Expression of the neural specific protein, GAP-43, dramatically lengthens the cell cycle in fibroblasts

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ABSTRACT

It has been demonstrated that during neurogenesis in the mammalian brain, cell-cycle lengthening in neuronal progenitors may cause them to switch from proliferation to neuron-generating division. However, little is known about the cellular mechanisms involved in lengthening of the cell cycle. Growth-associated protein-43 (GAP-43) is a nervous system-specific protein whose expression in proliferating neuroblasts is related to neurogenesis. In this study, we investigated the effect of GAP-43 on cell-cycle progression in transgenic fibroblast cells. Using cumulative bromodeoxyuridine labeling, cell-cycle kinetics in GAP-43-transgenic and control NIH 3T3 cells were analyzed. Our data demonstrate that expression of GAP-43 in fibroblasts results in lengthening of the cell cycle compared to control fibroblasts. The mechanism by which GAP-43 mediated this effect appeared to involve increasing the time spent by the cells in the G1 phase of the cell cycle.

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1. Introduction

Evidence increasingly suggests that cell-cycle regulation plays an important role in the differentiation of neural and other somatic stem cells (Fluckiger et al., 2006; Hodge et al., 2004; Legrier et al., 2001; Nguyen et al., 2006; Tarui et al., 2005; Iacopetti et al., 1999; Calegari et al., 2005; Georgopoulou et al., 2006; Gotz and Huttner, 2005). Several studies on cell-cycle kinetics have demonstrated that the cell cycle, in particular the G1 phase, is long in the differentiative and short in the proliferative divisions (Fluckiger et al., 2006; Calegari et al., 2005; Calegari and Huttner, 2003; Gotz and Huttner, 2005). In addition, it has been demonstrated by studies in vivo that lengthening of the G1 phase is sufficient to trigger a switch from proliferative to neuron-generating divisions in neural stem cells (Hodge et al., 2004; Calegari and Huttner, 2003). These findings raise the "cell-cycle length hypothesis" that lengthening of the G1 phase could causally contribute to neurogenesis (Hodge et al., 2004; Calegari et al., 2005; Calegari and Huttner, 2003; Gotz and Huttner, 2005). It is already known that the lengths of the cell cycle (Tc) and the G1 phase (Tg1) are regulated by the concerted actions of cyclins, cyclin-dependent kinases (CDK) and their inhibitors (Nguyen et al., 2006; Tarui et al., 2005). A major cell-cycle restriction point is located at the end of the G1 phase. The CDK inhibitor, p27kip1, is a critical negative modulator of the G1- to S-phase transition that binds to cyclin E:CDK2 and inhibits its activity (Nguyen et al., 2006; Tarui et al., 2005). It is tempting, therefore, to speculate that up-regulation of p27kip1 might contribute to the prolonged Tc1 observed during the process of neuronal determination/differentiation. However, data from a transgenic mouse model show that overexpression of p27kip1 increases the number of cells exiting the cell cycle (Tc) but not Tg1 (Tarui et al., 2005). Therefore, the relationship between the control of cell-cycle progression and neuronal differentiation is complex. Recent studies have identified a number of key factors controlling neural cell fate determination. For example, Tis21 and BM88, which can inhibit G1 progression, are selectively expressed during neurogenerating divisions (Iacopetti et al., 1999; Georgopoulou et al., 2006). This implicates that certain differentiation-inducing molecules are beginning to emerge as cell-cycle regulators at the onset of neural cell fate determination/differentiation.

