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Ribosomal Protein L10 Interacts With the SH3 Domain and Regulates GDNF-Induced Neurite Growth in SH-SY-5y Cells

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Abstract The 24.5 kDa ribosomal protein L10 (RP-L10), which was encoded by QM gene, was known to interact with the SH3 domain of Yes kinase. Herein, we demonstrate that RP-L10 interacts with the SH3 domain of Src and activates the binding of the Nck1 adaptor protein with skeletal proteins such as the Wiskott-Aldrich Syndrome Protein (WASP) and WASP interacting protein (WIP) in neuroblastoma cell line, SH-SY-5y. The RP-L10 was associated with the SH3 domains of Src and Yes. It is shown that two different regions of RP-L10 are associated with the Src-SH3. The effect of ectopic RP-L10 expression on neuronal cell scaffolding was explored in cells transiently transfected with QM. SH-SY-5y human neuroblastoma cells transfected with QM were considerably more susceptible to neurite outgrowth induced by glial cell line-derived neurotrophic factor (GDNF). However, RP-L10 did not directly interact with actin assembly. Taken together, these results suggest that the RP-L10 may positively regulate the GDNF/Ret-mediated signaling of neurite outgrowth in the neuroblastoma cell line, SH-SY-5y. J. Cell. Biochem. 99: 624–634, 2006.

Key words: ribosomal protein L10; Src; SH-SY-5y; neurite growth

Recently, SH3-containing c-Yes was screened by a yeast two hybrid system and the ribosomal protein L10 (RP-L10) was identified as a SH3-interacting protein [Oh et al., 2002]. The 24.5 kDa RP-L10 was encoded by QM gene. RP-L10 is highly homologous to the Jun-binding protein (Jif) in terms of its sequence [Monteclaro and Vogt, 1993], but it has been observed that RP-L10 and Jun have little relationship in vivo [Loftus et al., 1997]. Most RP-L10s are localized in the cytoplasm, and subcellular fractionation assays have shown that RP-L10 is peripherally localized to the endoplasmic reticulum [Nguyen et al., 1998a,b]. In some reports, it was suggested that yeast *QM* homologous genes, such as *GRC5* and *QSR1*, participate in the translational control of gene expression in yeast [Karl et al., 1999]. These genes are especially required for cell growth and differentiation throughout mRNA translation, because they take part in the recombination of the 60S and 40S RP subunits. The RP-L10 is known to be expressed in many different species, and to have highly conserved sequences throughout [Farmer et al., 1994]. Embryos and bone development studies indicate that RP-L10 is highly expressed in undifferentiated cells, while adult tissues tend not to maintain RP-L10 [Eisinger et al., 1993; Mills et al., 1999; Green et al., 2000]. It was found that RP-L10 was associated with the SH3 domain of c-Yes, resulting in the suppression of c-Yes kinase activity in HeLa cells [Oh et al., 2002]. RP-L10 was also found to interact with the SH3 domains of other Src family kinase (SFK) members, such as Fyn, Hck, Lyn, and Src [Oh et al., 2002]. However, the functional relevance of RP-L10 interaction with cellular events has rarely been addressed so far. We intended to know whether RP-L10 would be able to interact with SH3 domain of Src in neuronal cells and what cellular events could be brought about.

Src and Src-family protein kinases (SFKs) are known to play key roles in cell differentiation, motility, proliferation, and survival [Thomas

