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Cytoplasmic p21$^{Cip1/WAF1}$ regulates neurite remodeling by inhibiting Rho-kinase activity

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p21$^{Cip1/WAF1}$ has cell cycle inhibitory activity by binding to and inhibiting both cyclin/Cdk kinases and proliferating cell nuclear antigen. Here we show that p21$^{Cip1/WAF1}$ is induced in the cytoplasm during the course of differentiation of chick retinal precursor cells and N1E-115 cells. Ectopic expression of p21$^{Cip1/WAF1}$ lacking the nuclear localization signal in N1E-115 cells and NIH3T3 cells affects the formation of actin structures, characteristic of inactivation of Rho. p21$^{Cip1/WAF1}$ forms a complex with Rho-kinase and inhibits its activity in vitro and in vivo. Neurite outgrowth and branching from the hippocampal neurons are promoted if p21$^{Cip1/WAF1}$ is expressed abundantly in the cytoplasm. These results suggest that cytoplasmic p21$^{Cip1/WAF1}$ may contribute to the developmental process of the newborn neurons that extend axons and dendrites into target regions.

Introduction

A key event during terminal differentiation is a permanent withdrawal from the cell cycle. Much attention has focused on regulation of components known to control progression through the cell cycle, including cyclins, cyclin-dependent kinase (Cdk)* proteins, and Cdk inhibitors. The p21$^{Cip1/WAF1}$ gene was identified through the interaction with Cdk2 (Harper et al., 1993), and its expression is induced by activation of wild-type p53 (el-Deiry et al., 1993), and during cellular senescence (Noda et al., 1994) and differentiation (Jiang et al., 1994). An NH_2-terminal domain of p21$^{Cip1/WAF1}$ inhibits cyclin-Cdk kinases and a COOH-terminal domain of p21$^{Cip1/WAF1}$ inhibits proliferating cell nuclear antigen (Waga et al., 1994; Chen et al., 1995; Luo et al., 1995; Sherr and Roberts, 1995). These cell cycle inhibitory activities of p21$^{Cip1/WAF1}$ are attributable to its nuclear localization (Goubin and Ducommun, 1995; Sherr and Roberts, 1995). However, recent studies provide evidence that p21$^{Cip1/WAF1}$ has other biological activities in the cytoplasm. During the process of monocytic differentiation of U937 cells and HL60 cells by the treatment with vitamin D3, p21$^{Cip1/WAF1}$ expression was induced in the cytoplasm and this cytoplasmic p21$^{Cip1/WAF1}$ forms a complex with the apoptosis signal-regulating kinase 1 and inhibits the stress-activated MAPK cascade, thus contributing to the acquisition of resistance to various apoptogenic stimuli (Asada et al., 1999). Cytoplasmic localization of p21$^{Cip1/WAF1}$ was also observed in peripheral blood monocytes (Asada et al., 1999). Several reports propose possible mechanisms of translocation of p21$^{Cip1/WAF1}$ from the nucleus to the cytoplasm. It is reported that phosphatidylinositol-3 kinase/Akt phosphorylates threonine 145 in COOH-terminal NLS of p21$^{Cip1/WAF1}$ and phosphorylated p21$^{Cip1/WAF1}$ loses its ability to localize in the nucleus (Zhou et al., 2001). Another paper shows that truncation of the COOH-terminal of p21$^{Cip1/WAF1}$ by a member of the caspase family of proteases results in the loss of its NLS and the localization changes (Levkau et al., 1998).

During the course of differentiation of the neuronal cells, p21$^{Cip1/WAF1}$ also plays important roles in regulating the cell cycle. In several cell lines during differentiation after nerve growth factor treatment, the expression of p21$^{Cip1/WAF1}$ protein was increased (Decker, 1995; Dobashi et al., 1995; Yan and Ziff, 1995; Poluha et al., 1996; van Grunsven et al., 1996; Gollapudi and Neet, 1997; Erhardt and Pittman, 1998). However, neurons after differentiation seem to have special features, distinct from other cell types, as newborn neurons extend axons and dendrites to communicate with

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*Abbreviations used in this paper: Cdk, cyclin-dependent kinase; E, embryonic day.

Key words: p21$^{Cip1/WAF1}$; Rho-kinase; neurite outgrowth; differentiation; Rho

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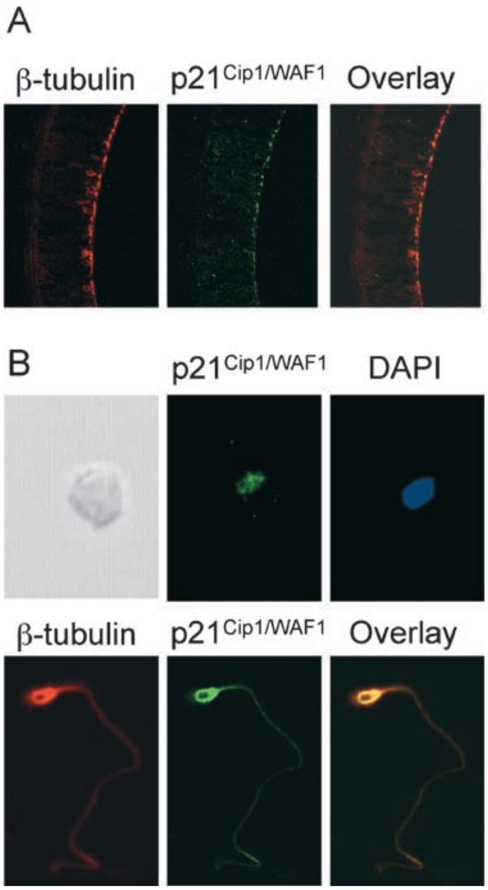

Figure 1. **Chick retinal neurons from E5 embryos display cytoplasmic p21$^{Cip1/WAF1}$ expression.** (A) Chick retinas from E5 embryos were immunostained with the anti-p21$^{Cip1/WAF1}$ antibody. In every panel, the right side is the vitreous body and the left side is the pigment epithelium. (B) p21$^{Cip1/WAF1}$ immunoreactivity in chick dissociated retinal cells from E5 embryos. The top panels are the cells devoid of β-tubulin immunoreactivity, and the bottom panels are the neurons.