The nervous system-specific protein, GAP-43, is a major component of intracellular signaling pathways (Benowitz and Routtenberg, 1997; Nakamura et al., 1998; Esdar et al., 1999). However, its complete spectrum of functions in the nervous system remains unclear. It has been reported that GAP-43 begins to be expressed in the central nervous system only after final cell division (Palacios et al., 1994). It is widely believed, therefore, that the function of GAP-43 is particularly related to axonal growth and nerve terminal plasticity (Benowitz and Routtenberg, 1997; Palacios et al., 1994). Recent studies have demonstrated, however, that GAP-43 is already expressed in proliferating neuroblasts (Esdar et al., 1999; Mani et al., 2001; Shen et al., 2004; Stricker et al., 2006). It was recently shown that GAP-43 is a molecular marker that correlates with the horizontal divisions (neuron-generating divisions) of neuronal progenitors may cause them to switch from proliferation to neuron-generating division. However, little is known about the cellular mechanisms involved in lengthening of the cell cycle. Growth-associated protein-43 (GAP-43) is a nervous system-specific protein whose expression in proliferating neuroblasts is related to neurogenesis. In this study, we investigated the effect of GAP-43 on cell-cycle progression in transgenic fibroblast cells. Using cumulative bromodeoxyuridine labeling, cell-cycle kinetics in GAP-43-transgenic and control NIH 3T3 cells were analyzed. Our data demonstrate that expression of GAP-43 in fibroblasts results in lengthening of the cell cycle compared to control fibroblasts. The mechanism by which GAP-43 mediated this effect appeared to involve increasing the time spent by the cells in the G1 phase of the cell cycle. 

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neuronal precursors during mammalian neurogenesis (Shen et al., 2004; Stricker et al., 2006). This neuronal differentiation was shown to be induced both in vitro and in vivo by the absence of GAP-43 expression, suggesting that GAP-43 is required for appropriate cell fate commitment during neurogenesis (Mani et al., 2001; Shen et al., 2004; Shen et al., 2008). This raises the issue of whether the differentiation-inducing activity of GAP-43 may influence cell-cycle progression, in particular by lengthening the G1 phase. To clarify this issue, we investigated whether GAP-43 may affect cell-cycle progression in a transgenic fibroblast cell line expressing GAP-43.

2. Materials and methods

2.1. Antibodies

The GAP-43 monoclonal antibody used for immunofluorescence and Western blotting analysis was purchased from Sigma–Aldrich. In the cell-cycle analysis experiments, BrdU was detected with a BrdU monoclonal antibody (Sigma–Aldrich). Anti-α-tubulin antisera and anti-actin antisera were both from Sigma–Aldrich. Anti-p27Kip1 polyclonal antibody was from Abcam. Anti-phosphohistone H3 (pH3) antibody (H3 9701) was from Cell Signaling Technology. Anti-mouse and anti-rabbit secondary antibodies conjugated-Alexa Fluor 594 (red) and Alexa Fluor 488 (green) were from Invitrogen.

2.2. Cell culture

NIH 3T3 and PT67 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen), low-glucose, supplemented with 10% fetal bovine serum (FBS, Invitrogen).

2.3. Plasmid construction and transfection

A full-length cDNA encoding rat GAP-43 was inserted into the plasmid pLNCX2. The plNCX2-GAP-43 and empty plNCX2 vectors were transfected into PT67 cells with Lipofectin. The transfected cells were selected in medium containing 400 μg/ml of G418 (invitrogen) to amplify and produce the retroviral in high-titer, which was then used to infect the NIH 3T3 cells.

2.4. Immunocytochemistry

Cells were washed twice with PBS and fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature. Fixed cells were incubated at room temperature for 25 min in PBS containing 0.1% Triton X-100, and then for 1 h in blocking solution (5% goat serum in PBS). The cells were then incubated with GAP-43 antibody or BrdU antibody (Sigma–Aldrich, 1:1000 dilution) overnight at 4°C. After further PBS washes, the cells were incubated for 1 h with goat anti-mouse secondary antibody conjugated with Alexa Fluor–488 (Invitrogen, 1:300) in the dark at room temperature. Coverslips were rinsed with PBS and mounted onto glass slides with 50% glycerin mounting media. Photographs were taken under a Leica DM 4000B microscope.