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and Brugge, 1997]. From the N- to C-terminus, Src contains an SH3 domain, an SH2 domain, a protein-tyrosine kinase domain, and a C-terminal regulatory tail. SFKs are controlled by receptor protein-tyrosine kinases, integrin receptors, G-protein coupled receptors, cytokine receptors, and steroid hormone receptors [Thomas and Brugge, 1997]. Src signals to a variety of downstream effectors including p85 (the regulatory subunit of phosphatidylinositol 3-kinase), RasGAP, Shc, phospholipase Cγ, several integrin signaling proteins (tensin, vinculin, cortactin, talin, and paxillin), and focal adhesion kinase [Brown and Cooper, 1996]. SFKs are activated by glial cell line-derived neurotrophic factor (GDNF) in neurons [Trupp et al., 1999], yet the role of SFKs in GDNF-dependent signaling has rarely been studied in neurons. It was previously reported that GDNF promotes the process of axonal regeneration process in the nervous system [Paveliev et al., 2004]. Neuroblastoma cell line, SH-SY-5y is derivatives of undifferentiated peripheral neuroblast cells that respond to GDNF and display a GDNF-responsive GFRa-RET receptor [Baloh et al., 1998]. We also observed the GDNF-induced neurite extension in SH-SY-5y cell lines. Therefore, using SH-SY-5y cell line, we investigated the effect of RP-L10 overexpressionon GDNF-induced neurite growth, where SFKs were entangled, to determine if responsive cells had a function of RP-L10 in neurite differentiation. In this study, we demonstrate that RP-L10 interacts with the SH3 domain of the Src protein and that the ectopic expression of RP-L10 markedly increases GDNF-induced neurite outgrowth in neuronal cells.

Neurite growth is dependent on receptor signaling events taking place in the tiny, specialized neuronal structures known as lamellipodia and filopodia [Mueller, 1999]. These extensions at the edge of neurons and growth cones contain a cross-linked F-actin meshwork. Thus, we additionally investigated the effect of RP-L10 on actin polymerization. Although a variety of signaling proteins has been identified to be involved in the regulation of actin networks, this work surveys on a terminal signaling component, Nck1 in linking to the organization of actin cytoskeleton. It has been reported that Nck1 bound with the Wiskott-Aldrich Syndrome Protein (WASP) and WASP interacting protein (WIP), which are regulators of the actin cytoskeleton [Ramesh et al., 1999; Zigmond, 2000]. WASP and its homologuegs, N-WASP and the Wave/Scar proteins, promote F-actin assembly by activating the ARP2/3 (actin-related protein) complex [Rohatgi et al., 1999]. Adaptor protein, Nck1 consist primarily of a single Src homology (SH)2 domain and SH3 domains and do not have other functional motifs [Mayer and Baltimore, 1993; Birge et al., 1996]. Here, we investigated the effect of RP-L10 on the interaction of Nck1 with WASP.

There have been few reports concerning the involvement of RP-L10 in the intracellular signaling pathway and its cellular function. Herein, we show for the first time that the RP-L10 regulates SH3-mediated protein signal transduction by acting as a new regulator of GDNF-induced neurite outgrowth in neuronal cells, suggesting that this signal involves the actin regulation pathway.

MATERIALS AND METHODS

DNA Construction

pEBG-QM was obtained by PCR using SpeI/ClaI-containing primers (aa 1-213, 5_-CGA-GGC-ACT-AGT-GTC-GCC-ATG-3_/5_-GG-CAG CAC-ATC-GAT-AGC-CCT-C-3_) and provided by Dr. H. Oh (Seoul National University). QM subclones encoding various portions of amino acid residues into pEBG vectors for mammalian transfections were constructed using adequate PCR primers as previously reported [Oh et al., 2002]. For the *Escherichia coli* expression of RP L10, a pGEX-4T (Amersham, Uppsala, Sweden) vector was used with the same inserted primers changing the restriction enzyme sites from SpeI or BamHI to EcoRI and from ClaI to XhoI. Src-SH3-SH3 domain containing pGEX vectors were kindly provided by Dr. Thomas E. Smithgall (School of Medicine, University of Pittsburgh) and pGEX-Yes-SH3 was cloned with (aa 77-134, 5_-AGT-CCA-GGA-TCC-AGTACT-TTA-ACA-G GT-3_/5_-A-CCA-CTC-TCG-AGC-TTG-AAT-GG AGTC-TG-3_) and provided by Dr. H. Oh (Seoul National University).

