000; Harada *et al*. 2001) and neuregulin (Fu *et al*. 2001) in cell cultures. Moreover, transcription factors such as δFosB (Chen *et al*. 2000) and Egr1 (Harada *et al*. 2001) are also important in regulating the transcription of Cdk5 and p35, respectively. NT2 cells with the characteristics of embryonic stem cells could provide a model system to enhance our understanding of the regulatory mechanism of Cdk5 signaling in the CNS.

In this study, the differentiation of NT2 cells using cell aggregation and RA was characterized by studying the expression of neuronal markers and the regulation of cell cycle proteins. Extension of neurites from NT2 aggregates was concomitant with the induction of Cdk5 activators, p35 and p39. The present study provided the first demonstration on the regulation of the expression of Cdk5 activators, p35 and p39, in differentiated NT2 neurons.

Materials and methods

Antibodies

Polyclonal antibodies specific for Cdk5 (C8), Cdk4 (H22), Cdk6 (C21), p35 (C19), cyclin A (H432), cyclin B1 (H433), cyclin D1 (H295), p21 (C-19), p27 (N-20) and Rb (IF8), as well as rat monoclonal cyclin D2 (34B1-3) and mouse monoclonal Cdc2(17), Cdk5 (DC17) and PCNA (PC10) antibodies were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Polyclonal antibody of p39 was raised against a synthetic peptide (KGRRPGGLPEE, a.a.14–24, Research Genetics, Huntsville, AL, USA) as previously described (Honjyo *et al*. 1999). Specificity of the antibody was confirmed by peptide competition (data not shown) and similar results were obtained using another p39 antibody. Monoclonal Cdk5 antibody (DC34) was purchased from NeoMarker (Lab Vision Corporation, Fremont, CA, USA), monoclonal NF200-P antibody (SMI 31) from Sternberger (Lutherville, MD, USA), and SV2, MAP1B and β-tubulin were obtained from Sigma (St Louis, MO, USA).

Cell culture

NT2 cells were cultured as previously described (Cheung *et al*. 1999). Briefly, NT2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; high glucose formulation; Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) in a 37°C incubator with 5% CO_2. For neuronal differentiation, cells were seeded at 1×10^6 cells/mL in an 85-mm bacteriological grade Petri-dish to initiate cell aggregation for 1 day prior to treatment with 5 μM all-*trans* RA (Sigma) for up to 3 weeks. Fresh medium was replenished every 2 days and cells were maintained in RA containing medium for 3 weeks.

Cell cycle analysis

For cell cycle analysis, aggregated cells were dissociated by trypsinization. The isolated cells were fixed in 70% and 95% ice-cold ethanol for 10 min, respectively. After washing with phosphate-buffered saline (PBS), the cells were filtered through a 70-μm nylon mesh and treated with RNase at 37°C for 20 min. They were stained with propidium iodide on ice for 30 min. The percentage of cells in G_1, S and G_2/M phases were determined using a FACscan flow cytometer (FACS Vantage, Becton-Dickinson, Franklin Lakes, NJ, USA).

Western blot analysis

Cells were washed with PBS and then lysed with RIPA buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 mM dithiothreitol (DTT) supplemented with protease inhibitors] on ice for 30 min. Protein concentration in the lysates was determined using Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were separated on SDS–polyacrylamide gel electrophoresis (SDS–PAGE) gels and then electro-transferred to nitrocellulose membranes. The blots were incubated with primary antibodies in TBS–Tween with 5% non-fat milk at room temperature for 2 h, washed and then incubated with horseradish peroxidase-conjugated secondary antibodies in TBS–Tween with 5% non-fat milk at room temperature for 1 h. The blots were developed using ECL reagent (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Kinase assay