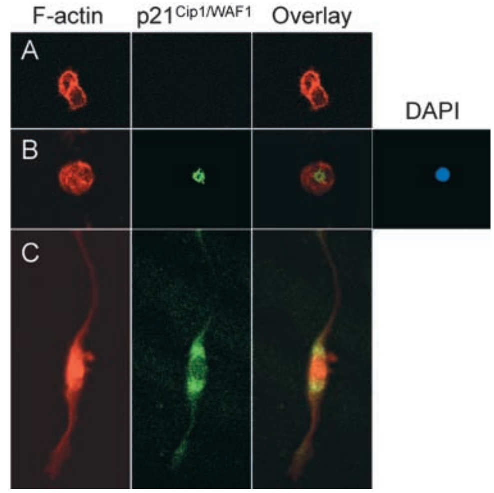

Figure 2. **Subcellular localization of p21$^{Cip1/WAF1}$** in DMSO-induced differentiating N1E-115 cells. (A–C) Immunocytochemical staining of p21$^{Cip1/WAF1}$ with the anti-p21$^{Cip1/WAF1}$ antibody. Representative features of N1E-115 cells incubated without DMSO (A), or with DMSO for 1 d (B), and 4 d (C).

appropriate targets. For example, dorsal root ganglion neurons up to postnatal day 3 to 4 or embryonic retinal ganglion neurons can extend their neurites rapidly on myelin-associated glycoprotein, which is an effective neurite outgrowth inhibitor for adult neurons (Johnson et al., 1989; Mukhopadhyay et al., 1994; De Bellard et al., 1996; Cai et al., 2001). These findings suggest that immature neurons may have intrinsic mechanisms that confer resistance to the inhibitory molecules.

Here we show a novel function of cytoplasmic p21$^{Cip1/WAF1}$. Cytoplasmic expression of p21$^{Cip1/WAF1}$ was observed in newborn neurons that extensively extend neurites. As p21$^{Cip1/WAF1}$ binds to Rho-kinase and inhibits its activity, changes in the cytoskeletal organization are at least partly attributable to the nonenzymatic protein inhibitor.

Results

Chick retinal neurons from E5 embryos display cytoplasmic p21$^{Cip1/WAF1}$ expression

During the period of active neurogenesis, some neuroblasts enter the postmitotic state and then start migrating to their final destination. In the embryonic chick retina, ganglion cells are actively generated around embryonic day 5 (E5) (Frade et al., 1997). We examined expression of p21$^{Cip1/WAF1}$ in these cells to test whether p21$^{Cip1/WAF1}$ was associated with differentiation and morphogenesis of these cells. Using immunohistochemistry it was found that retinal neurons immediately after neurogenesis were migrating into deep layers (Fig. 1 A). p21$^{Cip1/WAF1}$ immunoreactivity was detected in the cells at the vitreous surface of the central neural retina using a monoclonal antibody against p21$^{Cip1/WAF1}$ (Fig. 1 A). These p21$^{Cip1/WAF1}$-positive cells were immature retinal neurons before migration. Therefore, it is suggested that p21$^{Cip1/WAF1}$ is involved in the differentiation of retinal precursor cells in vivo.

Next, we isolated neural precursor cells from E5 retinas to assess more precisely the subcellular localization of p21$^{Cip1/WAF1}$. Dissociated retinal cells cultured on laminin-1 extended neurites rapidly (Frade et al., 1996b). Cells were cultured on laminin-1 in a chemically defined medium containing 1 μM insulin. Insulin used in the micromolar range is likely to be acting on insulin-like growth factor-I receptors, thus mimicking the differentiative effect of insulin-like growth factor-I on the E5 retinal cells (Frade et al., 1996a). In almost all the immature cells devoid of immunoreactivity for β-tubulin, the expression of p21$^{Cip1/WAF1}$ was predominantly seen in the nucleus (Fig. 1 B). p21$^{Cip1/WAF1}$ in the nucleus may contribute to a change in the cell cycle in these cells. On the other hand, in most neurons that had relatively long neurites with immunoreactivity for neuron-specific β-tubulin, p21$^{Cip1/WAF1}$ was mainly localized in the cytoplasm (Fig. 1 B). These findings suggest that cytoplasmic expression of p21$^{Cip1/WAF1}$ is induced in the newborn neurons.

In vitro differentiation of N1E-115 cells is associated with p21$^{Cip1/WAF1}$ expression in the cytoplasm

We next used neuroblastoma N1E-115 cells to examine whether neuronal differentiation was associated with cyto-