Cell Line Culture

The SH-SY-5y (human neuroblastoma) cells were cultured in Dulbecco's modified Eagle's medium (DMEM: Gibco, Rockville, MD) supplemented with 10% fetal bovine serum. The cell line was purchased from KCLB (Korean Cell Line Bank). All cell types were incubated in a 5% CO_2 humidified incubator at 37°C.

Transfection

The SH-SY-5y cell line was maintained in DMEM supplemented with 10% fetal bovine serum, in a 5% CO_2 humidified incubator. For the GST pull-down assays and the immunoprecipitation, Src-SH3 gene fragments fused pFLAG vectors and pEBG-QM derivatives were co-transfected in 100-mm plates and cultured for 2 days. For neurite outgrowth assay, SH-SY-5y cells were transfected with pCMV-QM or pCMV. All the transfections were performed with Lipofectamine Plus and LipofectAMINE Reagent (Invitrogen, Calsbad, CA) following the manufacturer's instructions.

GST Pull-Down Assay

After 2 days of incubation, co-transfected SH-SY-5y cells were washed twice with PBS and treated with 2 ml of ice-cold lysis buffer [1% Nonidet P-40, 1% sodium deoxycholate, 0.15 M NaCl, 10 mM sodium phosphate (pH 7.0), 100 μM sodium vanadate, 50 mM NaF, 50 μM leupeptin, 1% aprotinin, 2 mM EDTA, and 1 mM dithiothreitol]. The cell plate was incubated for 20 min at 4°C with agitation. The cells were scraped and centrifuged at 12,000 rpm for 30 min. The cell lysates (1 ml) were added to previously prepared 50% slurry GST-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). Following incubation for 3–4 h at 4°C with gentle rocking, the samples were centrifuged at 6,000 rpm for 3 min and then washed five times with 1 ml of lysis buffer. Five times SDS loading buffer was added to the beads, boiled for 5 min, and subjected to 12% SDS–PAGE. The binding of the two proteins was detected by Western blot analysis.

Co-Immunoprecipitation

The cells were washed in PBS and lysates were prepared as described above. 200–500 μg of the pre-cleared lysates were diluted to 0.2 mg/ml with lysis buffer supplemented with 1% Triton X-100 and 5 μg of anti-RP L10 rabbit polyclonal antibody (Santa Cruz Biotechnology) or anti-FLAG M2 monoclonal antibody (Sigma, St. Louis, MO). Incubation was performed for 2 h at 4°C on a rocker, and then 25 μl of pre-washed protein A/G-agarose (Santa Cruz Biotechnology) was added. After 1 h of incubation, the agarose was centrifuged and washed three times with lysis buffer containing 1% Triton X-100. The proteins were eluted with 50 μl of elution buffer (0.1 M glycine/HCl buffer, pH 2.5) three times. Then, Western blot analysis was carried out to detect the protein interactions.

Protein Interactions Under Native Conditions

For the detection of the endogenous protein–protein interactions, 2×10^6 SH-SY-5y cells were incubated for 2 days, and the lysates were prepared according to the above method. We used 1 mg of the precleared cell lysate and 10 μg of the anti-Src or anti-RP L10 (C-17) rabbit polyclonal antibody (Santa Cruz Biotechnology) for co-immunoprecipitation. GST pull-down assays of each GST-SH3 (Src-SH3, Yes-SH3, and Src) were also performed with native SH-SY-5y cell lysate and overexpressed GST-SH3s from *E. coli*. We incubated 30 μl of GST-SH3 bound to GST-agarose and 1 mg of the SH-SY-5y cell lysate for 4 h at 4°C. They were washed four times with lysis buffer. Native RP L10 was immunoprecipitated with 5 μg of the anti-RP L10 antibody from the 0.5 mg of precleared cell lysate. After 1 h of incubation, 0.5 mg of the *E. coli* cell lysates containing GST-SH3 and 40 μl of the pre-washed protein A/G agarose were added and co-immunoprecipitated for 3 h.

