

Overexpression of cdk4/cyclin D1 induces apoptosis in PC12 cells in the presence of trophic support

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Abstract The induction of apoptosis by cell cycle regulator molecules under conditions optimal for exponential growth was examined in rat pheochromocytoma PC12 cells by overexpression of cyclins and cyclin-dependent kinases (cdks). By flow cytometry and by immunofluorescence, only cells overexpressing cdk4 or cyclin D1 underwent apoptosis, which was not associated with G1-arrest. Cdk4 kinase activity was significantly higher in cdk4-, or cyclin D1-expressing cells. Furthermore, induction of apoptosis by cdk4 was abrogated by co-transfection of p16^{INK4}, or dominant negative cdk4. These results suggest that upregulation of cdk4 kinase activity is a primary and critical mediator of apoptosis in PC12 cells under physiological conditions. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Cdk4; Cyclin D1; PC12 cell; Apoptosis; Cdk inhibitor; Dominant negative cdk4

1. Introduction

The cell cycle is strictly regulated by a complex set of mechanisms. The G1–S transition is controlled by the specific interaction of several combinations of G1 cyclins and cyclin-dependent kinases (cdks) [1,2]. Recently, several proteins that function as positive regulators of the cell cycle have been demonstrated to induce apoptosis when overexpressed. These include cyclin D1, c-myc, E2F1 and human papilloma virus (HPV) oncoproteins [3–6].

We have been investigating the involvement of cell cycle regulator molecules in the apoptosis of rat pheochromocytoma PC12 cells and described the following [7]: apoptosis induced by serum withdrawal was accompanied by the upregulation of cdk4 protein expression and kinase activity. Conversely, constitutive overexpression of cdk4 significantly promoted apoptosis induced by serum withdrawal, whereas

overexpression of other cyclins, cdk2 or cdc2 did not [7]. Although these results suggest that cdk4 plays a crucial role in apoptosis induced by serum withdrawal, the apoptotic function of cdk4 under more physiological conditions has not been elucidated.

To further clarify this issue, we examined the potential effect of cell cycle regulator molecules on cell fate by transient overexpression of their cDNAs in PC12 cells cultured in the presence of serum, a condition that closely resembles the physiological condition.

2. Materials and methods

2.1. Cell lines and plasmids transfection

PC12 cells were grown as described [7]. The cDNAs encoding cyclins, cdks and dominant negative cdk4 (cdk4dn) were subcloned into the pME vector [8,9]. The cdk4dn consists of the inactive mutant (K35M) with a hemagglutinin (HA) tag fused to the amino-terminus of the cdk4 [9]. Transient transfection was performed by electroporation as described [10]. For double transfection of cdk4/p16 or cdk4/cdk4dn, 50 µg of each cDNA (total 100 µg) was used. After 24 h of incubation in the normal culture conditions, bromo-deoxyuridine (BrdU, Sigma) was added to a final concentration of 50 µM. At 24, 48, 72, 96 and 120 h after transfection, cells were harvested and used for further experiments.

2.2. Flow cytometry by fluorescein-activated cell sorter (FACS)

The cells were incubated with primary antibodies at 4°C for 2 h followed by fluorescein-isothiocyanate (FITC)-conjugated secondary antibody and propidium iodide (PI) staining [7]. FITC and PI fluorescence were measured using a FACS flow cytometer (Becton-Dickinson) [7]. To analyze the cells overexpressing introduced cDNA products, the gate was set for the fractions manifesting a significantly higher level of FITC fluorescence (between channel 10¹ and 10²) and the cells in those fractions were collected. For cdk4 kinase assay, cells in those fractions were recovered by a cell sorter.

2.3. Antibodies

Primary antibodies, cyclins A, E, D1, and cdk2, cdk4 and cdc2 were used at the dilutions as previously described [7,10,11]. Other antibodies were used at the following dilutions: cyclin B1 (Novocastra), 1:100; p16 (Neomarker), 1:200; HA-probe (Santa Cruz), 1:100 and anti-BrdU antibody (Takara), 1:100. The secondary antibodies were FITC-conjugated, or rhodamine-conjugated goat anti-mouse IgG, or swine anti-rabbit IgG (Jackson Immuno Research Lab.); 1:80 dilutions.

2.4. Immunofluorescence and morphological observation

Cells were stained with the same primary antibodies used for flow cytometry [12]. The specimens were examined and images were captured by a CCD camera (Photometrics). For tentative evaluation of protein expression levels by image analysis, semiquantification of

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Abbreviations: cdk, cyclin-dependent kinase; INK4, inhibitor of kinase 4; HPV, human papilloma virus; cdk4dn, dominant negative cdk4; PI, propidium iodide; FITC, fluorescein-isothiocyanate; HA, hemagglutinin; FACS, fluorescein-activated cell sorter

FITC intensity was performed by IP-Lab software (Scanalytics). The values in positively stained cells were measured and expressed as ratios relative to the endogenous levels in vector-transfected cells.