2.5. Western blot analysis

Cells were cultured for 48 h, washed twice with PBS, harvested with a cell scraper, and resuspended in homogenization buffer [50 mM Tris–Cl (pH 7.5) containing 2 mM dithiothreitol (DTT), 2 mM EDTA, 2 mM EGTA, 50 mM 4-(2-aminoethyl)-benzenesulfonfonylfluoride hydrochloride, 5 μg/ml each of leupeptin, aprotinin, pepstatin A and chymostatin, 50 mM potassium fluoride (KF), 50 mM okadaic acid, 5 mM sodium pyrophosphate, 2% sodium dodecyl sulfate (SDS)]. Cells were sonicated to completely dissolve the cell pellet. The protein concentration of each sample was determined using a BCA kit (Pierce, USA). Protein samples (30 μg) were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes (Schleicher and Schuell, USA), which were then blocked in 10 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.1% Tween 20 for 1 h. The membranes were then incubated with anti-GAP-43 monoclonal antibody at 1:3000 or anti-p27Kip1 antibody at 1:2000 in TBST for 3 h, rinsed three times with TBST, and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, USA) at 1:5000 in TBST for 1 h. After incubating in ECL Western blotting reagent (Perkin-Elmer Life Science, USA), the membranes were exposed to ECL Hyperfilm (Amerham Biosciences). The amount of protein was determined densitometrically using Quantity One software (Gel Doc 2000 Image System, Bio-Rad Inc., USA).

2.6. Cell synchronization

Synchronization of cells was accomplished by the starve-block method. Cells were blocked in the G0 phase by incubation in DMEM containing 1% FBS for 48 h.

2.7. Cell proliferation assays

Cell proliferation was measured using the MTT cell viability assay. The cells were plated in a 96-well plate at a density of 2 × 103 cells/well, synchronized for 48 h and then returned to normal culture medium. After growth at each time point (0, 12, 24, 36, 48, 72 and 84 h), the cell proliferation was assessed by colorimetric measurement of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT). Growth rates were determined by calculating the increase in absorbance at each time point compared to that at time = 0 h. The experiment was repeated in triplicate.

2.8. Cell-cycle analysis

Cumulative BrdU labeling was used to measure the length of cell cycle and S-phase (Fluckiger et al., 2006; Alexiades and Cepko, 1996). Four hours after plated in 24-well plates (1 × 104 cells/well), BrdU (Sigma) was added to the 10% FBS medium to a final concentration of 10 μM for the indicated times (2, 4, 6, 8, 10, 12, 16, 20, 28 h). At the end of each time point, cells were fixed with 4% paraformaldehyde (PFA). For BrdU immunodetection, cells were treated with 4N HCl for 4 min to denature DNA, neutralized with sodium borate, and then processed for immunostaining as described above. Cell-cycle kinetics, i.e., the total length of the cell cycle, length of the S-phase and the growth fraction (GF, maximum labeling index), were calculated from the cumulative labeling index (LI) values. The percentage of labeled mitotic figures (PLM labeling) (Fluckiger et al., 2006; Hodge et al., 2004; Alexiades and Cepko, 1996; Calegari et al., 2005) was used to determine the G2/M duration. Forty-eight hours after plating, cultures were pulse-labeled for 1 h with BrdU (30 μM). After incubation for different time periods (1, 2, 3, 4, 5, 5.5, 5.75, 6 h), cultures were fixed in 4% PFA and immunolabeled with primary antibodies, mouse anti-BrdU (Sigma–Aldrich, 1:1000) and rabbit anti-phosphohistone H3 (Cell Signaling Technology, 1:100), followed by anti-mouse (594) and anti-rabbit (488) secondary antibodies (Invitrogen, 1:300). The length of G2/M phase of the cell cycle was determined as the time required to labeling all mitotic figures.

Mitotic index (MI), defined as the proportion of the total cell population in mitosis, was determined for both transgenic and control cells following 48 h in culture. Mitotic figures were counted following immunofluorescence cell staining with an antibody to α-tubulin (1:1000) and Hoechst 33258 (Merck, 200 ng/ml).

2.9. Statistical analysis

The data are given as mean ± S.E.M. The effects of GAP-43 on cell growth rate were assessed using t-test. Two-way ANOVA was used to analysis the PLM labeling between transgenic and control cell groups. A P-value less than 0.05 was assumed to denote a significant difference and levels of significance are indicated by symbols: *P < 0.05, **P < 0.01.