Immunofluorescence

Cells (1×10^5) were plated on coverslips. Fixation was performed by a slightly modified methanol fixation method. The fixed cells were permeabilized for 3 min at 4°C in PBS supplemented with 0.1% Triton X-100. After washing three times with PBS, the coverslips were soaked in blocking buffer (0.5 g/100 ml of bovine serum albumin in PBS) for 15 min at room temperature. The cells were then incubated with the anti-Src mouse and the anti-RP L10 rabbit polyclonal antibody (each 1:300 dilution in blocking buffer) for 1 h. Following the third successive 5-min washing, the cells were incubated for 45 min with rhodamine-conjugated goat anti-rabbit (1:100, Santa Cruz Biotechnology) and fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (1:100, Sigma). After the third 5-min wash with PBS, the coverslips were mounted, and visualization was performed using a BX51 fluorescent microscope (Olympus, Tokyo, Japan).

Fluorescence Dye Staining

The cultures were stained for neurite growth with FM 1-43 (Molecular Probes-Invitrogen,

Carlsbad, CA) and for F-actin with phalloidin–Alexa568 (Molecular Probes-Invitrogen). The FM 1–43 dye labeling was carried out as described [Wang et al., 2002]. Briefly, the culture coverslips were treated with high K^+ loading solution containing (KCl, 60 mM; NaCl, 57.6 mM; $CaCl_2$, 3.5 mM; Hepes, 10 mM, pH 7.6; FM 1–43, 2 μM) for 2 min. Cells were then washed with PBS, lightly fixed (4% paraformaldehyde, for 10 min), and washed again. The culture coverslips were mounted onto glass slide, and imaged under a fluorescence microscope with a GFP filter set. For F-actin staining, the fixed cells were permeabilized for 3 min at room temperature in PBS supplemented with 0.05% Triton X-100. After washing with PBS, the coverslips were treated with 5 U/ml phalloidin–Alexa568 and imaged under a fluorescence microscope with a WG filter set equipped with Photometrics CoolSNAP camera (Roper Scientific, Duluth, GA).

Morphometry

To measure the length of neurite, digital images of 30 neurons per sample were taken with IX70 Inverted microscope (Olympus) using 10×, 20×, and 40× objectives, and neurite length was measured with Image-ProPlus software (Media Cybernetics, Silver Springs, MD). In those cases when a neuron had several neurites, all lengths were measured and averaged.

For the manual quantification of branch number and axon length, axon lengths were measured from the cell body to the distal extent of the central region of the growth cone. As described [Dent et al., 2004], the axon was defined as the process that remained parallel to the axon segment proximal to the branch point. Branches were defined as processes extending at orthogonal angles to the axon. The counts were performed blind. Three hundred to five hundred neurons per sample were counted to measure the neurons bearing neurite growth.

In all experiments, statistical significance was calculated for data from three independent experiments using one-way ANOVA (Excel, Microsoft, Redmond, WA). Error bars represent standard deviation of mean; symbols * and ** represent $P < 0.05$ and 0.01, respectively.

RESULTS

RP-L10 Interacts With the Src-SH3

Immunoprecipitation and the GST-pull down assay showed that RP-L10 was bound to Src-SH3 (Fig. 1). The co-immunoprecipitation analysis carried out with in vitro-translated proteins showed that GST-RP-L10, but not GST itself, co-precipitated with Flag-Src-SH3. A subsequent GST pull-down analysis confirmed that RP-L10 was associated with Src-SH3, suggesting that RP-L10 and Src protein interact