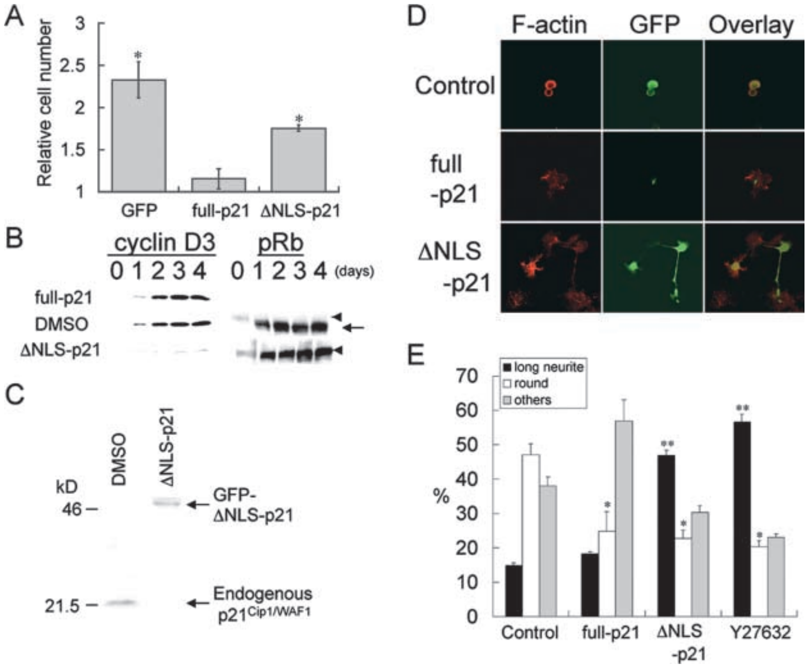

Figure 3. **Morphological changes of N1E-115 cells by overexpression of p21$^{Cip1/WAF1}$.** (A) Growth of N1E-115 cells. Cells were seeded in 6-cm dishes, transfected, and were counted 1 and 2 d after transfection. The relative increases in the number of the cells are shown. The values are means ± SEM of 3 independent experiments. *, $P < 0.01$ compared with full-p21 (Student's *t* test). There is no significant difference between GFP and GFP-ΔNLS-p21 transfected cells. (B) Western blot analysis of cyclinD3 and pRb. N1E-115 cells were treated with DMSO, or transfected with GFP-full-p21 or GFP-ΔNLS-p21, then were harvested at 1, 2, 3 and 4 d. Arrowheads indicate hyperphosphorylated pRb, and the arrow indicates underphosphorylated pRb. (C) Expression levels of p21$^{Cip1/WAF1}$ in N1E-115 cells treated with DMSO for 4 d or transfected with GFP-ΔNLS-p21. (D) N1E-115 cells were transfected with GFP (control), GFP-full-p21 or GFP-ΔNLS-p21. Shown are photomicrographs of the cells transfected with each construct. (E) Quantification of the morphology of the cells. N1E-115 cells exposed to Y-27632 (10 μM) for 30 min or expressing GFP, GFP-full-p21, or GFP-ΔNLS-p21 were categorized into three groups; the cells with long neurites (long neurite), cells with a round form (round), and cells with other forms (others). Data represent means ± SEM of three independent experiments. *, $P < 0.05$ compared with control. **, $P < 0.01$ compared with control as well as full-p21 (Student's *t* test).

plasmic expression of p21$^{Cip1/WAF1}$. N1E-115 cells, which were induced to differentiate by DMSO, were immunostained with the anti-p21$^{Cip1/WAF1}$ antibody. After 24 h of DMSO treatment, p21$^{Cip1/WAF1}$ was induced in the nucleus (Fig. 2 B). However, after 4 d, a time point when the extensive neurite genesis was well evident, p21$^{Cip1/WAF1}$ was mainly localized in the cytoplasm (Fig. 2 C). In this regard, the differentiation-associated cytoplasmic expression of p21$^{Cip1/WAF1}$ is not restricted to chick retinal precursor cells.

Ectopic expression of p21$^{Cip1/WAF1}$ affects the morphology of N1E-115 cells

As the cells with cytoplasmic expression of p21$^{Cip1/WAF1}$ extended long neurites, and those devoid of cytoplasmic p21$^{Cip1/WAF1}$ did not (Figs. 1 and 2), we hypothesized that cytoplasmic p21$^{Cip1/WAF1}$ was associated with neurite elongation. Therefore, we next asked if relocalization of p21$^{Cip1/WAF1}$ to the cytoplasm elicited the extension of the neurites. To address this question, the mammalian expression vector for p21$^{Cip1/WAF1}$ with loss of nuclear localization signal (ΔNLS-p21; aa 1–140) as well as the full-length p21$^{Cip1/WAF1}$ (full-p21; aa 1–164) was made (Asada et al., 1999). The cells transfected with ΔNLS-p21 or GFP proliferated until 48 h after transfection (Fig. 3 A), although those with full-p21 stopped proliferation. In the cells transfected with full-p21 or treated with DMSO, the protein level of cyclin D3 strongly increased (Kranenburg et al., 1995), whereas no change in the expression was found in those with ΔNLS-p21 (Fig. 3 B). Furthermore, although underphosphorylated pRb, retinoblastoma gene product, was induced and hyperphosphorylated pRb became undetectable by DMSO treatment, hyperphosphorylated pRb remained predominant in ΔNLS-p21–transfected cells during the observation period (Fig. 3 B). These data demonstrate that ΔNLS-p21 has no differentiation inducing activity in N1E-115 cells, as shown in U937 cells (Asada et al., 1999), thus enabling us to estimate the effects of p21$^{Cip1/WAF1}$ without taking the differentiation effect on the cells into account. The expression level of ΔNLS-p21 in N1E-115 cells was comparable with that of endogenous p21$^{Cip1/WAF1}$ in the cells with DMSO treatment for 4 d (Fig. 3 C). N1E-115 cells were transfected with these constructs and the morphological changes were assessed 48 h later. The cells with the full-length p21$^{Cip1/WAF1}$ expression showed a somewhat flattened and enlarged appearance and decreased cell rounding (Fig. 3 D) compared to those with GFP expression or no transfection, whereas there was no increase in the cell population that had long neurites (Fig. 3 E). These changes may be caused by the differentiation of N1E-115 cells expressing p21$^{Cip1/WAF1}$ in the nucleus (Kranenburg et al., 1995), as we observed a similar phenotype when the cells were induced to be differentiated by DMSO treatment (Kimhi et al., 1976) (unpublished data). The cells with the full-length p21$^{Cip1/WAF1}$ expression extended long neurites 4 d later, a time point when the signal for p21$^{Cip1/WAF1}$ was also seen in the cytoplasm (unpublished data). On the other hand, >45% of the cells transfected with ΔNLS-p21 extended long neurites (3.1-fold increase compared with the control; Fig. 3 E). This result suggests that cytoplasmic p21$^{Cip1/WAF1}$ regulates neurite remodeling in N1E-115 cells.