3. Results

3.1. Identification of GAP-43 expression in transgenic NIH 3T3 cells

Following NIH 3T3 cell transduction with plNCX2-GAP-43 and the empty vector plNCX2, and subsequent selection in G418-containing culture medium for 3 weeks, Western blotting and immunocytochemistry were used to detect the expression of GAP-43 protein. Expression of GAP-43 in the GAP-43-transgenic cells was confirmed by Western blotting, which also confirmed the absence of GAP-43 expression in the control cells (expressed the empty vector) (Fig. 1A). Immunocytochemistry also showed that GAP-43 expression was readily detectable in GAP-43-trangenic cells but was absent in the control cells (Fig. 1B and C). The GAP-43 protein was strongly expressed in the cells and often associated with varicosities and the plasma membrane of processes (Fig. 1C). The cells with GAP-43 expression appeared with ruffled membranes and numerous filopodia or long, thin processes (Fig. 1C).

3.2. Effect of GAP-43 on cell morphology and p27Kip1 expression

Normally, the NIH 3T3 cell is from the fibroblast cell line, which usually exhibits a bipolar or shuttle-like shape with two processes. In our study, it was shown under light microscopy that the GAP-43-transgenic cells appeared with larger and thin cell body, ruffled membranes, and thin processes or numerous filopodia, while majority of control cells exhibit typical bipolar shape (Fig. 2A and B). Expression level of p27Kip1 in the GAP-43-transgenic cells and
Fig. 1. Analysis of GAP-43 protein expression. Western blot (A) of cell extracts prepared from 3T3 fibroblasts stably-expressing and control cells (infected with empty vector). β-Actin were used as a positive control of GAP-43 expression. The Immunocytochemical characterization of GAP-43 expression in control (B) and transgenic (C) cells, demonstrating that sustained expression of GAP-43 in transgenic 3T3 cells. Scale bar: 50 μm.

Fig. 2. Effect of GAP-43 on cell morphology and p27kip1 expression. Light microscope image of control (A) and GAP-43-transgenic cells (B) demonstrate the effect of GAP-43 expression on cell morphology. Scale bar: 20 μm. Western blot (C) demonstrate the p27kip1 expression level of transgenic and control cells (P > 0.05). Error bar represents S.E.M. of three independent experiments.
control cells were detected by Western blotting, which demonstrated that there is no significant difference between GAP-43 expressing and control cells (Fig. 2C).

3.3. Effect of GAP-43 on cells growth rate

Growth curves for both GAP-43-transgenic (wild type) cells and control cells (empty vector), cultured in normal serum-containing growth medium (Fig. 3A), showed that they all exhibited exponential proliferation but with the appreciable differences in their growth rates. In the early parts of the growth curves (≤36 h), no difference in the growth rate of the GAP-43-transgenic (wild type) and control cells (empty vector) was observed. For GAP-43-expressing (wild type) cells, however, an obvious arrest in growth rate occurred after 48 h. We also established another control with NIH 3T3 cells transfected with pseudo-phosphorylated form of GAP-43, Asp41 mutant protein (data not shown). The results of proliferation assay showed that GAP-43 mutant expressing has no effect on slowdown growth rate of the NIH 3T3 cells (Fig. 3B).

3.4. Tc is increased in transgenic cells

Detailed analyses of cell-cycle kinetics were performed in the GAP-43 expressing cells and control cells. To determine whether GAP-43 influences overall cell-cycle length, a cumulative BrdU-labeling protocol was used to generate LI values for the transgenic cells and control cells. In these experiments, the data set indicating the change in labeling over time (from 2 to 28 h post BrdU application) was used to determine the regression line (Fig. 4). The r² values (GAP-43-expressing cells: r² = 0.9817; control cells: r² = 0.9636) indicated the significance of closeness between experimental and expected values. In this plot (Fig. 3), GF, Tc, and Tg can be calculated from the equation (y-intercept = Tg/ Tc × GF) and from the time at reaching the inflection point, which is Tc – Tg (i.e., at Tc – Tg, LI = GF). Individual phase lengths for Tc and Tg (Table 1) were derived from the graphs of LI versus time in culture (Fig. 4) and the equations cited above. Consequently, the time for GAP-43 expressing cells to reach GF increased significantly compared to control groups. This suggested that the length of one or more phases of the cell cycle was increased by GAP-43 expression. In addition, the maximum LI value (GF) of transgenic cells was higher than control cells.