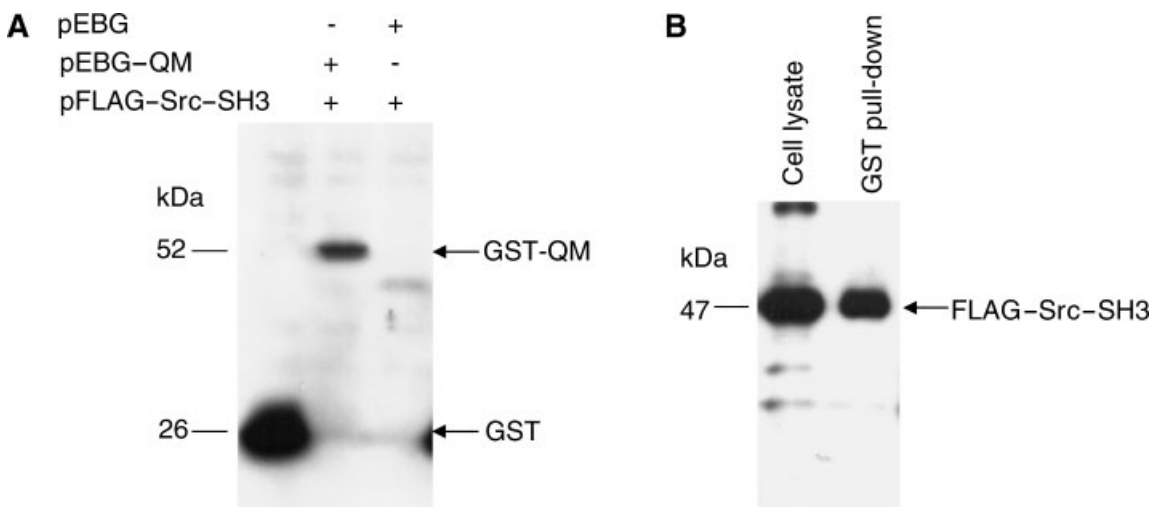

Fig. 1. RP-L10 interacts with Src-SH3. **A**: Immunoprecipitation was performed with anti-FLAG antibody and detected with anti-GST antibody by Western blot analysis (**lanes 2** and **3**). **Lane 1**, GST protein; **lane 2**, SH-SY-5y cells were co-transfected with pEBG-QM and pFLAG-Src-SH3; **lane 3**, SH-SY-5y cells were co-transfected with pEBG and pFLAG-Src-SH3. **B**: Co-transfected SH-SY-5y cell lysates were pulled down and detected with anti-FLAG antibody by Western blot analysis. **Lane 1**, lysates from cells co-transfected with pFLAG-Src-SH3 and pEBG-QM; **lane 2**, GST pull-down data of pFLAG-Src-SH3 and pEBG-QM co-transfected cell lysates.