For Cdk5 kinase activity analysis, NT2 cells were extracted with lysis buffer containing 20 mM Tris, pH 7.6, 150 mM NaCl, 0.5% NP40, 1 mM EDTA and protease inhibitors. Protein lysates were then incubated with 40 μL of protein-G sepharose pre-conjugated with 5 μL Cdk5 antibody (C8; 1 μg) or 5 μL p35 antibody (C19; 1 μg) at 4°C for 1 h. The immunoprecipitates were washed three times with lysis buffer and twice with kinase buffer (20 mM MOPS, pH 7.4, 30 mM $MgCl_2$, 100 μM cold ATP). The *in vitro* kinase reaction was then performed at 30°C for 30 min with kinase buffer containing 100 μM [γ-^{32}P] ATP as described (Ching *et al*. 2000). [γ-^{32}P] phosphate incorporated into the substrate (histone H1) was separated by SDS–PAGE and then exposed to X-ray autoradiography.

Immunocytochemical staining

For immunocytochemical analysis, NT2 aggregates were replated on poly-D-lysine (Sigma) and laminin (Life Technologies, Rockville, MD, USA) coated tissue culture dishes following 21 days of RA treatment. Twenty-four hours after replating, cells were fixed with 4% paraformaldehyde in PBS on ice for 20 min. After permeabilization with 0.1% Triton X-100 (Sigma), 4% goat serum and 1% bovine serum albumin for 20 min, cells was incubated with primary antibodies at 4°C for 1 day. Cells were then incubated with FITC- or rhodamine-conjugated secondary antibodies (Cappel, Turnhout, Belgium) at room temperature (22°C) for 1 h. There was no detectable signal in the absence of primary antibodies. The staining was subjected to confocal microscopy.

Results

Neuronal differentiation and cell cycle analysis of NT2 cells

We have recently developed a rapid protocol based on cell aggregation to produce enriched human CNS neurons following RA treatment (Cheung *et al*. 1999). Using this protocol, extension of long neurites from NT2 aggregates could be readily observed after treatment with RA for 14 days (Fig. 1). NT2 cells migrating from the aggregates also showed neuronal morphology with phase bright cell bodies that are immunopositive with neurofilament antibody (NF-160 and NF-200). The differentiation status of NT2 cells following this treatment paradigm was examined by western blot analysis using neuronal markers. Induction of NF160-P expression was detected in NT2 aggregates following 14 days of treatment with RA, and the increase was sustained at 21 days (Fig. 2). Similar to the induction of NF200-P, expression of axonal and dendritic markers such as tau isoforms 1 and 2, MAP1B, and synapsin I was also increased following 21 days of RA treatment and sustained at 24 days (Fig. 2). When compared with the method previously developed to produce CNS neurons (Pleasure and Lee 1993; Pleasure *et al*. 1992), our cell aggregation protocol significantly reduced the time normally required for the induction of neuronal differentiation. Enriched CNS neurons could be produced in 14 days (compared with 60 days using the conventional protocol), as revealed by the expression of neuronal markers such as NF160-P, NF200-P, tau, MAP1b and synapsin I.

To examine the distribution of NT2 cells in different phases of the cell cycle during neuronal differentiation, FACS analysis of DNA content in NT2 cells following RA