Effects of cytoplasmic p21$^{Cip1/WAF1}$ on the cytoskeletal organization

Overexpression of a dominant active mutant of RhoA or p160ROCK, an isoform of Rho-kinase, induced cell rounding in N1E-115 cells (Hirose et al., 1998), but the expression of a dominant negative mutant of p160ROCK or treatment with Y-27632 (Fig. 3 E), chemical compounds with

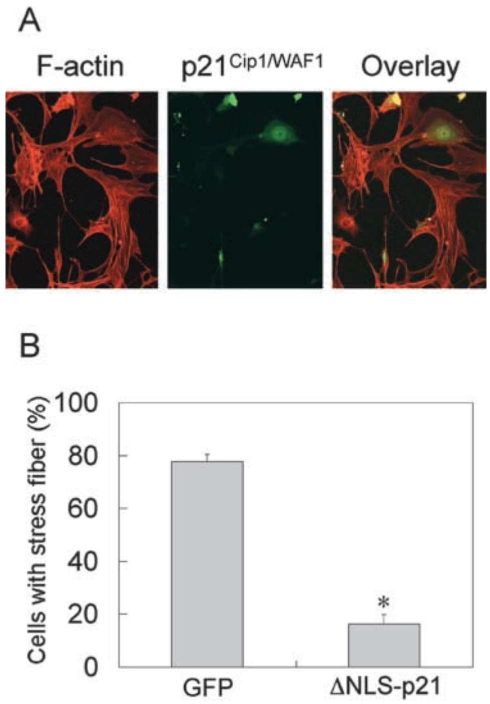

Figure 4. **Effects of cytoplasmic p21$^{Cip1/WAF1}$ on the cytoskeletal organization.** (A) NIH3T3 cells were transfected with GFP-ΔNLS-p21. After serum starvation for 16 h, the cells were treated with 10% fetal bovine serum, fixed, and stained with rhodamine-conjugated phalloidine. (B) Quantification of the cells containing stress fibers. Data represent means ± SEM of three independent experiments. *, $P < 0.01$ compared with GFP (Student's *t* test).

specific inhibitory activity of Rho-kinase (Uehata et al., 1997), induced significant neurite formation (Hirose et al., 1998). Our findings in N1E-115 cells, in combination with these previous reports, suggest that the neurite-promoting activity of cytoplasmic p21$^{Cip1/WAF1}$ may be associated with Rho/Rho-kinase. Therefore, we next used NIH3T3 cells to examine whether p21$^{Cip1/WAF1}$ would regulate actin cytoskeleton mediated by Rho. NIH3T3 cells were transfected with ΔNLS-p21, and then were serum-starved for 16 h. Incubation with serum for 10 min induced the formation of actin stress fibers, preferentially through activation of Rho (Ridley and Hall, 1992). However, NIH3T3 cells transfected with ΔNLS-p21 had little stress fiber formation after the addition of serum, whereas prominent stress fibers were found in nontransfected cells (Fig. 4, A and B). Extensive actin stress fibers were observed in the cells with the full-length p21$^{Cip1/WAF1}$ expression (unpublished data). These results suggest that Rho-induced actin reorganization in NIH3T3 cells may be blocked by the cytoplasmic expression of p21$^{Cip1/WAF1}$.

p21$^{Cip1/WAF1}$ binds to Rho-kinase in the cytoplasm

Rho-kinase was shown to work with mDia1 to elicit the Rho induced phenotype in the fibroblast (Watanabe et al., 1999). As the serum is one of the most potent activators of Rho (Ridley and Hall, 1992), loss of stress fiber formation by the expression of cytoplasmic p21$^{Cip1/WAF1}$ in serum stimulated cells may result from the blockade of the downstream pathway of Rho. Morphological changes of N1E-115 cells by the expression of ΔNLS-p21 were comparable with those by Y-27632 (Fig. 3 E). Given that p21$^{Cip1/WAF1}$ inhibits the activity of the apoptosis signal-regulating kinase 1 (Asada et al., 1999) as well as cyclin-Cdk kinases that are serine threonine kinases (for review see Pines, 1995), we speculated that p21$^{Cip1/WAF1}$ might inhibit the activity of Rho-kinase, which is also a serine threonine kinase. To test the possibility that cytoplasmic p21$^{Cip1/WAF1}$ forms a complex with Rho-kinase in the cytoplasm, coimmunoprecipitation studies were performed using the 293T cells cotransfected with GFP-ΔNLS-p21 and myc-tagged Rho-kinase. Cytoplasmic expression was well evident in the 293T cells transfected with GFP-ΔNLS-p21 (Fig. 5 A). When the lysates were immunoprecipitated with the anti-p21$^{Cip1/WAF1}$ antibody, p21$^{Cip1/WAF1}$ efficiently precipitated myc-tagged Rho-kinase (Fig. 5 B). In an attempt to test if the interaction of ΔNLS-p21 with Rho-kinase depends on its cellular