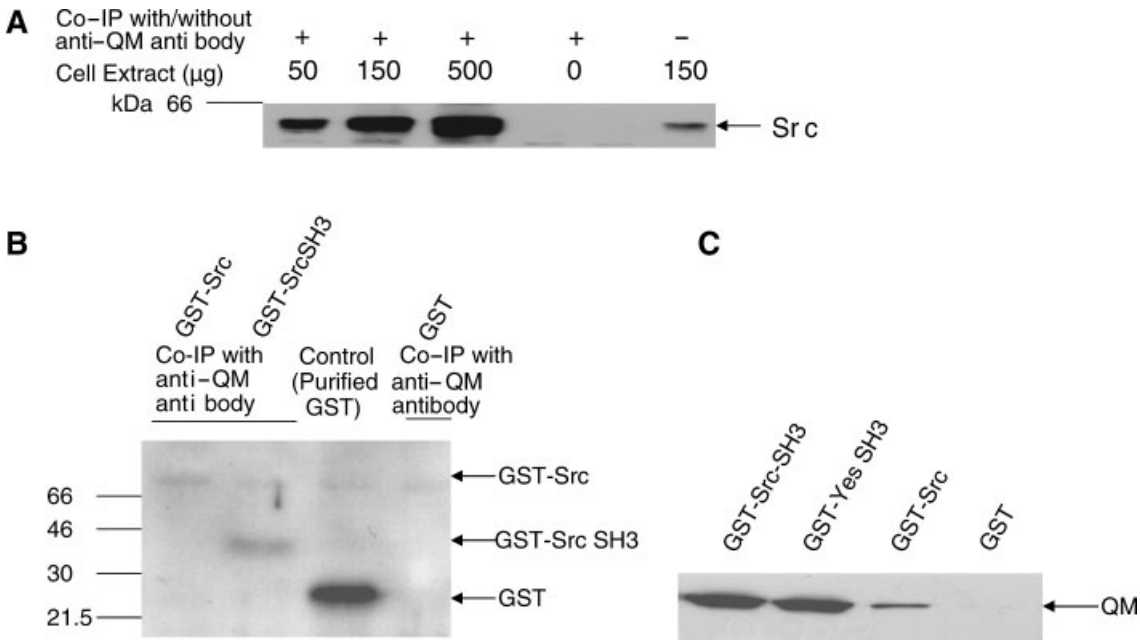

Fig. 2. RP-L10 interacts with Src under native conditions. Endogenous protein binding assays were performed between the RP-L10 and Src proteins in SH-SY-5y neuronal cells. **A**: **Lanes 1–3**, 50, 150, and 500 μg of native SH-SY-5y cell extracts were co-immunoprecipitated with anti-RP-L10 antibody and detection with anti-Src antibody; **lane 4**, control without cell extracts; **lane 5**, control without anti-RP L10 antibody. **B**: SH-SY-5y cells transfected with GST-fused protein [**lane 1**, GST-Src; **lane 2**, GST-Src-SH3; **lane 4**, GST] were co-immunoprecipitated with anti-RP L10 antibody followed by lysis and detected with GST antibody. **Lane 3**, purified GST. **C**: GST-pull-down assays were performed using SH-SY-5y cells transfected with GST-fused SH3 [**lane 1**, GST-Src-SH3; **lane 2**, GST-Yes-SH3; **lane 3**, GST-Src; **lane 4**, GST]. SH-SY-5y cell lysates were incubated with pulled-down GST-SH3 of Yes and Src-SH3, and detected with RP-L10 antibody.

with each other in the SH-SY-5y cell line under native conditions (Fig. 2). Not only did RP-L10 bind to an SH3 domain of Src, but also to the Src protein, although their binding intensities were different (Fig. 2B,C). As shown in Figure 2C, the SH3 domain of Src showed comparable binding intensities to those of c-Yes.

RP-L10 Binds to Src-SH3 Through More Than One Binding Site

To verify the binding region more specifically, several QM subclones were constructed and used for this purpose (Fig. 3). The SH3 domain is one of the well-known protein binding domains and several consensus amino acid sequences have been reported that normally contain prolin-rich residues such as PXXP [Kay et al., 2000]. The RP L10 sequence analysis showed that there are eight proline residues, but that PYP (aa16–18), which is known to be a conserved SH3 binding sequence, is the only proline-rich site of RP-L10. It has been proven in several crystallographic studies that a PYP amino acid fragment is not suitable for SH3 hydrophobic pockets [Oh et al., 2002]. There should be two amino acids between prolines to match the proline-rich binding groove of the SH3 domain [Feng et al., 1995; Nguyen et al., 1998]. The most feasible SH3 binding peptide consensus of RP-L10 is the RPARCYR (aa 4–10) fragment. It was reported that the VPMRLR peptide of YAP (Yes-associated protein) interacts with the SH3 domain of p53BP-2 (p53-binding protein-2) [Espanel and sudol, 2001]. The SPOT test revealed that the critical amino acids for binding to the SH3 domain are the valine, proline, and the arginine of the fourth residue, and that they are conserved in the R**P**A**R**CYR peptide except for the valine.