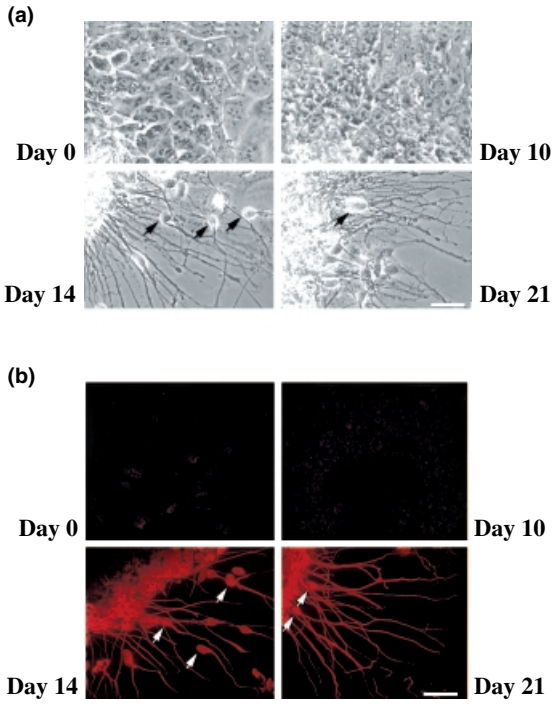

Fig. 1 Phase micrograph (a) and neurofilament staining (b) of cultured NT2 cells in aggregates. Aggregated NT2 cells were treated with RA for 0, 10, 14 and 21 days as described in Methods. The aggregates were then replated on culture plates coated with poly D-lysine and laminin and maintained for 1 day in the absence of RA. Extensive neurites were extended from NT2 aggregates following treatment with RA for 14–21 days. Arrows indicate migrating neurons. Magnification, 32×; scale bar, 10 μm.

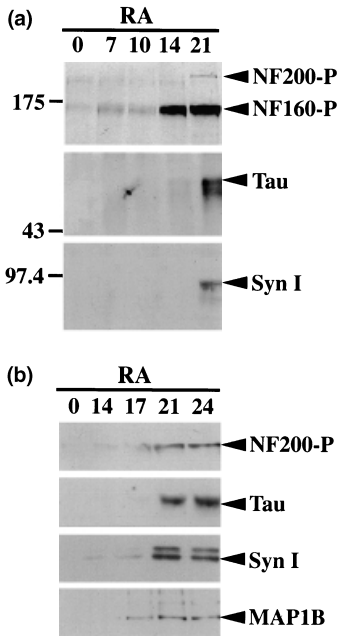

Fig. 2 Western blot analysis of the expression of specific neuronal markers in NT2 aggregates following RA treatment. Aggregated NT2 cells were treated with RA for various time periods (a, 0–21 days; b, 0–24 days) as indicated. Neuronal markers including the phosphorylated middle and heavy forms of neurofilament (NF160-P and NF200-P), tau isoform 1 and 2 (Tau), MAP1B and synapsin I (Syn I) were detected in NT2 cells following RA treatment.

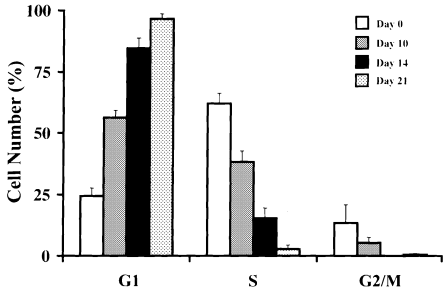

Fig. 3 Distribution of NT2 cells in different phases of the cell cycle (G_1, S and G_2/M) during the process of neuronal differentiation. NT2 cells were aggregated in the presence of RA for 0, 10, 14 and 21 days. The percentage of cell population in various phases of the cell cycle was analyzed using flow cytometer. Each data point represented the mean ± SEM of one representative experiment; $n = 3$.

treatment was performed. Prior to the addition of RA, majority of NT2 cells were in the process of proliferation, as evident by ~60% of the cells in S phase. Following treatment with RA for 10–14 days, cells in S phase were drastically reduced, while ~50–80% of the cells entered into G_1 phase. With the period of RA treatment extended to 21 days, > 95% of NT2 cells became arrested in G_1 phase (Fig. 3).

Expression of cell cycle regulatory proteins in NT2 cells during the course of neuronal differentiation

Complexes of Cdks and their regulatory subunits cyclins play a pivotal role in the regulation of cell cycle progression. We have examined the expression of Cdks, cyclins and Cdk inhibitors in NT2 cells during neuronal differentiation. While the expression of Cdks (Cdc2, Cdk4 and Cdk6) decreased after 21 days of RA treatment (Fig. 4a), cyclin A and cyclin D2 exhibited a biphasic profile of expression, i.e. induced after 10 days of RA treatment and then decreased following prolonged RA treatment (Fig. 4b). Abundant level of cyclin B1 and cyclin D1 proteins could be detected in undifferentiated NT2 aggregates, the expression of which decreased gradually to barely detectable level after RA treatment for 21 days. Low level of Cdk inhibitors, p21 and p27, were detected in undifferentiated aggregated NT2 cells and the expression was induced after 10 days of RA treatment (Fig. 4c), consistent with the withdrawal of NT2 cells from the cell cycle. Other cell cycle regulatory proteins such as PCNA and Rb also play a key role in controlling the cell cycle arrest during neuronal differentiation. The expression of Rb, which is normally involved in regulating the transcription factors important for the induction of cell cycle genes (Chellappan *et al*. 1991), increased after RA treatment and then decreased in differentiated NT2 aggregates (Fig. 4c). On the other hand, the protein expression of PCNA, a DNA synthesis marker (Tsurimoto 1999), was reduced in NT2 cells after 21 days of RA treatment (Fig. 4c).

