Cytoplasmic \( p21^{\text{Cip1/WAF1}} \) regulates neurite remodeling by inhibiting Rho-kinase activity

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\( p21^{\text{Cip1/WAF1}} \) has cell cycle inhibitory activity by binding to and inhibiting both cyclin/Cdk kinases and proliferating cell nuclear antigen. Here we show that \( p21^{\text{Cip1/WAF1}} \) is induced in the cytoplasm during the course of differentiation of chick retinal precursor cells and N1E-115 cells. Ectopic expression of \( p21^{\text{Cip1/WAF1}} \) lacking the nuclear localization signal in N1E-115 cells and NIH3T3 cells affects the formation of actin structures, characteristic of inactivation of Rho. \( p21^{\text{Cip1/WAF1}} \) 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 \( p21^{\text{Cip1/WAF1}} \) is expressed abundantly in the cytoplasm. These results suggest that cytoplasmic \( p21^{\text{Cip1/WAF1}} \) 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 \( p21^{\text{Cip1/WAF1}} \) 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 NH2-terminal domain of \( p21^{\text{Cip1/WAF1}} \) inhibits cyclin-Cdk kinases and a COOH-terminal domain of \( p21^{\text{Cip1/WAF1}} \) 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 \( p21^{\text{Cip1/WAF1}} \) are attributable to its nuclear localization (Goubin and Ducommun, 1995; Sherr and Roberts, 1995). However, recent studies propose possible mechanisms of translocation of \( p21^{\text{Cip1/WAF1}} \) from the nucleus to the cytoplasm. It is reported that phosphatidylinositol-3 kinase/Akt phosphorylates threonine 145 in COOH-terminal NLS of \( p21^{\text{Cip1/WAF1}} \) and phosphorylated \( p21^{\text{Cip1/WAF1}} \) loses its ability to localize in the nucleus (Zhou et al., 2001). Another paper shows that truncation of 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, \( p21^{\text{Cip1/WAF1}} \) also plays important roles in regulating the cell cycle. In several cell lines during differentiation after nerve growth factor treatment, the expression of \( p21^{\text{Cip1/WAF1}} \) 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...
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<sup>Cip1/WAF1</sup>. Cytoplasmic expression of p21<sup>Cip1/WAF1</sup> was observed in newborn neurons that extensively extend neurites. As p21<sup>Cip1/WAF1</sup> 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<sup>Cip1/WAF1</sup> 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<sup>Cip1/WAF1</sup> in these cells to test whether p21<sup>Cip1/WAF1</sup> 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<sup>Cip1/WAF1</sup> immunoreactivity was detected in the cells at the vitreous surface of the central neural retina using a monoclonal antibody against p21<sup>Cip1/WAF1</sup> (Fig. 1 A). These p21<sup>Cip1/WAF1</sup>-positive cells were immature retinal neurons before migration. Therefore, it is suggested that p21<sup>Cip1/WAF1</sup> 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<sup>Cip1/WAF1</sup>. 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<sup>Cip1/WAF1</sup> was predominantly seen in the nucleus (Fig. 1 B). p21<sup>Cip1/WAF1</sup> 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<sup>Cip1/WAF1</sup> was mainly localized in the cytoplasm (Fig. 1 B). These findings suggest that cytoplasmic expression of p21<sup>Cip1/WAF1</sup> is induced in the newborn neurons.

**In vitro differentiation of N1E-115 cells is associated with p21<sup>Cip1/WAF1</sup> expression in the cytoplasm**

We next used neuroblastoma N1E-115 cells to examine whether neuronal differentiation was associated with cyto-
Neurite outgrowth by cytoplasmic p21Cip1/WAF1

plasmic expression of p21\textsuperscript{Cip1/WAF1} induced N1E-115 cells, which were induced to differentiate by DMSO, were immunostained with the anti-p21\textsuperscript{Cip1/WAF1} antibody. After 24 h of DMSO treatment, p21\textsuperscript{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\textsuperscript{Cip1/WAF1} was mainly localized in the cytoplasm (Fig. 2 C). In this regard, the differentiation-associated cytoplasmic expression of p21\textsuperscript{Cip1/WAF1} is not restricted to chick retinal precursor cells.

**Ectopic expression of p21\textsuperscript{Cip1/WAF1} affects the morphology of N1E-115 cells**

As the cells with cytoplasmic expression of p21\textsuperscript{Cip1/WAF1} extended long neurites, and those devoid of cytoplasmic p21\textsuperscript{Cip1/WAF1} did not (Figs. 1 and 2), we hypothesized that cytoplasmic p21\textsuperscript{Cip1/WAF1} was associated with neurite elongation. Therefore, we next asked if relocation of p21\textsuperscript{Cip1/WAF1} to the cytoplasm elicited the extension of the neurites.

To address this question, the mammalian expression vector for p21\textsuperscript{Cip1/WAF1} with loss of nuclear localization signal (\textDelta NLS-p21; aa 1–140) as well as the full-length p21\textsuperscript{Cip1/WAF1} (full-p21; aa 1–164) was made (Asada et al., 1999). The cells transfected with \textDelta NLS-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 \textDelta NLS-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 \textDelta NLS-p21-transfected cells during the observation period (Fig. 3 B). These data demonstrate that \textDelta NLS-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\textsuperscript{Cip1/WAF1} without taking the differentiation effect on the cells into account. The expression level of \textDelta NLS-p21 in N1E-115 cells was comparable with that of endogenous p21\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{Cip1/WAF1} expression extended long neurites 4 d later, a time point when the signal for p21\textsuperscript{Cip1/WAF1} was also seen in the cytoplasm (unpublished data). On the other hand, >45% of the cells transfected with \textDelta NLS-p21 extended long neurites (3.1-fold increase compared with the control; Fig. 3 E). This result suggests that cytoplasmic p21\textsuperscript{Cip1/WAF1} regulates neurite remodeling in N1E-115 cells.