2.5. DNA fragmentation assay

At each time point after transfection, 10^5 cells were harvested which overexpressed introduced gene products. DNA was extracted with extraction buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.2% Triton X-100) as previously described [7] and 10 μ g of DNA was loaded onto a 2% agarose gel. Independent experiments were performed in duplicate.

2.6. In vitro kinase reactions and densitometric analysis

Cdk4 kinase activity was assayed as previously described by independent duplicate experiments [7,10]. Densitometric quantification of the data was done using NIH images [13].

3. Results and discussion

Apoptosis was defined by the presence of subdiploid DNA content, nuclear morphology and internucleosomal DNA fragmentation [7,14].

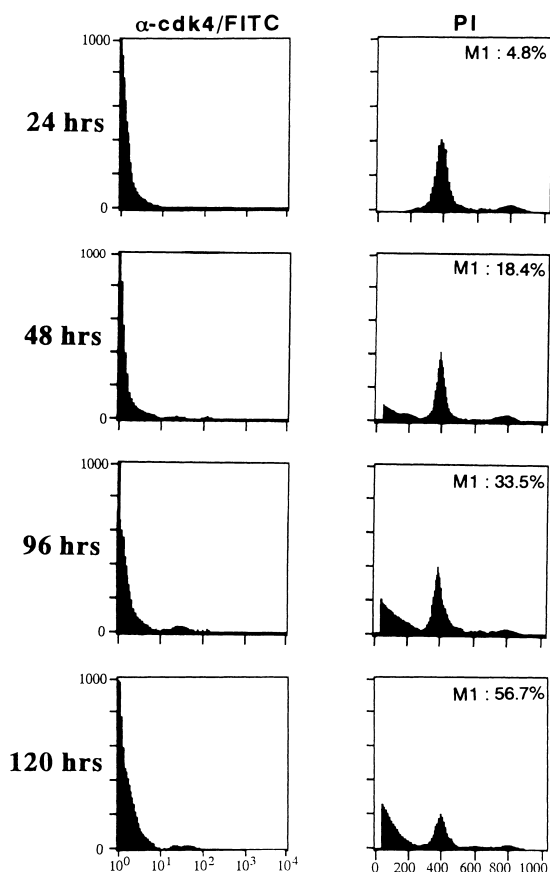


Fig. 1. Time-course experiment of flow cytometric analysis of cdk4 protein overexpression and induction of apoptosis. Cells were transfected with cDNA expressing cdk4 protein and stained with the anti-cdk4 antibody (left columns) and PI (right columns). Results at 24, 48, 96 and 120 h after transfection are shown. The percentages of cells in the subdiploid fraction are indicated as M1 in the right columns.