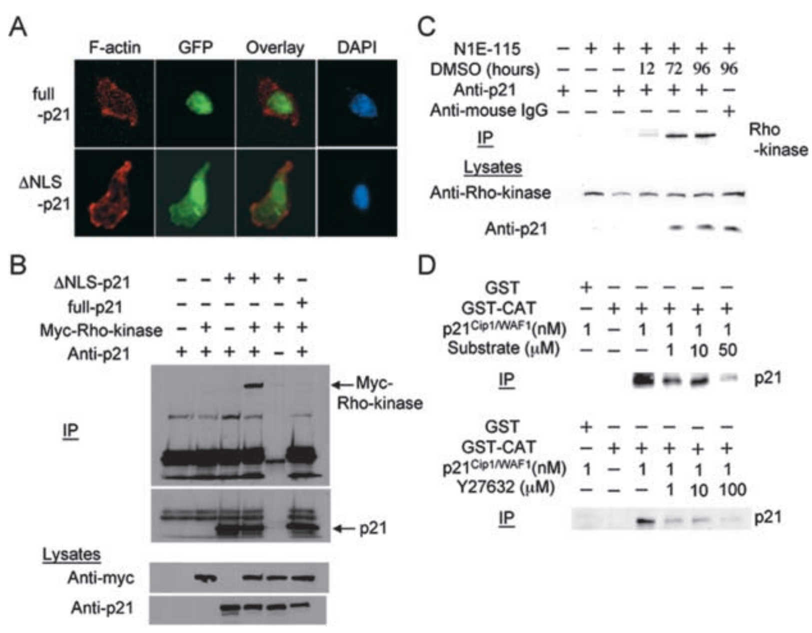

Figure 5. **Cytoplasmic p21$^{Cip1/WAF1}$, but not p21$^{Cip1/WAF1}$ in the nucleus, precipitates Rho-kinase.** (A) Subcellular localization of ectopically expressed proteins in 293T cells. Note the difference in the localization between GFP-full-p21 and GFP-ΔNLS-p21. (B) 293T cells were cotransfected with myc-Rho-kinase in combination with GFP-full-p21 or GFP-ΔNLS-p21. The lysates were immunoprecipitated with the anti-p21$^{Cip1/WAF1}$ antibody. Immunocomplexes were electrophoresed and blotted with anti-myc antibody. Expression of Rho-kinase and p21$^{Cip1/WAF1}$ in the lysates was determined. (C) Interaction of p21$^{Cip1/WAF1}$ with Rho-kinase using lysates prepared from differentiating N1E-115 cells with DMSO treatment. Immunoprecipitated p21$^{Cip1/WAF1}$ was electrophoresed and immunoblotted with anti-Rho-kinase antibody. Anti–mouse IgG antibody was used as a negative control. (D) In vitro interaction of recombinant full-length p21$^{Cip1/WAF1}$ and the catalytic domain of Rho-kinase (GST-CAT). S6 kinase substrate peptide (AKRRRLSSLRA) and Y-27632 at the indicated concentrations were coincubated.

localization, we then tested the interaction of Rho-kinase with GFP-full-p21, which was expressed predominantly in the nucleus (Fig. 5 A). In contrast to ΔNLS-p21, only a faint signal could be detected (Fig. 5 B), despite comparable expression of the full-length and truncated forms of $p21^{Cip1/WAF1}$ in the 293T cells.

Interaction of the proteins artificially overexpressed may be difficult to detect in natural cells. Employing the anti-p21 antibody, we examined the interaction of endogenous proteins using lysates prepared from differentiating N1E-115 cells. N1E-115 cells expressed $p21^{Cip1/WAF1}$ in the cytoplasm after treatment with DMSO for 3 to 4 d (Fig. 2). In the p21 immunoprecipitates, the anti Rho-kinase antibody revealed the presence of a protein corresponding to Rho-kinase (Fig. 5 C).

The lack of an interaction of the full-length-p21 with Rho-kinase may be attributable to the difference of the localization in the cells. Therefore, we tested the in vitro interaction of the recombinant full-length $p21^{Cip1/WAF1}$ and Rho-kinase. These proteins in vitro bound to each other (Fig. 5 D). As GST fused to the fragment of Rho kinase used here corresponds to the catalytic region of Rho-kinase (GST–CAT; aa 6–553), $p21^{Cip1/WAF1}$ may directly bind to the catalytic region of Rho-kinase. This is substantiated by our finding that S6 kinase substrate peptide (AKRRRLSSLRA) as well as Y-27632 inhibited the interaction of $p21^{Cip1/WAF1}$ with Rho-kinase dose dependently (Fig. 5 D). These results suggest that $p21^{Cip1/WAF1}$ associates with Rho-kinase in the cytoplasm.

$p21^{Cip1/WAF1}$ inhibits Rho-kinase activity

We next investigated whether $p21^{Cip1/WAF1}$ could inhibit the activity of Rho-kinase in vitro. The kinase assay was carried out using S6 kinase substrate peptide and [γ-^{32}P] ATP. By using a scintillation counter, the quantity of ^{32}P-labeled substrate peptide on the phosphocellulose paper was determined. This kinetic analysis revealed that $p21^{Cip1/WAF1}$ inhibited the Rho-kinase activity toward S6 kinase substrate peptide in a dose-dependent manner (Fig. 6 A), and the IC_{50} value estimated was 1.43 nM.

These results prompted us to examine whether the Rho-kinase activity was inhibited by the expression of ΔNLS-p21 in vivo. 293T cells were transfected with myc-Rho-kinase with or without ΔNLS-p21. The kinase assay was carried out using the lysates from the cells in the same method as the in vitro assay. The results show that the Rho-kinase activity was inhibited to 48.1% on average in the cells expressing ΔNLS-p21 compared with the control (Fig. 6 B). This inhibitory effect was comparable with that of Y-27632 (51.9% inhibition), although expression of the full-length $p21^{Cip1/WAF1}$ had no significant effect. Our data clearly demonstrate that the activity of Rho-kinase was inhibited by $p21^{Cip1/WAF1}$ in vivo as well as in vitro.