Figure 4 shows that Src-SH3 interacted with all of the RP-L10 fragments except for one fragment containing 52–90 amino acids. The more noteworthy result from our data is that the region containing 141–161 amino acid sequences promoted the binding intensity of RP-L10 with Src-SH3, as shown in lane 1 of Figure 4. Based on these results, we believe that there is more than one binding site involved in

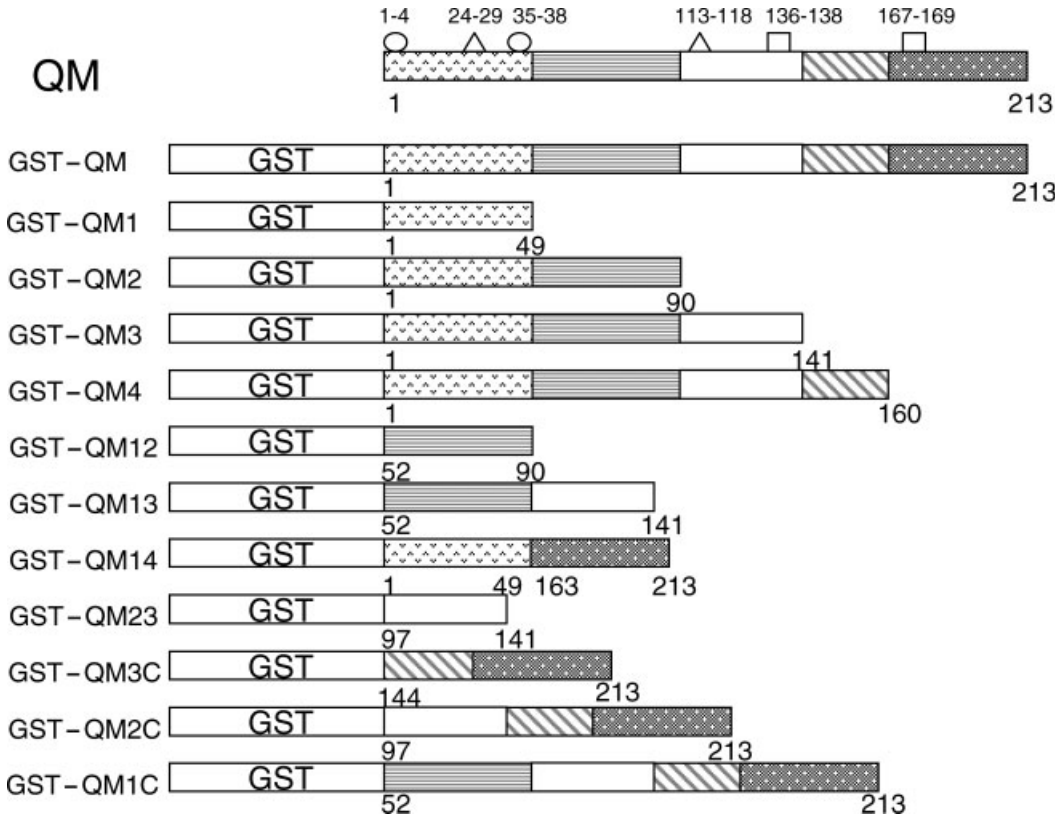

Fig. 3. Schematic diagram of RP-L10 constructs. The RP-L10 contains two PKC phosphorylation sites (□, 136–138 and 167–168), two N-myristoylation sites (Δ, 24–29 and 113–118) and two amidation sites (○, 1–4 and 35–38) [Oh et al., 2002]. All the numbers in the above figure indicate amino acid sequence numbers.

the association of RP-L10 with Src-SH3. It is likely that the RP-L10 region containing 141–160 amino acid sequences plays an important role in the binding of RP-L10 with Src-SH3, while the region containing 52–90 amino acids does not function as a binding motif. This result is in agreement with the report that PXR plays a critical role in the interaction of protein with SH3 domain [Espanel and sudol, 2001].