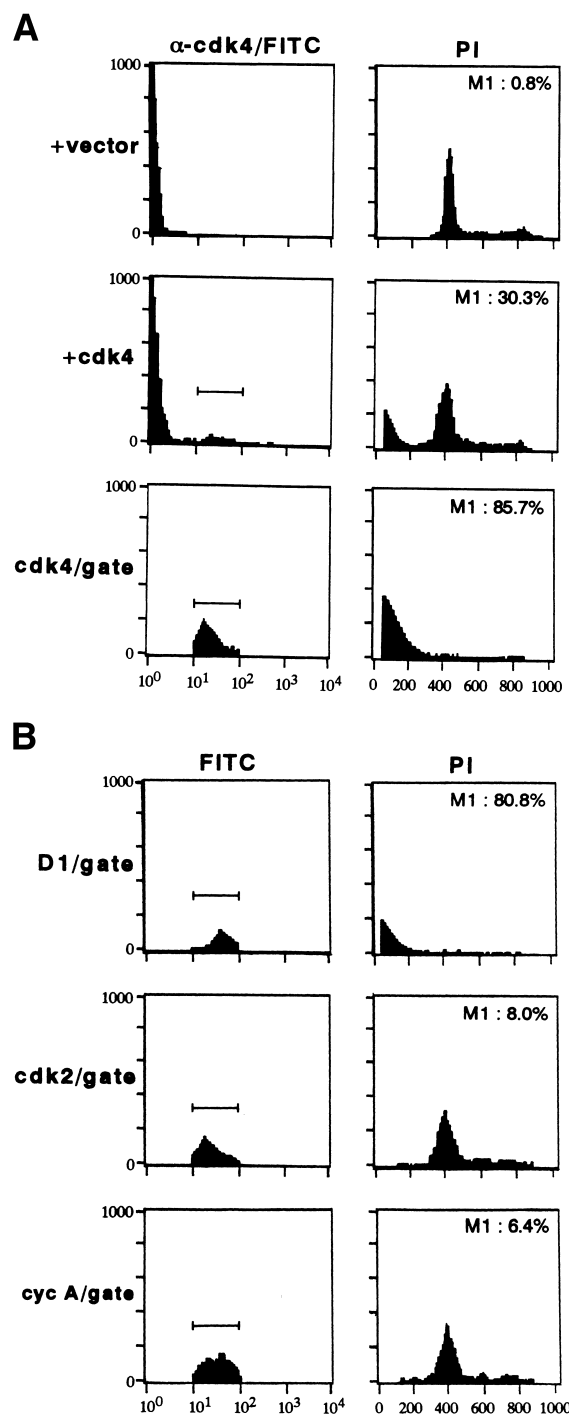


Fig. 2. Flow cytometric analysis of overexpressed proteins and induction of apoptosis. A: Cells were transfected with empty vector (top row) or with cDNA expressing cdk4 protein (middle and bottom rows). Cells were stained with the antibody (left columns) and PI (right columns). Cells overexpressing cdk4 protein were collected by setting the gate (middle and bottom rows), and the DNA content of these cells was analyzed (bottom row). B: For the results of cyclin D1 (top row), cdk2 (middle) and cyclin A (bottom), only the data obtained by collecting overexpressing cells are shown. The results from cells overexpressing cyclins B1 and E, and cdc2 were similar to those of cdk2 or cyclin A. The percentages of cells in the subdiploid fraction are indicated as M1 in the right columns.

Table 1
Apoptotic fractions in flow cytometry

	M1 \pm 2 S.D. (%) ^a	M1/gate \pm 2 S.D. (%) ^b
vector	0.84 \pm 1.5	–
cyclin A	1.86 \pm 1.8	6.35 \pm 1.3
cyclin B1	2.45 \pm 1.2	5.35 \pm 4.5
cyclin D1	32.5 \pm 12.6 ^c	80.8 \pm 16.2 ^d
cyclin E	2.33 \pm 1.5	7.05 \pm 3.2
cdc2	5.20 \pm 2.2	8.40 \pm 4.4
cdk2	1.26 \pm 1.4	7.98 \pm 3.8
cdk4	30.3 \pm 15.1 ^c	85.7 \pm 20.0 ^d

^aM1: subdiploid fraction in total cells.

^bM1/gate: subdiploid fraction in gated cells.

^cSignificantly higher than others: $P < 0.001$.

^dSignificantly higher than others: $P < 0.0001$.

3.1. Overexpression of cell cycle regulators and induction of apoptosis

Cells were transfected with cDNAs encoding cyclins A, B1, D1 or E, cdk2, cdk4 or cdc2, as well as empty vector and the presence of subdiploid DNA content was examined by flow cytometry. Preliminary experiments revealed that higher efficiencies of transfection were obtained by 40–100 μ g of cDNA, and no clear dose-dependency was observed. For the time-course experiment, the extent of apoptosis was evaluated by flow cytometric analysis at 24, 48, 96 and 120 h of incubation in the normal culture conditions after transfection. The inten-

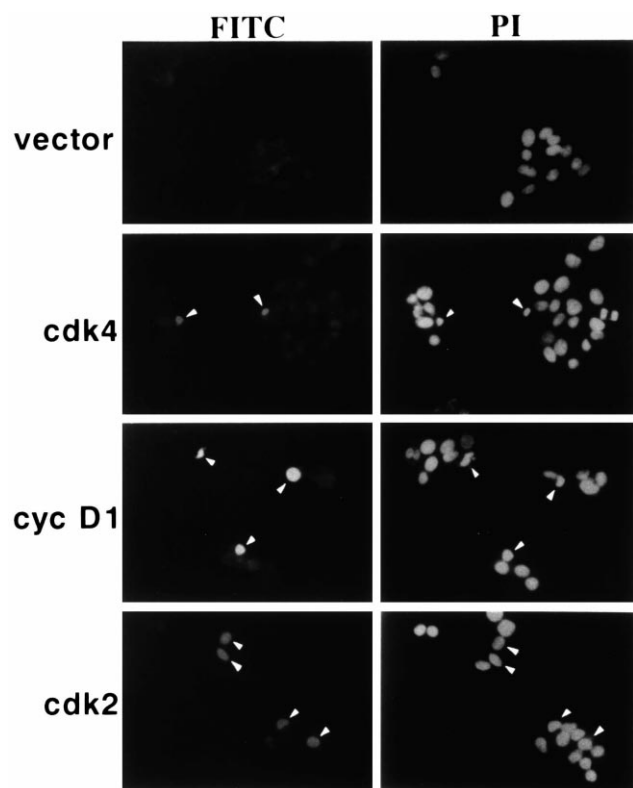


Fig. 3. Morphological observations of protein-overexpressing cells. Cells were transfected with pME ('vector'), cDNA encoding cdk4 ('cdk4'), cyclin D1 ('cyc D1') or cdk2 ('cdk2'). The cells were stained by the respective antibodies ('FITC', left column) and PI (right column). Vector-transfected cells were stained with anti-cdk4 antibody. Arrows indicate cells with intense positive staining for the introduced proteins. Since the results from cells overexpressing cyclins A, B1 and E, and cdc2 were similar to those of cdk2, only the data from cdk2-overexpressing cells are shown.