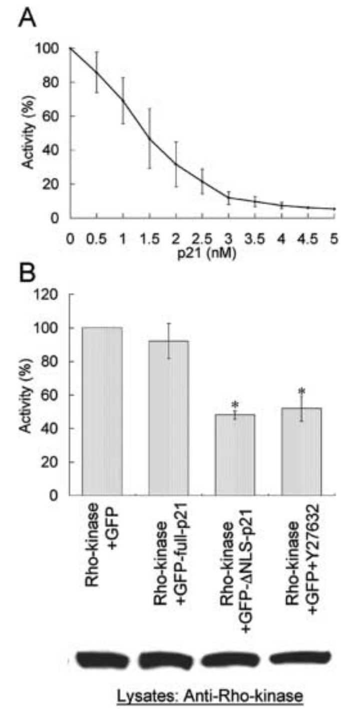

Figure 6. **$p21^{Cip1/WAF1}$ inhibits Rho-kinase activity.** (A) The activity of Rho-kinase was assayed in the presence of the indicated concentrations of $p21^{Cip1/WAF1}$. The percentage was quantified compared to CPM in the absence of $p21^{Cip1/WAF1}$. Data represent means ± SEM of three independent experiments. (B) The activity of Rho-kinase was assayed with the cells exposed to Y-27632 (10 μM) for 30 min or cotransfected with myc-Rho-kinase and $p21^{Cip1/WAF1}$ constructs. The expression of Rho-kinase was determined by Western blot to normalize the relative activities. The relative activities were quantified compared to CPM in the control cells cotransfected with myc-Rho-kinase and GFP. Data represent means ± SEM of 3 independent experiments. *, $P < 0.001$ compared with control (Student's *t* test).

Cytoplasmic $p21^{Cip1/WAF1}$ promotes neurite outgrowth and branching of the hippocampal neurons

To investigate the relevance of our findings that the cytoplasmic $p21^{Cip1/WAF1}$ acts on Rho-kinase, we assessed the effects on neurons. Cultures of the hippocampal neurons from rat E18 embryos were used. We chose these neurons, as they did not express endogenous $p21^{Cip1/WAF1}$ enough to be detected by immunocytochemistry using the anti-$p21^{Cip1/WAF1}$ antibody (unpublished data). Dissociated hippocampal neurons were incubated for 48 h and transfected with ΔNLS-p21. 24 h after transfection, the cells were fixed and immunolabeled with β-tubulin III. The total neurite length per neuron, the axonal length, defined as the length of the longest neurite per neuron, the number of primary processes originating from the neuronal somata, and the number of branch points per neuron were determined (Neumann et al., 2002). The neuronal morphology of the cells expressing ΔNLS-p21 was apparently different from the control cells without transfection or expressing GFP (Fig. 7 A). The cells with the ΔNLS-p21 expression extended longer neurites and had more branch points than the control cells (GFP-expressing cells or no transfection). Ectopic expression of ΔNLS-p21 increased the total neurite length per neuron from 135.9 μm (±7.2 μm SEM) to 307.2 μm (±34.0 μm SEM), the axonal length from 66.3 μm (±3.2 μm SEM) to 162.9 μm (±18.6 μm SEM), and the number of branch points per neuron from 1.3 (±0.2 SEM) to 2.6 (±0.3 SEM). However, no change in the number of primary processes was found by overexpression of cytoplasmic $p21^{Cip1/WAF1}$ (Fig. 7 B). These results indicate that cytoplasmic $p21^{Cip1/WAF1}$ regulates neurite remodeling in the embryonic hippocampal neurons.

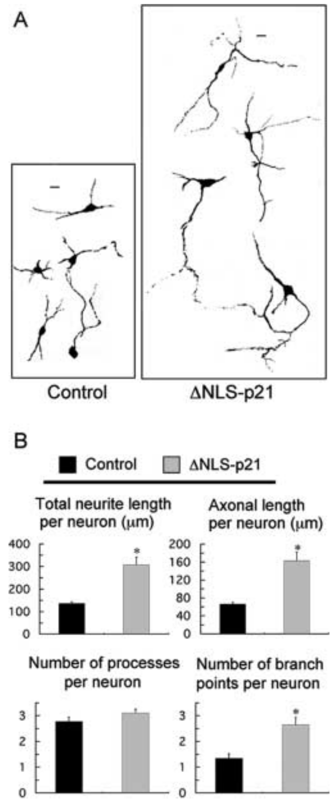

Figure 7. **Neurite outgrowth and branching of hippocampal neurons by overexpression of cytoplasmic p21$^{Cip1/WAF1}$.** (A) Morphology of hippocampal neurons transfected with GFP or GFP-ΔNLS-p21 by computer tracing. Primary hippocampal neurons were transfected with GFP (control) or GFP-ΔNLS-p21 (ΔNLS-p21). Neurons were immunostained with anti–β-tubulin III antibody, and were traced with image analysis computer software. Bars, 10 μm. (B) Morphological analysis of primary hippocampal neurons transfected with GFP or GFP-ΔNLS-p21. In neurons transfected with ΔNLS-p21, the total neurite length, the axonal length and the number of branch points per neuron were increased compared with those transfected with GFP. Data represent means ± SEM of three independent experiments. *, $P < 0.001$ compared with control (Student's *t* test).

Discussion

In the developmental stage of chick retina, p21$^{Cip1/WAF1}$ was shown to be associated with the differentiation of the neurons. The newborn neurons from E5 chick neural retina in vitro and differentiating N1E-115 cells express p21$^{Cip1/WAF1}$ in the cytoplasm. Ectopic expression of p21$^{Cip1/WAF1}$ without the NLS suppresses stress fiber formation in the serum-stimulated NIH3T3 cells and promotes neurite outgrowth from N1E-115 cells and E18 hippocampal neurons. These effects may be mediated by the inhibition of Rho-kinase, as p21$^{Cip1/WAF1}$ inhibits Rho-kinase activity in vitro and in vivo.

Cytoplasmic expression of p21$^{Cip1/WAF1}$

There have been several reports that show cytoplasmic expression of p21$^{Cip1/WAF1}$ and its possible mechanisms. In peripheral blood monocytes and differentiating U937 cells, p21$^{Cip1/WAF1}$ was detected in the cytoplasm (Lubbert et al., 1991; Asada et al., 1999). U937 cells can differentiate into monocytes with vitamin D3 treatment, and 1 d after the treatment p21$^{Cip1/WAF1}$ was induced in the nucleus. However, it was mainly localized in the cytoplasm after 3 d, a time point when the monocytic differentiation was well evident. As we noticed that young neurons which express β-tubulin display cytoplasmic p21$^{Cip1/WAF1}$ expression, whereas the precursor cells not expressing β-tubulin did not (Fig. 1 B), it is suggested that cytoplasmic p21$^{Cip1/WAF1}$ is induced during the developmental stage after differentiation in neurons as well as monocytic cells and has relevant roles.