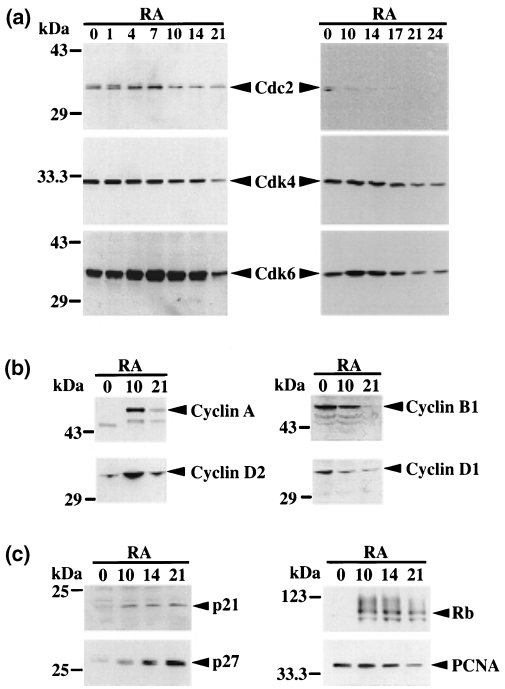

Fig. 4 Western blot analysis of the expression of cell cycle related proteins in NT2 cells during neuronal differentiation. (a) Protein expression of Cdc2, Cdk4 and Cdk6 in NT2 cells following RA treatment for different periods (0–24 days). Protein expression of cyclin A, cyclin B1, cyclin D1 and cyclin D2 in NT2 cells following 10–21 days of treatment with RA was depicted in (b). The expression of Cdk inhibitors (p21 and p27), as well as Rb and PCNA in NT2 cells following RA treatment (0–21 days) was depicted in (c).

Expression of Cdk5 and its activators in NT2 cells

Cdk5 and its activators exhibited an expression profile distinct from those of the other Cdks and cyclins in NT2 cells during neuronal differentiation. Basal level of Cdk5 protein was detected in untreated NT2 aggregates, and the protein expression gradually increased upon treatment with RA (Fig. 5a). While p35 protein expression was barely detectable in NT2 aggregates, it was significantly induced following 14 days of RA treatment (Fig. 5a). The induction of another Cdk5 activator, p39, lagged behind that of p35, suggesting that it might have functional role distinct from that of p35 (Fig. 5b).

As described above, the aggregation protocol greatly enhanced the neuronal differentiation of NT2 cells when compared to the conventional plating method. Consistent with the appearance of NF200-P in aggregates following RA treatment, prominent induction of p35 protein expression could be detected in differentiated NT2 aggregates at 14 days (data not shown) and 21 days (Fig. 6a). Because the conventional plating method requires longer periods of time (about 60 days) to produce enriched neurons, the induction of p35 could not observed on day 21. Significant difference in Cdk5 protein expression could not be observed in NT2

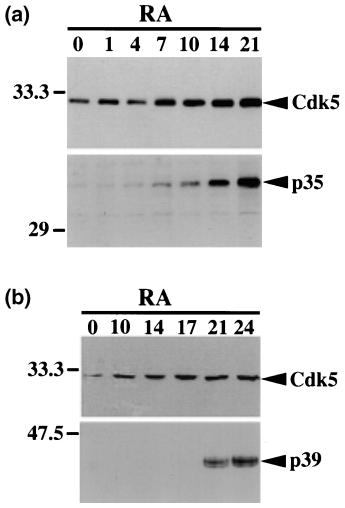

Fig. 5 Western blot analysis of the expression of Cdk5 and its activator, p35 and p39, in NT2 cells treated with RA. Protein expression of Cdk5 and p35 (a) and p39 (b) was examined in NT2 cells aggregated for various periods of time (a, 0–21 days; b, 0–24 days) in the presence of RA.