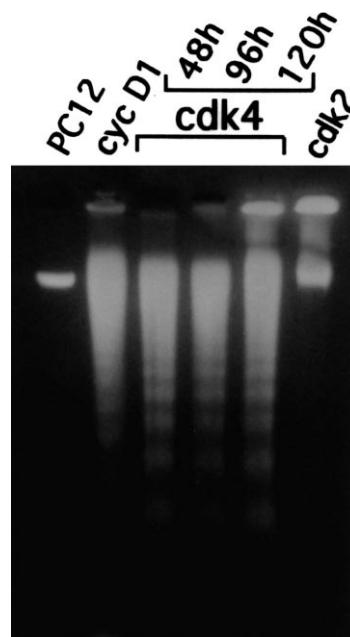


Fig. 4. DNA fragmentation assay. Agarose gel electrophoresis of DNA extracted from parental cells ('PC12'), cells overexpressing cyclin D1 ('cyc D1'), and cdk2 ('cdk2') as well as those overexpressing cdk4 ('cdk4') during the time-course experiments. Cells were harvested at 96 h or at the indicated times after transfection, and 10 μ g of extracted DNA was electrophoresed.

sity of the FITC fluorescence varied over time: FITC fluorescence was below detectable level until 36 h after transfection, and these levels were the highest around 90–110 h (Fig. 1). Again, the levels remarkably decreased after 120 h, although apoptotic fractions could be continuously detected after 48 h when cDNA encoding cdk4 or cyclin D1 was introduced (Fig. 1). Thus, transfection was performed using 50 μ g of cDNA and transfected cells were cultured for 96 h.

In untreated parental and vector-transfected cells, the apoptotic subdiploid fraction was minimal up to 0.8% of total cells (Fig. 2 and Table 1). In contrast, in cdk4-transfected cells, the percentage of cells exhibiting subdiploid DNA content rose to 30.3% (Fig. 2A). When cdk4-overexpressing cells were separated by gating for high FITC intensity, 85.7% had subdiploid DNA content. Cells transfected with cyclin D1 exhibited a subdiploid DNA content in 32.5% of total cells and 80.8% of cyclin D1-overexpressing cells (Fig. 2B). However, cells transfected with cdk2 or other cDNAs were similar to vector-transfected cells with regard to DNA content (Fig. 2B). The apoptotic fractions in the total cells, as well as in cells overexpressing cdk4 or cyclin D1, were higher to a statistically significant extent ($P < 0.001$ and $P < 0.0001$, respectively) (Table 1).

3.2. Morphological change

To confirm whether the observed increase of the subdiploid fraction was due to apoptosis, we examined the nuclear features by immunofluorescence. No remarkable nuclear morphological changes were observed in either the parental or vector-transfected cells. In cdk4-transfected cells, intense positive staining was detected in the nucleus of 7.2% of the cells. In 100 of cdk4-positive cells, 89% (89/100 cells) exhibited marked shrinkage and condensation of the nuclear chromatin (Fig. 3). In cells transfected with cyclin D1, 8.8% showed

intense nuclear staining, and 71% (71/100 cells) of those were accompanied by nuclear bleb formation, fragmentation, nuclear shrinkage, and chromatin condensation (Fig. 3). However, in cdk2-transfected cells, only 4% (4/100 cells) of cdk2-overexpressing cells exhibited apoptotic change (Fig. 3). Similarly, in cells transfected with other cDNAs, most of the positively stained cells remained unchanged (data not shown). These results demonstrate that overexpression of cdk4 or cyclin D1 can induce apoptosis even in the presence of serum.

3.3. Internucleosomal DNA fragmentation assay

Next, in an attempt at further confirmation of apoptosis, we examined the occurrence of the internucleosomal DNA fragmentation characteristic of apoptosis during the time course. Since the number of cdk4-overexpressing cells was not so remarkable until 48 h after transfection as shown in Fig. 1, and enough cells were not recovered by cell sorting, we analyzed the extracted DNA from the gated cells obtained at 48, 96 and 120 h. As shown in Fig. 4, DNA fragmentation was observed in cdk4- or cyclin D1-overexpressing cells collected by sorting at any time points and the pattern of DNA fragmentation in those cells was similar and extensive. However, DNA extracted from cdk2-overexpressing cells did not show this internucleosomal fragmentation except for slight gel smearing (Fig. 4).