Zhou et al. (2001) previously reported a possible mechanism of translocation of p21$^{Cip1/WAF1}$, which was triggered by Akt-induced phosphorylation of p21$^{Cip1/WAF1}$ at residues Thr^{145}. As Thr^{145} is in the NLS of p21$^{Cip1/WAF1}$, phosphorylation of p21$^{Cip1/WAF1}$ may result in the loss of its nuclear localization ability. However, another group casts doubt on this finding, as they could not confirm translocation of p21$^{Cip1/WAF1}$ by this phosphorylation (Rossig et al., 2001). More investigation will be required to address this discrepancy; therefore, we did not use the constitutive phosphorylated mutant of p21$^{Cip1/WAF1}$ in our study. Truncation of the nuclear localization signal is also the mechanism of regulation of subcellular localization of p21$^{Cip1/WAF1}$. At an early phase during DNA damage-induced apoptosis, the COOH-terminal of p21$^{Cip1/WAF1}$ is truncated by a member of the caspase family of proteases (Gervais et al., 1998; Levkau et al., 1998; Zhang et al., 1999), and after cleavage p21$^{Cip1/WAF1}$ loses its NLS and exits from the nucleus (Levkau et al., 1998). The ΔNLS-p21 construct we used here was similar to this truncated p21$^{Cip1/WAF1}$ and worked well in our system. However, as we observed the signals for GFP-ΔNLS-p21 also in the nucleus of transfected cells and the hippocampal neurons, GFP-ΔNLS-p21 would enter the nucleus by passive diffusion (Lang et al., 1986).

Cytoplasmic p21$^{Cip1/WAF1}$ inhibits Rho-kinase activity

Rho-kinase plays important roles in, for example, stress fiber, and focal adhesion formation (Leung et al., 1996; Amano et al., 1997), smooth muscle contraction (Kureishi et al., 1997), cytokinesis (Yasui et al., 1998), and neurite retraction (Amano et al., 1998), as a downstream effector of Rho (Matsui et al., 1996). Some chemical compounds have been shown to inhibit Rho-kinase activity (Uehata et al., 1997). Staurosporine, HA1077 and Y-32885 inhibited the activity of Rho-kinase as well as protein kinase N, one of the targets of Rho, and the IC_{50} values of these toward Rho-kinase were ~7 nM, 1.7 μM, and 0.4 μM, respectively (Amano et al., 1999). In this study, p21$^{Cip1/WAF1}$ inhibited Rho-kinase activity in a dose-dependent manner, and the IC_{50} value was 1.43 nM, suggesting the strong inhibitory effect.

Rho/Rho-kinase and the neurite outgrowth

A number of factors that regulate Rho activity are implicated in neurite outgrowth and growth cone guidance (for review see Luo, 2000). We showed previously that the axonal outgrowth was facilitated by the ligand binding to the neurotrophin receptor p75 presumably through inactivation of Rho (Yamashita et al., 1999). In addition, our observation that myelin-associated glycoprotein as well as tumor necrosis factor elicited inhibition of neurite outgrowth and branching seems to be mediated by the activation of Rho (Neumann et al., 2002; Yamashita et al., 2002). Taking these findings into consideration, blocking the activity of Rho-kinase would be a good molecular target, as the axonal outgrowth should be promoted by blocking the downstream pathway even if Rho is activated by some cytokines or guidance molecules. In fact, Rho-kinase was shown to be a possible therapeutic target for central nervous system axon regeneration (Lehmann et al., 1999).

However, not all the neuronal cells respond to various stimuli in the same way. In PC12 cells during differentiation after nerve growth factor treatment, ectopic expression of constitutively active Rho does not cause the disappearance of neurites (Sebok et al., 1999). In dorsal root ganglion neurons up to postnatal day 3 to 4 or embryonic retinal ganglion neurons, axonal outgrowth was not significantly inhibited by myelin-associated glycoprotein, which activates Rho (Johnson et al., 1989; Mukhopadhyay et al., 1994; De Bellard et al., 1996; Cai et al., 2001; Yamashita et al., 2002). Although these reports suggest that the responses of the neurons to Rho depend on the cell context, another interpretation of the data is that immature or young neurons may have intrinsic mechanisms to overcome the inhibitory effects mediated by Rho. The molecular mechanisms that govern these phenomenon remain to be elucidated, however, our notion that cytoplasmic $p21^{Cip1/WAF1}$ promotes neurite outgrowth through inactivation of Rho-kinase may be an interesting hypothesis to explain the loss of responses to Rho activation. Future studies will address these issues.

Materials and methods

Preparation of chick retina and retinal cells

Whole chick E5 embryos (White Leghorn) were fixed with 4% paraformaldehyde in PBS overnight and immersed in 30% sucrose. Cryosections (30 μm in thickness) of retinas were cut on the coronal plane, thaw mounted onto slides, and dried at room temperature. For retinal neuron culture, retinas from E5 embryos were dissected free from the pigment epithelium and dissociated as described previously (Rodriguez-Tebar et al., 1989; de la Rosa et al., 1994). Dissociated cells were plated (20,000 cells/cm^2) on 4-well chamber slides (Nalge Nunc International K.K.), which were previously coated with poly-L-ornithine/laminin (Sigma-Aldrich) (Collins, 1978). Cells were cultured in DME/F12 mixture (1:1) with N2 supplement (Bottenstein and Sato, 1979), and maintained at 37°C in a water-saturated atmosphere containing 5% CO_2 for 12 h and fixed with 4% paraformaldehyde in PBS.