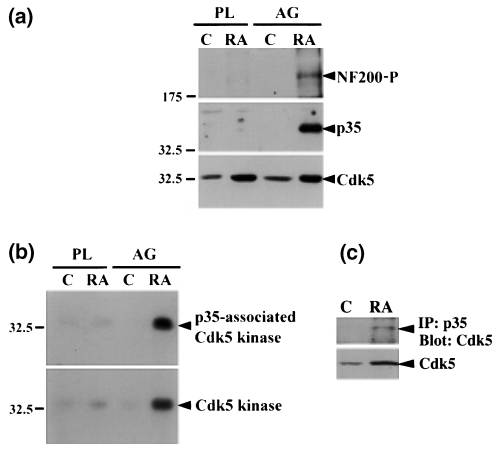

Fig. 6 Aggregation induced the expression of p35 and Cdk5 kinase activity. (a) Western blot analysis to compare the expression of p35, Cdk5 and NF200-P protein in NT2 cells. NT2 cells were differentiated using the plating protocol (PL) or the aggregation protocol (AG) in the absence (C) or presence of RA (RA). (b) p35-associated Cdk5 kinase activity (top) and total Cdk5 kinase activity (bottom) were examined in NT2 cells cultured using the two differentiation methods. (c) Co-immunoprecipitation analysis of Cdk5 and p35 in differentiated NT2 aggregates (RA) and undifferentiated NT2 aggregates (C).

cells differentiated using the two protocols. The kinase activity of Cdk5 in NT2 cells using these two differentiation methods was also examined by measuring the incorporation of γ-^{32}P into the substrate histone H1. While a very low level of Cdk5 kinase activity was induced in RA-treated NT2 cells using the normal plating method, the Cdk5 kinase activity increased significantly in aggregated NT2 cells treated with RA for 21 days when compared to untreated NT2 aggregates (Fig. 6b). Similarly, p35-associated Cdk5 kinase activity was induced in differentiated NT2 aggregates (Fig. 6b). These findings suggested that most of the Cdk5 kinase activity in NT2 neurons was contributed by the association of the kinase with p35. Co-immunoprecipitation experiment showed that Cdk5 associated with p35 in differentiated NT2 neurons (Fig. 6c).

Subcellular localization of Cdk5 activators in differentiated NT2 neurons

Previous findings from our laboratory have demonstrated that NT2 neurons expressed different neurotransmitters such as tyrosine hydroxylase, substance P and GABA (Cheung *et al*. 1999). We also examined the subcellular localization of Cdk5 and its activators in differentiated NT2 neurons by immunocytochemical analysis. Previous studies have demonstrated the co-localization of Cdk5 protein and p35 in the axon shafts and growth cones, suggesting that they played important roles in neuronal migration and neurite outgrowth. Neurofilaments, in particular the phosphorylated high molecular weight form (NF200-P), β-tubulin, and SV2, a protein present in varicosity-like structures, have been shown to be the markers of differentiated neurons. We have examined the localization of Cdk5 activators at the NT2 neurites, and compared them with these markers. While staining for both NF200-P and SV2 could be observed in the neurites of NT2 neurons, SV2 was detected in discrete puncta in some NT2 neurites (Fig. 7). Similar to NF200-P and SV2, p35 was localized to the neurites of NT2 neurons; moreover, p35 was also localized to the varicosity-like structures (Fig. 7). Double immunostaining of Cdk5 and p35 demonstrated that they were co-localized along the axon shaft and in the growth cones of NT2 neurons (Fig. 7), consistent with the previous report on the localization of Cdk5 at the neurites in primary rat cortical neurons (Nikolic *et al*. 1996). Similar to p35, p39 was also expressed along the axons of NT2 neurons that showed strong staining with Tau, β-tubulin and MAP1B (Fig. 8) and NF200-P (data not shown). Like SV2, p39 was prominently expressed in the varicosity-like structures (Fig. 8). A previous study has suggested that the presence of p39 at the synapses might implicate a potential role in synaptogenesis (Humbert *et al*. 2000). Double immunostaining also revealed the co-localization of p39 with Cdk5 along the axon shaft, as well as the filopodia and lamellipodia region of NT2 neurites (Fig. 8).