3.4. Image analysis of FITC intensity

We next examined a correlation between the expression levels of the introduced gene products and the occurrence of

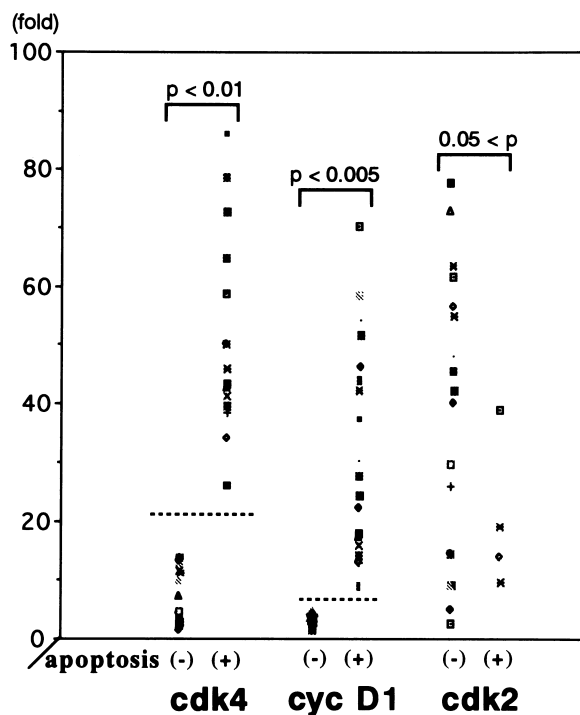


Fig. 5. Semiquantification of image analysis. The intensity values of the FITC signal in stained cells relative to endogenous levels are shown. All the data in cdk4, cyclin D1 and cdk2 stainings are expressed as dots. The presumed thresholds of expression at which apoptosis is induced are indicated as broken lines. Since the results from cells overexpressing cyclins A, B1 and E, and cdc2 were similar to those of cdk2, only the data from cdk2-overexpressing cells are shown.

Table 2
Apoptotic fractions in co-transfection

	M1 \pm 2 S.D. (%) ^a	M1/gate \pm 2 S.D. (%) ^b
vector (100 μ g)	5.08 \pm 4.4	–
cdk4/p16	13.5 \pm 5.2 ^c	–
cdk4/gate	–	40.8 \pm 16.3 ^d
p16/gate	–	6.50 \pm 4.9
cdk4/cdk4dn	12.9 \pm 4.3 ^c	–
cdk4/gate	–	36.5 \pm 10.7 ^d
cdk4dn/gate	–	0.61 \pm 1.3

^aM1: subdiploid fraction in total cells.

^bM1/gate: subdiploid fraction in gated cells.

^cSignificantly lower than M1 in cdk4 transfection alone: $P < 0.001$.

^dSignificantly lower than M1 in cdk4 transfection alone: $P < 0.005$.

apoptosis. FITC intensity in cdk4-transfected cells ranged from 2.1-fold to 86.4-fold higher than endogenous levels, and cells displaying an FITC intensity more than 25.7-fold higher exhibited morphological changes characteristic of apoptosis (Fig. 5). There was a statistically significant difference in FITC intensities of cdk4 immunofluorescence between apoptotic and non-apoptotic cells ($P < 0.01$). The intensity of cyclin D1 staining ranged from 1.4- to 70.6-fold higher than endogenous level, and apoptosis was observed in cells displaying intensities higher than 8.1-fold (Fig. 5). There was also a statistically significant difference in cyclin D1 intensities between apoptotic and non-apoptotic cell groups ($P < 0.005$). In cells transfected with cdk2 and other cDNAs, there were few apoptotic cells, and FITC intensity of the positively stained cells did not show clear correlation with apoptosis (Fig. 5).

3.5. Abrogation of cdk4-induced apoptosis by cdk4 inhibitor protein, p16^{INK4A} and by cdk4dn

To confirm that induction of apoptosis was due to the action of cdk4 and cyclin D1, we co-expressed p16, inhibitor of cdk4 and cdk6, together with cdk4 [2]. Whereas the total cell apoptotic fraction of cells transfected with cdk4 alone was 30.3% (Fig. 2A), this fraction significantly declined to 13.5% in cells transfected with both cdk4 and p16 ($P < 0.001$, Fig. 6A and Table 2). When cells were gated for high cdk4 expression, the apoptotic fraction was 40.8% for cells transfected with both cdk4 and p16 (Fig. 6A), which is significantly lower than the 85.7% apoptosis for cells transfected with cdk4 alone ($P < 0.005$, Table 2, Fig. 2A). When gated for high p16 expression, only 6.5% of cells were found in the apoptotic fraction (Fig. 6A). Conversely, when apoptotic cells were collected, these cells exhibited a low level of p16 expression (Fig. 6B). Thus, the 40.8% of cells found in the apoptotic fraction represent those in which p16 expression is not dominant against cdk4.