3.5. Tg2/M is normal in GAP-43-transgenic cells

The combined length of the G2 and M phases of the cell cycle was determined by the PLM method (Fluckiger et al., 2006; Hodge et al., 2004; Alexiades and Cepko, 1996; Calegari et al., 2005). After exposure to BrdU, the cells were incubated for 1.0, 2.0, 3.0, 4.0, 5.0, 5.5, 5.75 or 6.0 h, and the labeled mitotic cells were identified by co-labeling with phosphohistone H3 antibody (Fig. 5). The percentage of labeled mitotic figures (PH3 positive) labeled with BrdU of the GAP-43-transgenic and control cells. The plots illustrate the linear advance of LI toward maximum levels in transgenic (open circle) and control (filled circle) cells as the number of BrdU applications increased over the experimental period. As maximum labeling is reached, LI values level off accordingly in each group. LI values plateau at an earlier time in control cells, indicating that LI progresses to a maximum at a faster rate in control cells and that Tc – Tg is increased in transgenic cells. Results are presented as mean of three independent experiments.

### Table 1

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<th></th>
<th>Tc (h)</th>
<th>Tg (h)</th>
<th>GF</th>
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<tr>
<td>Controls cells</td>
<td>17.41</td>
<td>5.31</td>
<td>89.39</td>
</tr>
<tr>
<td>Transgenic cells</td>
<td>25.47</td>
<td>5.55</td>
<td>89.39</td>
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**P < 0.05.**  
***P < 0.01.
from S-phase to G₂-phase. The data showed that 0% of the mitotic figures were labeled by BrdU at 5.5 h, while nearly 100% of the mitotic figures were labeled at 6 h. Statistical analysis showed that the slopes were identical \( (F = 1.52, P > 0.05) \). Therefore, \( T_{\text{M}} \) was about 0.5 h in both transgenic and control cells. Individual estimates of \( T_{\text{G2}} \), which were determined by subtracting \( T_{\text{M}} \) values from \( T_{\text{G2/M}} \) at 6 h, did not differ greatly between transgenic and control cells.

### 3.6. Reduced mitotic index in GAP-43 expressing cells

Another method to estimate \( T_{\text{M}} \) is to calculate the mitotic index (MI). If the proliferating cells are asynchronously distributed through the cell cycle, the number of cells in mitosis (\( N_{\text{M}} \)) as a fraction of the total cell population (\( N_{\text{T}} \)) will equal \( T_{\text{C}} \) divided by \( T_{\text{C}} \). In other words, \( T_{\text{M}} \) can be computed by the formula \( T_{\text{M}} = T_{\text{C}} \times N_{\text{M}}/ N_{\text{T}} \). The MI data showed a decrease of 50% in transgenic cells (1.463 ± 0.425%) compared with the controls (2.845 ± 0.833%) (Fig. 7). However, the computed results showed that \( T_{\text{M}} \) values were quite similar in transgenic and control cells (29.49 ± 6.50 and 22.37 ± 8.52 min, respectively, \( P > 0.05 \)), indicating that \( T_{\text{M}} \) did not differ substantially between the groups. These data confirmed the findings of the PLM experiments. Individual estimates of \( T_{\text{M}} \) and \( T_{\text{G2}} \) were not substantially different in transgenic and control cells.

### 3.7. G₁ phase lengthening accounts for increased \( T_{\text{C}} \) in GAP-43 expressing cells

Data from the cumulative and PLM experiments demonstrated that \( T_{\text{C}} \) was increased in transgenic cells, whereas \( T_{\text{S}} \) and \( T_{\text{G2/M}} \) were invariant. Using the equation, \( T_{\text{G1}} = T_{\text{C}} - (T_{\text{S}} + T_{\text{G2/M}}) \), \( T_{\text{G1}} \) was calculated in control and GAP-43-transgenic cells and it was shown that \( T_{\text{G1}} \) was nearly 8 h longer in GAP-43 expressing cells compared with the controls. This lengthening in \( T_{\text{G1}} \) accounts for the observed lengthening of \( T_{\text{C}} \) in transgenic cells. Therefore, the lengthening of the G₁ phase in transgenic cells is attributable entirely to the increase of cell-cycle length.