Discussion

In the present study, we have used a rapid protocol based on cell aggregation to produce mature human neurons following 21 days of RA treatment. The mature neurons can be characterized by the expression of specific neuronal markers, cell cycle analysis and the differential regulation of cell cycle regulatory proteins. The detection of neuronal phenotype and

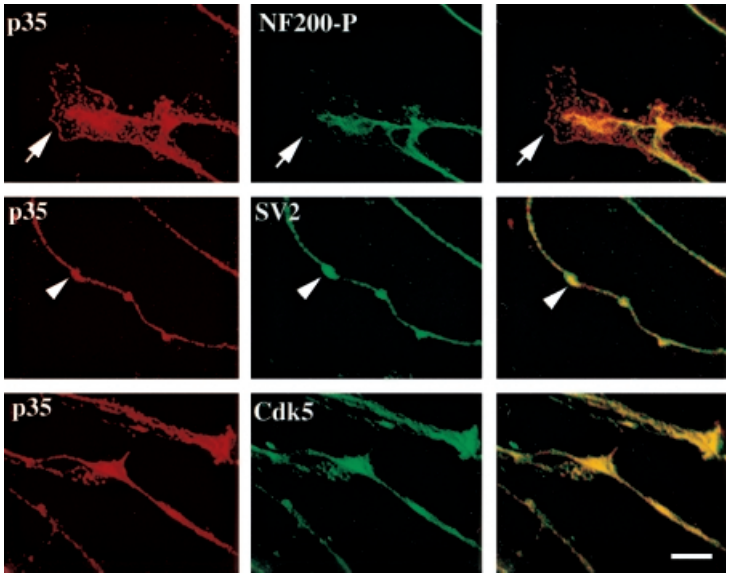

Fig. 7 Cellular localization of p35 in differentiated NT2 neurons. Double immunostaining of p35 (left panels) with NF200-P, SV2 and Cdk5 (middle panels) followed by confocal imaging were performed in differentiated NT2 aggregates following RA treatment for 21 days. The right side is the superimposed image of p35 with other neuronal proteins. Arrows indicate the filopodia and lamellipoda structures of the growth cones, while arrowheads indicate the region of varicosity-like structures. Scale bars, 25 μm.

the expression of neuronal markers correlate with the exit from cell cycle as demonstrated by FACS analysis. The neuronal markers used in this study are normally expressed in the neuronal cells during later stages of development, suggesting that the differentiated NT2 neurons are mature and are capable of forming functional synapses.