We next used cdk4dn that specifically inhibits wild-type cdk4 activity by competing for binding to cyclin D [9]. The apoptotic fraction in the cdk4/cdk4dn co-transfected cells was 12.9% of total cells, which is significantly lower than that in cdk4 alone ($P < 0.001$) (Figs. 2A and 6C, and Table 2). When cells were gated for high cdk4 expression, the apoptotic fraction was 36.5% of cdk4-overexpressing cells, which was significantly lower ($P < 0.005$) than that in cdk4 alone (Figs. 2A and 6C, and Table 2). Gating for cdk4dn-expressing cells revealed that only 0.6% had a subdiploid DNA content (Fig. 6C). When the apoptotic cells were separated, those cells revealed a lower level of cdk4dn expression (Fig. 6D). There-

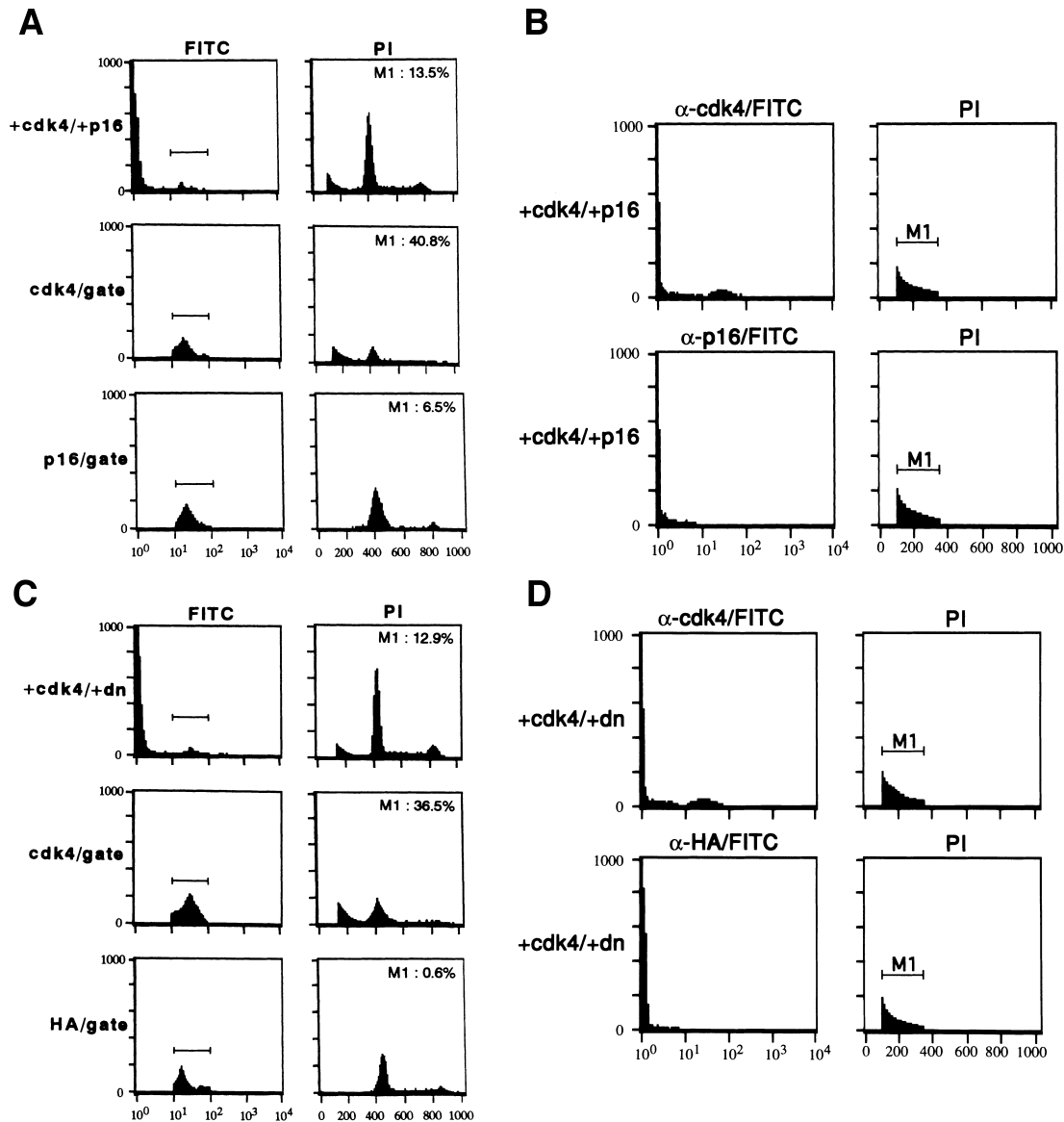


Fig. 6. Analysis of cells co-transfected with cdk4/p16^{INK4A}, and cdk4/cdk4dn. Cells were co-transfected with cDNA encoding cdk4 and p16 (A, B) or with cdk4 and cdk4dn (C, D). Cells overexpressing introduced proteins were collected by setting the gate for cdk4 (A and C, middle rows), p16 (A, bottom row) or for HA (for cdk4dn, C, bottom row), and the DNA content of the collected cells was analyzed. The percentages of cells in the subdiploid fraction are indicated as M1 in the right columns. (B, D) Cells in the subdiploid fraction were collected by the gate for M1, and the FITC intensities of cdk4, p16 and cdk4dn in the collected cells were analyzed.

fore, upregulation of cdk4 and cyclin D1 seems to be a cause, rather than a consequence, of apoptosis in PC12 cells.