### 4. Discussion

Many studies have demonstrated that GAP-43 has important functions in postmitotic cells (neurons) (Benowitz and Routtenberg, 1997; Esdar et al., 1999), but the role of its expression in proliferating cells has not yet been determined. As recent reports, GAP-43 already expressed at early stages of neuronal commitment; when GAP-43 is absent, neural differentiation in vitro and in vivo is not initiated appropriately (Mani et al., 2001; Mishra et al., 2008; Shen et al., 2004; Shen et al., 2008). These results indicate a causative link between GAP-43 and neural cell fate determination. However, there are still important questions regarding the mechanism of the GAP-43 as an important determinant that...
It has long been known that cell-cycle arrest in G1 is sufficient to determine the cell fate (Hodge et al., 2004; Calegari and Huttner, 2003). So it has been speculated that a number of key factors implicated in cell fate determination might emerge as cell-cycle regulators at the onset of neural cell fate determination (Georgopoulou et al., 2006). One approach to demonstrating a direct effect of GAP-43 on cell-cycle progression would be to express GAP-43 gene in proliferating cells, which do not normally express GAP-43. We employed retroviral vectors to mediate sustained expression of GAP-43 in NIH 3T3 cells, which was usually used for studies of cell proliferation and cell-cycle regulation. The NIH 3T3 cells expressing GAP-43 have been used for studies for the cellular mechanism of the regulation of neurite outgrowth and the degradation of GAP-43 protein (De Moliner et al., 2005). Our results show that expression of GAP-43 in fibroblasts promoted a striking change in their morphology, consistent with previous reports that GAP-43 regulates neurite outgrowth (De Moliner et al., 2005; Verhaagen et al., 1994). This indicates that we have successfully established GAP-43 expressing cell model. Next, we compared the growth rate of the transgenic cells with that of control cells and demonstrated that GAP-43 expression slowed the growth rate of NIH 3T3 cells.

The two cytokinetic parameters, growth fraction (GF) and overall length of the cell cycle ($T_C$), are critical determinants of cell growth rate (Alexiades and Cepko, 1996). GF represents the proportion of actively proliferating cells within the total cell population. A reduction in GF may cause the growth rate of a cell population to slow down, because the number of actively proliferating cells is fewer. On the other hand, the growth rate of proliferative cells is primarily dependent on the length of the cell cycle. In normally proliferating cells, both decreased GF and increased cell-cycle length may lead to cell growth rate arrest. Our data from cytokinetic analysis indicate that GAP-43 acts to slow down the rate of cell proliferation by lengthening $T_C$, rather than decreasing GF. The results of a previous study showed that the absence of GAP-43 also led to an increase in cell–cycle length (Mishra et al., 2008). This increase was particularly due to S-phase lengthening, which was a consequence of arrest at the transition from S-phase to G2/M phase (Mishra et al., 2008). The lengthening of the cell cycle observed in the present study seems due mainly to an increase in the length of the G1 phase, while the duration of the S and G2/M phases changed little. Many reports suggest that GAP-43 is involved in the regulation of cell-cycle progression (Mani et al., 2001; Mishra et al., 2008;...
Shen et al., 2004). In addition, the data presented here also show that GF is increased in cells expressing GAP-43, suggesting that GAP-43 lengthens the cell cycle but does not arrest it (i.e. decrease the proportion of cycling cells). As we know, the p27Kip1 is the most important CKI for controlling the G1 to S-phase transition and therefore it has been speculated to be involved in prolonging T_{G1} (Nguen et al., 2006; Tarui et al., 2005). The results of Western blot in our study did not show any difference of p27Kip1 expression levels between GAP-43 expressing and control cells. As previous finding, overexpression of p27Kip1 increases the number of cells exiting the cell cycle (Q) but not T_{G1} (Tarui et al., 2005). Combined with our result, it suggests therefore that GAP-43 may influence cell-cycle progression through another pathway independent of the p27Kip1.