Plasmid constructs

pEGFP-full-p21 (aa 1–164) and pEGFP-ΔNLS-p21 (aa 1–140) are mammalian expression vectors for GFP fused proteins (Asada et al., 1999). Myc-Rho-kinase in pEF-BOS was provided by Dr. K. Kaibuchi (Nagoya University, Nagoya, Japan).

Cell culture and transfection

NIH3T3 cells, N1E-115 cells, and 293T cells were maintained in DME containing 10% fetal bovine serum. Lipofectamine 2000 (Invitrogen) was used for transfection. For the stress fiber formation assay, NIH3T3 cells were cultured in serum-free medium for 16 h after transfection. Stress fiber formation was evoked by incubating the cells with 10% serum for 10 min. Hippocampal neurons were prepared from 18-d-old Sprague-Dawley rats, as previously described (Neumann et al., 1995). Briefly, hippocampi were dissected and the meninges removed. The trimmed tissue was dissociated by trituration. The dissociated cells were plated on dishes precoated with poly-L-lysine (Sigma-Aldrich), and cultured in DME containing 10% fetal bovine serum for 24 h. Then, the medium was replaced with DME with B27 supplement (Invitrogen), and the cells were transfected with GFP or GFP-ΔNLS-p21. Neuronal morphology was estimated at 24 h after the transfection.

Morphological analysis of N1E-115 cells

N1E-115 cells were transfected with GFP, GFP-full-p21, or GFP-ΔNLS-p21, and cultured in serum-starved condition for 5 h. Then, the medium was replaced with DME containing 10% fetal bovine serum. The cells were fixed at 48 h after transfection. The morphology of the cells was categorized into three groups; neurite-positive cells, round cells, and the other cells. The cells with longer neurites than their soma were defined as neurite positive cells. The other cells had various features including microspikes, ruffles and a flattened appearance.

Coimmunoprecipitation of ΔNLS-p21 and Rho-kinase

293T cells were transfected with myc-Rho-kinase in combination with GFP-full-p21 or GFP-ΔNLS-p21. At 48 h after transfection, the cells were lysed with 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 0.5% Nonidet-P40 including protease inhibitor cocktail tablets; Roche). The cell lysates were centrifuged at 13,000 *g* for 20 min, and the supernatant was collected. Immunoprecipitations were performed for 2 h at 4°C using an anti-$p21^{Cip1/WAF1}$ mouse monoclonal antibody (Santa Cruz Biotechnology) and 0.75 ml of the supernatant. The immunocomplexes were collected with protein G-Sepharose (Amersham Pharmacia Biotech) slurry (50% vol/vol), washed four times with lysis buffer, and subjected to SDS-PAGE. They were transferred to the polyvinylidene difluoride membranes and probed with the anti-myc rabbit polyclonal antibody (Santa Cruz Biotechnology). Interaction of endogenous proteins in N1E-115 cells was assessed in the same way using anti–Rho-kinase antibody.

In vitro binding assay

Recombinant full-length $p21^{Cip1/WAF1}$ (1–164, >98% purity, 1 nM; Santa Cruz Biotechnology) and purified GST fused protein of a fragment of Rho-kinase (GST-CAT; aa 6–553) were incubated in 1 ml of the buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 1 mM EDTA including protease inhibitor cocktail tablets) for 2 h, and GST-CAT was precipitated using glutathione sepharose (Amersham Pharmacia Biotech). The resultant precipitates were electrophoretically transferred to polyvinylidene difluoride membranes after SDS/PAGE with 10% gels and were immunoblotted with the anti-$p21^{Cip1/WAF1}$ antibody.

Kinase assay

The kinase reaction for Rho-kinase was carried out using a S6 Kinase Assay Kit (Upstate Biotechnology) according to the manufacturer's instructions. Briefly, for in vitro assay, 10 μl of assay dilution buffer (ADB: 20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate and 1 mM dithiothreitol), 10 μl of substrate cocktail (250 μM substrate peptide [AKRRRLSSLRA] in ADB), 10 μl of the inhibitor cocktail, 10 μl of the [γ-^{32}P] ATP mixture (magnesium/ATP cocktail including 10 μCi of the [γ-^{32}P] ATP) and 20 mU of Rho kinase fragment (aa 1–543; Upstate Biotechnology) were mixed. After incubation with $p21^{Cip1/WAF1}$ protein for 10 min at 30°C, the reaction mixtures were spotted onto the P81 phosphocellulose paper and quantified using a scintillation counter.

For the in vivo assay, 293T cells were cotransfected with myc-Rho-kinase in combination with GFP or $p21^{Cip1/WAF1}$ constructs. Cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Nonidet-P40 and protease inhibitor cocktail). The kinase assay was carried out using the lysates.

Immunostaining

For immunohistochemistry, sections of chick retinas were permeabilized and blocked with the blocking buffer (0.1% Triton X-100, 0.1% BSA, and 5% goat serum in PBS) for 30 min at room temperature. For immunocytochemistry, cells were permeabilized and blocked with the buffer containing 0.2% Triton X-100. They were incubated overnight at 4°C with the anti-$p21^{Cip1/WAF1}$ antibody (1:1000) and an anti–β-tubulin class III rabbit polyclonal antibody (TuJ1) (1:2,000; Research Diagnostic, Inc.), followed by incubation for 1 hour with Alexa 488-labeled goat anti–mouse IgG antibody (Molecular Probes) and Alexa 568-labeled goat anti–rabbit IgG antibody (Molecular Probes). Tetramethyl rhodamine isothiocyanate-labeled phalloidin (1:1,000; Sigma-Aldrich) was used to detect F-actin in NIH3T3 cells and N1E-115 cells. Hippocampal neurons were immunostained with the anti-TuJ1 antibody. When necessary, DAPI (300 nM; Wako) was used to stain the nucleus. Samples were examined under a confocal laser-scanning microscope (Carl Zeiss).

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