3.6. Cdk4 kinase activity

We next examined cdk4 kinase activity by sorting cells with a higher expression of introduced gene products. Cdk4 kinase activities in cells overexpressing cyclins A, B1 or E, cdc2 or cdk2 were similar to those of parental and vector-transfected cells (Fig. 7A). In cdk4-expressing cells, significantly higher cdk4 kinase activity (4.8-fold of parental cells) was present. In addition, cyclin D1-expressing cells manifested higher cdk4 kinase activity (3.1-fold of parental cells) (Fig. 7A). When the cells co-transfected with cdk4/p16 after sorting by higher cdk4 expression were examined, cdk4 kinase activity was still high, 2.2-fold of vector-transfected cells, probably due to the mix-

ture of p16-positive and -negative fractions in cdk4-expressing cells (Fig. 7B). However, when co-transfected cells were sorted with high p16 expression, kinase activity was as low as 0.3-fold of control cells. Thus, inhibition of cdk4-induced apoptosis correlated with inhibition of cdk4 kinase activity.

3.7. Analysis of G1-arrest by BrdU incorporation

To investigate whether apoptosis induced by cdk4/cyclin D1 is caused by or associated with G1-arrest, the relationship between apoptosis and BrdU incorporation was analyzed by immunofluorescence. In vector-transfected cells, 78% of cells showed incorporated BrdU (data not shown). When we analyzed cdk4- or cyclin D1-overexpressing cells, most of which revealed morphological changes of apoptosis in the nuclei, 88% or 91% of those showed positive reactivity by anti-

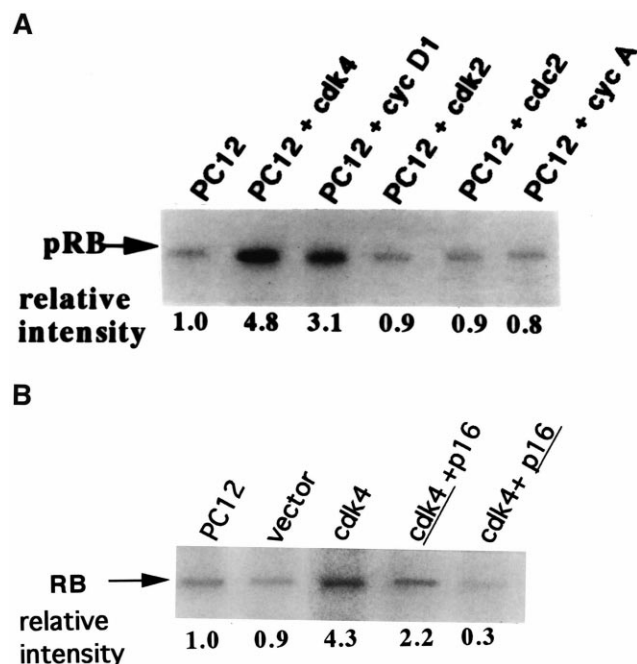


Fig. 7. Kinase activities associated with cdk4. A: Cdk4-associated kinase activity in parental cells ('PC12') and cells expressing cdk4 ('PC12+cdk4'), cyclin D1 ('PC12+cyc D1'), cdk2 ('PC12+cdk2'), cdc2 ('PC12+cdc2') and cyclin A ('PC12+cyc A') is shown. B: Cdk4 kinase activity in co-transfected cells with cDNAs of cdk4 and p16 after sorting cells by anti-cdk4 (cdk4/p16) or anti-p16 (cdk4/p16) antibodies in relation to parental cells ('PC12'), vector-transfected cells (100 µg, 'vector') and cells expressing cdk4 (100 µg, 'cdk4') is shown. The intensities of the radioactive signals were expressed as ratios relative to that obtained in parental cells.

BrdU staining, respectively (Fig. 8). Similarly, in cdk2-over-expressing cells, although they did not show morphological change of apoptosis, 83% showed BrdU incorporation. In contrast, in p16-expressing cells, only 17% showed BrdU incorporation, suggesting G1-arrest caused by overexpressed p16 protein (Fig. 8). These results indicate that apoptosis induced by cdk4/cyclin D1 is not associated with G1-arrest.

Recent reports have shown that the 'inappropriate' activation of cyclins and cdk4 is a critical event during apoptosis in neuronal cells [3,7,15]. We previously demonstrated that cyclins and cdk4 differ in their abilities to induce apoptosis and concluded that cdk4 is a critical regulator of apoptosis in PC12 cells [7]. However, there are several reports implicating cdc2, cyclins B and E, in the apoptosis of neuronal cells [15,16]. The reason for these diverse results seems to be that experiments were performed under non-physiological conditions: (i) the establishment of cell lines constitutively over-expressing a potential apoptosis-inducing protein [7], (ii) induction of apoptosis by various triggers, such as serum withdrawal [7,15], or exposure to anti-cancer drugs [17–19]. In these cases, it is uncertain whether the upregulations of cyclin/cdk are primary events directly involved in induction of apoptosis. In turn, the involvement of cyclins/cdk complexes in apoptosis has been suggested by studies using cdk inhibitors. For example, flavopiridol and olomoucine provide long-term rescue of neuronal cells from apoptosis [16,18,19]. However, to our knowledge, the direct effect of cyclins/cdk4 on apoptosis has not been clearly demonstrated. Herein, we examined apoptosis in PC12 cells: (i) cultured in the presence