It is known that GAP-43 interacts with several components of signal transduction pathways, including PKC and calmodulin (Benowitz and Routtenberg, 1997; Nakamura et al., 1998; Esdar et al., 1999). In its unphosphorylated state, GAP-43 can bind calmodulin in the absence of Ca^{2+}, while PKC is able to phosphorylate GAP-43 at serine residue 41 (Benowitz and Routtenberg, 1997). It has been demonstrated that up-regulation of MARCKS (another major PKC substrate) expression in dividing cells prompts a lowering of the free calmodulin concentration and thus inhibits cell-cycle progression at the G1/S boundary (Herget et al., 1993). This suggests that the levels of GAP-43-like proteins are inversely correlated to cell-cycle progression (Herget et al., 1993). On the other hand, our result from cell proliferation assay demonstrated that pseudo-phosphorylated form of the protein (Asp41 mutant GAP-43) had no effect on cell proliferation rate, which also implied the suggestion that the GAP-43 might involve in regulating cell-cycle progression through calmodulin.

According to previous studies of neural development, the expression of GAP-43 is gradually increased from the onset of neurogenesis (Benowitz and Routtenberg, 1997; Legrier et al., 2001; Mani et al., 2001; Shen et al., 2004). Here, we have demonstrated that GAP-43 markedly lengthens the cell cycle by extending the G1 phase, further supporting the ‘cell-cycle length hypothesis’ of neuronal cell fate determination. It is possible therefore that increase in the length of the G1 phase by GAP-43 might be prerequisite for promoting neuronal differentiation.

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References


Legrier, M.E., Ducray, A., Propper, A., Chao, M., Kastner, A., 2003. Selective lengthening of T_{G1} phase transition and therefore it has been speculated to be involved in prolonging T_{G1} (Nguen et al., 2006; Tarui et al., 2005). The results of Western blot in our study did not show any difference of p27Kip1 expression levels between GAP-43 expressing and control cells. As previous finding, overexpression of p27Kip1 increases the number of cells exiting the cell cycle (Q) but not T_{G1} (Tarui et al., 2005). Combined with our result, it suggests therefore that GAP-43 may influence cell-cycle progression through another pathway independent of the p27Kip1.

It is known that GAP-43 interacts with several components of signal transduction pathways, including PKC and calmodulin (Benowitz and Routtenberg, 1997; Nakamura et al., 1998; Esdar et al., 1999). In its unphosphorylated state, GAP-43 can bind calmodulin in the absence of Ca^{2+}, while PKC is able to phosphorylate GAP-43 at serine residue 41 (Benowitz and Routtenberg, 1997; Nakamura et al., 1998; Esdar et al., 1999). Phosphorylation of GAP-43 at Ser41 can release calmodulin and prevent calmodulin from binding to it (Benowitz and Routtenberg, 1997; Nakamura et al., 1998; Esdar et al., 1999). It has been proposed that GAP-43 could serve as calmodulin ‘sponge’ to sequestering calmodulin (Benowitz and Routtenberg, 1997). It has been demonstrated that up-regulation of MARCKS (another major PKC substrate) expression in dividing cells prompts a lowering of the free calmodulin concentration and thus inhibits cell-cycle progression at the G1/S boundary (Herget et al., 1993). This suggests that the levels of GAP-43-like proteins are inversely correlated to cell-cycle progression (Herget et al., 1993). On the other hand, our result from cell proliferation assay demonstrated that pseudo-phosphorylated form of the protein (Asp41 mutant GAP-43) had no effect on cell proliferation rate, which also implied the suggestion that the GAP-43 might involve in regulating cell-cycle progression through calmodulin.

According to previous studies of neural development, the expression of GAP-43 is gradually increased from the onset of neurogenesis (Benowitz and Routtenberg, 1997; Legrier et al., 2001; Mani et al., 2001; Shen et al., 2004). Here, we have demonstrated that GAP-43 markedly lengthens the cell cycle by extending the G1 phase, further supporting the ‘cell-cycle length hypothesis’ of neuronal cell fate determination. It is possible therefore that increase in the length of the G1 phase by GAP-43 might be prerequisite for promoting neuronal differentiation.

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