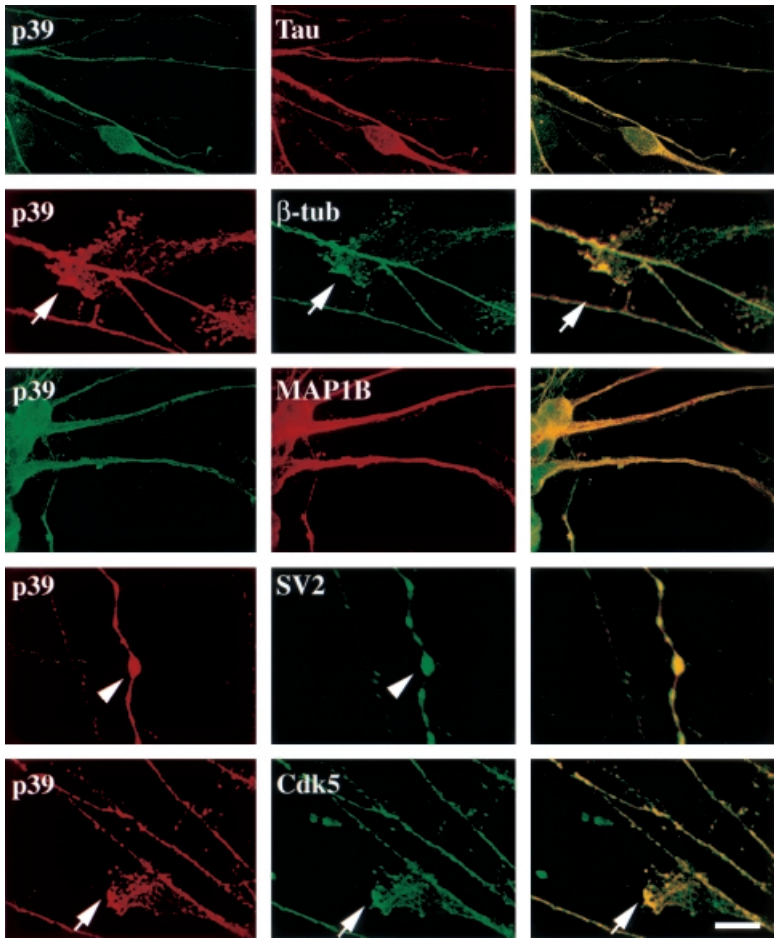

Fig. 8 Cellular localization of p39 in differentiated NT2 neurons. Double immunostaining of p39 (left panels) with Tau, β-tubulin, MAP1B, SV2 and Cdk5 (middle panels) followed by confocal imaging were performed in differentiated NT2 aggregates following RA treatment for 21 days. The right side is the superimposed image of p39 with other neuronal proteins. Arrows indicate the filopodia and lamellipoda structures of the growth cones, while arrowheads indicate the region of varicosity-like structures. Scale bars, 25 μm.

The differential regulation of cell cycle-related proteins contributes to the withdrawal of NT2 cells from the cell cycle and the commitment to neuronal fate. Prominent expression of mitotic kinase, Cdc2, and DNA synthesis marker, PCNA, is consistent with the notion that NT2 cells are in the process of cell proliferation during the initial period of RA treatment (before day 10). Cell cycle analysis reveals that large proportion of NT2 cells enter the G_1 phase after 10 days of RA treatment, suggesting that the cells start to differentiate into neurons as revealed by the morphological phenotype and neuronal markers. The expression profile of the cell cycle proteins is similar to that observed with other neuroblastoma cell line such as PC12 (Yan and Ziff 1995). For example, the Cdk proteins, Cdc2, Cdk4 and Cdk6, as well as their regulatory partners, cyclin A, cyclin B and cyclin D family are down-regulated while Cdk inhibitors such as p21 and p27 are induced. Although detectable level of Cdks is observed in NT2 neurons, it is likely that their kinase activities are inhibited by binding with Cdk inhibitors such as p21 and p27 (Harper *et al*. 1993). Because Rb has been suggested to play a role in biochemical and morphological development of young neurons as well as the permanent exit of neurons from cell cycle (Lee *et al*. 1994), the induction of Rb in NT2 cells is consistent with their commitment to neuronal fate. Taken together, the differential expression of cell cycle regulated proteins suggests that NT2 cells undergo a complicated network of protein–protein interaction during the regulation of exit from cell cycle and initiation of terminal neuronal differentiation.