**Effects of cytoplasmic p21\textsuperscript{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
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 and myc-tagged Rho-kinase. Coimmunoprecipitation studies were used to test the interaction of ΔNLS-p21 with Rho-kinase depending on its cellular location. The interaction between GFP-full-p21 and ΔNLS-p21 was confirmed in the lysates of 293T cells cotransfected with GFP-full-p21 and ΔNLS-p21. The lysates were immunoprecipitated with the anti-p21\(^{Cip1/WAF1}\) antibody. Immuno- complexes 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. Immune-precipitated 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 S6 kinase substrate peptide and the activity of Rho-kinase in vitro. The kinase assay was carried using a scintillation counter, the quantity of 32P-labeled S6 kinase substrate peptide and branching of the hippocampal neurons. Cultures of the hippocampal neurons from rat E18 embryos were used. We chose these neurons, as they did not express endogenous p21Cip1/WAF1 enough to be detected by immunocytochemistry using the anti-p21Cip1/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 p21Cip1/WAF1 (Fig. 7 B). These results indicate that cytoplasmic p21Cip1/WAF1 regulates neurite remodeling in the embryonic hippocampal neurons.

Figure 6. p21Cip1/WAF1 inhibits Rho-kinase activity. (A) The activity of Rho-kinase was assayed in the presence of the indicated concentrations of p21Cip1/WAF1. The percentage was quantified compared to CPM in the absence of p21Cip1/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 p21Cip1/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).
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with GFP or GFP-Morphological analysis of primary hippocampal neurons transfected experiments. *, P with GFP. Data represent means points per neuron were increased compared with those transfected the total neurite length, the axonal length and the number of branch were traced with image analysis computer software. Bars, 10

were transfected with GFP (control) or GFP-GFP-Morphology of hippocampal neurons transfected with GFP or

Figure 7. Neurite outgrowth and branching of hippocampal neurons by overexpression of cytoplasmic p21Cip1/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, p21Cip1/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 p21Cip1/WAF1 in the cytoplasm. Ectopic expression of p21Cip1/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 p21Cip1/WAF1 inhibits Rho-kinase activity in vitro and in vivo.

Cytoplasmic expression of p21Cip1/WAF1

There have been several reports that show cytoplasmic expression of p21Cip1/WAF1 and its possible mechanisms. In peripheral blood monocytes and differentiating U937 cells, p21Cip1/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 p21Cip1/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 p21Cip1/WAF1 expression, whereas the precursor cells not expressing β-tubulin did not (Fig. 1 B), it is suggested that cytoplasmic p21Cip1/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 p21Cip1/WAF1, which was triggered by Akt-induced phosphorylation of p21Cip1/WAF1 at residues Thr145. As Thr145 is in the NLS of p21Cip1/WAF1, phosphorylation of p21Cip1/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 p21Cip1/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 p21Cip1/WAF1 in our study. Truncation of the nuclear localization signal is also the mechanism of regulation of subcellular localization of p21Cip1/WAF1. At an early phase during DNA damage-induced apoptosis, the COOH-terminal of p21Cip1/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 p21Cip1/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 p21Cip1/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 p21Cip1/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 IC50 values of these toward Rho-kinase were ~7 nM, 1.7 μM, and 0.4 μM, respectively (Amano et al., 1999). In this study, p21Cip1/WAF1 inhibited Rho-kinase activity in a dose-dependent manner, and the IC50 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; Ya-
mashita 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 p21Cip1/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/cm²) on 4-well chamber slides (Nalge Nunc International K.K.), which were previously coated with poly-L-lysine/aminin (Sigma-Aldrich) (Collins, 1978). Cells were cultured in DME/F12 mixture (1:1) with N2 supplement (Bottenstein and Singer, 1979), and maintained at 37°C in a water-saturated atmosphere containing 5% CO₂ 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 pEG-F-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-lysine (Sigma-Aldrich), and cultured in DME containing 10% fetal bovine serum for 24 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 micropinocytosis, 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-p21Cip1/WAF1 mouse monoclonal antibody (Santa Cruz Biotechnology) and 0.75 ml of the supernatant. The immunocomplexes were collected with protein G-Sepharose (Amersham Pharmaica 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 p21Cip1/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) was incubated in 1 ml of the buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, and 1 mM EDTA including protease inhibitor cocktail tablets) for 2 h, and GST-CAT was precipitated using glutathione sepharose (Amersham Pharmaica Bio-tech). The resultant precipitates were electrophoretically transferred to polyvinylidene difluoride membranes after SDS-PAGE with 10% gels and were immunoblotted with the anti-p21Cip1/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-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate and 1 mM dihydrothreitol), 10 μl of substratecocktail (250 μM substrate peptide [AKRRRLSSLRA] in ADB), 10 μl of the inhibitor cocktail, 1 μl of the [γ-32P] ATP mixture (magnesium/ATP cocktail including 1 μCi of the [γ-32P] ATP and 20 μl of Rho kinase fragment (aa 1–543; Upstate Biotechnology) were mixed. After incubation with p21Cip1/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 p21Cip1/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-p21Cip1/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 h 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 phallolidin (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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