of serum, (ii) via transient assay and (iii) by specifically analyzing cells overexpressing the protein of interest by cell sorting. Since the cell properties of PC12 cells and culture conditions have been well characterized, the experimental conditions used in the present study are relatively close to physiological conditions [3,7,8,16]. Our results suggest that upregulation of cdk4 activity plays a causative role in initiating apoptosis in PC12 cells, probably by directing the cells to early cell cycle transition, rather than leaving the cell in G1-arrest, as was observed in the experiment of BrdU incorporation [3,5–7]. This interpretation is supported by the previous notion that apoptosis in PC12 could occur at any phase of the cell cycle [20]. However, these effects seem to be specific for cdk4 and cyclin D1, since overexpression of the other proteins did not cause apoptosis, and this apoptosis was inhibited by simultaneous introduction of p16^{INK4A}, or cdk4dn.

The amount of both endogenous cdk4 and cyclin D1 may be mutually limiting for formation of active cdk4/cyclin D1 complex, and ectopic expression of cdk4 or cyclin D1 may drive the formation of active cdk4/cyclin D1 complexes, leading to the induction of apoptosis. Thus, the cdk4 kinase complex may function in a dose-dependent manner and show the differential effects on cells: endogenous levels of cdk4/cyclin D1 are necessary to promote cell proliferation, whereas overexpression induces apoptosis even under otherwise physiological conditions. This idea is supported by the results in image analysis.

The physiological substrate of cdk4/cyclin D1 is presumed to be the underphosphorylated, active form of pRB [1,2], a protein that plays a crucial role in protecting cells from apo-

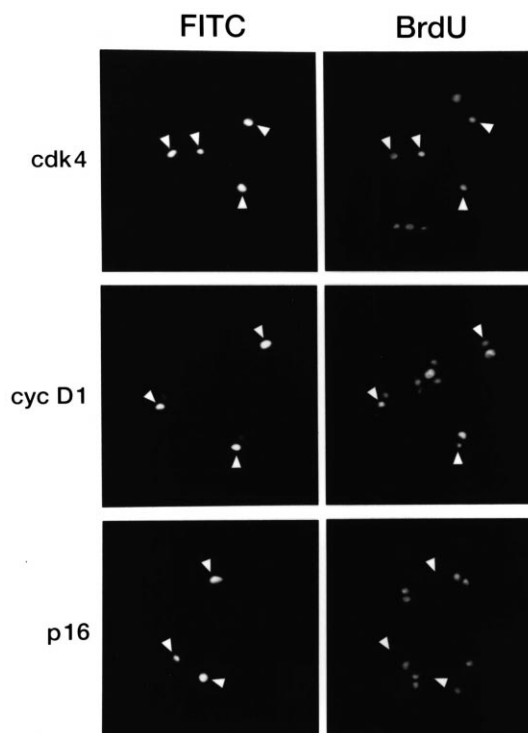


Fig. 8. Effect of protein overexpression on cell cycle progression. PC12 cells were transfected with cDNA expressing cdk4, cyclin D1 or p16. At 24 h after transfection, BrdU was added to the medium. Cells were double-stained with antibodies against respective proteins ('FITC', left columns) and anti-BrdU antibody followed by rhodamine-conjugated secondary antibody ('BrdU', right columns).

ptosis [21,22]. Accordingly, ectopic overexpression of cyclin D1, HPV E7, adenovirus E1A, E2F1, or c-myc, all of which associate with and/or inactivate pRB, has been shown to induce apoptosis [3–6,23,24]. Our results may also be the result of increased pRB phosphorylation caused by higher cdk4/cyclin D1 kinase activity. However, overexpression of other major pRB kinases, cdk2 and cdc2, has not been shown to induce apoptosis in the present study even though introduction of cdk2 slightly enhanced S-phase entry in the experiment of BrdU incorporation [1,2,25,26]. One possible explanation may be that different pRB kinases phosphorylate different sites, with different functional consequences: pRB phosphorylated at Ser⁶⁰¹, Ser⁷⁷³, Ser⁷⁸⁰ and Ser⁷⁹⁵, sites of cdk4 phosphorylation, may be more effective in inducing downstream molecules in the apoptotic cascade than pRB phosphorylated by cdk2 or by cdc2 [27–29]. Finally, the possibility still remains that other critical regulators of apoptosis exist downstream of cdk4/cyclin D1.

In summary, we have demonstrated that overexpression of cdk4 or cyclin D1 induces apoptosis in PC12 cells in the presence of serum. Since it may be attributable to inactivation of pRB, a detailed analysis of the status of pRB and the cloning of other cdk4 substrates which regulate downstream death factors are underway in our laboratory.

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