The present study provides the first demonstration on the induction of Cdk5 activators, p35 and p39, as well as Cdk5 kinase activity in NT2 neurons differentiated by RA together with aggregation method. As increasing evidence suggests a role of Cdk5 in neuronal patterning and synapse formation in CNS, it is important to study the regulation of expression and activities of Cdk5 in CNS (Sharma *et al*. 1999a; Chen *et al*. 2000; Zukerberg *et al*. 2000). However, the early death of Cdk5(–/–) mutants in embryonic stages complicates the analysis of Cdk5 signaling pathway in early CNS development *in vivo* (Ohshima *et al*. 1996). With progressive appearance of neuronal properties and prominent induction of p35 and p39 in NT2 cells during the course of RA-induced differentiation, NT2 cells provides a useful system to investigate the early signaling pathway of Cdk5/p35 and Cdk5/p39 in CNS development.

In this study, the expression pattern of Cdk5, p35 and p39 is differentially regulated during neuronal differentiation of aggregated NT2 cells. While Cdk5 is present in the undifferentiated NT2 cells, its activators are only prominently expressed in differentiated NT2 neurons. Furthermore, the protein expression of p39 lags behind that of p35 during the process of neuronal differentiation of NT2 cells. Interestingly, using the protocol of cell aggregation which significantly reduces the time normally required for neuronal differentiation, the expression of p35 and p39 proteins in NT2 neurons is prominently induced. Because neurotrophins have been demonstrated to induce the p35 transcript in cultured neurons (Tokuoka *et al*. 2000; Harada *et al*. 2001), and the expression of neurotrophin receptors is induced during neuronal differentiation of NT2 cells (Cheung *et al*. 1996), it is possible that the induction of expression of Cdk5 activators in NT2 aggregates might be mediated by the increase in Trk expression.

The expression pattern of Cdk5 and its activators during neuronal differentiation of NT2 cells is reminiscent of the profile observed during rat brain development (Tsai *et al*. 1993). Cdk5 protein is gradually increased in the rat brain during development, while the expression of p35 can be detected earlier than p39. Furthermore, the subcellular localization of Cdk5 and p35 in NT2 cells is consistent with the finding observed in primary cortical neurons (Nikolic *et al*. 1996). They are both localized along the entire length of differentiated NT2 neurite, consistent with the notion that Cdk5 plays an important role in neurite outgrowth. In addition to the localization in the axons and growth cones, we show here that p35 and p39 are also detected at the varicosity-like structures that supposed to contain synaptic vesicles. The differential temporal expression of p35 and p39 following RA treatment suggests that they may have different functions in regulating Cdk5 activity during neuronal development of NT2 cells. It is noteworthy that similar differential regulation of p35 and p39 in denervated muscle is also recently observed in our laboratory (unpublished observation). The differential expression of p35 and p39 observed in NT2 neurons allows for studying the distinct and overlapping functions of Cdk5/p35 and Cdk5/p39 during neuronal development.

The extension of neurites from replated NT2 aggregates is dependent on the extracellular substrates such as laminin (Cheung *et al*. 1999). Through the activation of integrin receptors, laminin has been shown to enhance Cdk5 activity (Li *et al*. 2000). Previous *in vitro* studies have demonstrated that Cdk5 can phosphorylate NF160 and NF200 (Qi *et al*. 1998; Sharma *et al*. 1999b) and microtubule-associated proteins such as MAP1B (Pigino *et al*. 1997) and tau (Wada *et al*. 1998). These proteins are the major neurocytoskeletal proteins that are important for the growth of neurites and maintenance of neuronal polarity. Taken together, our findings suggest that the increase in Cdk5 kinase activity in NT2 aggregates replated on laminin substrate plays a pivotal role in the phosphorylation of substrates that are involved in neurite outgrowth.

Aberrant phosphorylation of neurofilament (Julien and Mushynski 1998) and tau (Imahori and Uchida 1997) has been observed in brains with Alzheimer's disease. One of the candidate kinases that have been proposed to be involved in such aberrant phosphorylation is Cdk5 (Baumann *et al*. 1993; Illenberger *et al*. 1998). Taken together, our findings

not only demonstrate the regulation of expression of Cdk5 activators during neuronal differentiation of NT2 cells, but also provide a good *in vitro* model system to study the signaling pathway of Cdk5 activation as well as to elucidate the roles of Cdk5 in neuronal differentiation and neruo-degenerative diseases.

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