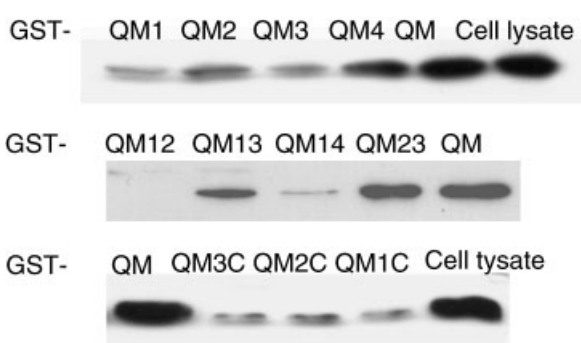

Fig. 4. The interacting regions of RP-L10 with Src-SH3. Subcloned RP-L10 in pEBG and pFLAG-Src-SH3 were co-transfected into the SH-SY-5y cell line and immunoprecipitation was performed with anti-GST antibody and detected with anti-Src antibody.

RP-L10 and Src-SH3 Protein are Endogenously Expressed in SH-SY-5y

The immunofluorescence data indicated that RP-L10 and the Src proteins were endogenously expressed in the human neuroblastoma cell line (Fig. 5). Our data also showed that RP-L10 and the Src proteins were localized in the cytoplasm.

Modulation of RP-L10 Expression Affects Neurite Outgrowth

To investigate the effects of RP-L10 on neuronal cell function, we used SH-SY-5y cells transfected with the pCMV-QM gene as an up-regulated model. In the case of RP-L10 transfectant and a matching control transfected with pCMV vector, we observed axon growth using optical microscopy in conjugation with FM1–43 fluorescent dye. As shown in Figure 6A, the overexpression of RP-L10 was detected by the observation of green fluorescence in the neuron transfectant. The overexpression of RP-L10

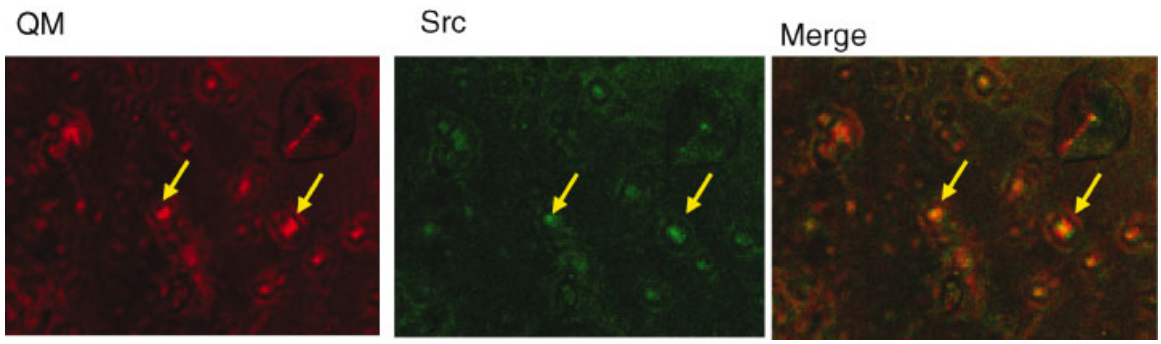

Fig. 5. Co-localization of RP-L10 and Src protein. Co-immunofluorescence was performed as indicated in the Materials and Methods section. RP-L10 is indicated by red-fluorescence and Src, by green fluorescence. Yellow indicates the co-localization of RP L10 and Src protein in the cytoplasm of the SH-SY-5y cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

did not lead to an obvious change in the axon length, but did seem to increase the amount of branching (Fig. 6B,C). Some of the QM-transfectant axons had three or more branches, whereas this was rarely seen in the controls. Next we investigated whether RP-L10 influences GDNF-induced neuonal axon growth. GDNF was applied at a concentration of 50 ng/ml to the control and QM-transfectant cultures for periods of 12 h up to 48 h. In the RP-L10 transfectant and matching control, we also observed axon growth. As shown in Figure 7A, in the QM-transfectant cultures which were treated with GDNF, the length of the axons averaged (37 ± 11) μm (n = 200) after 48 h, whereas the control neurons which were

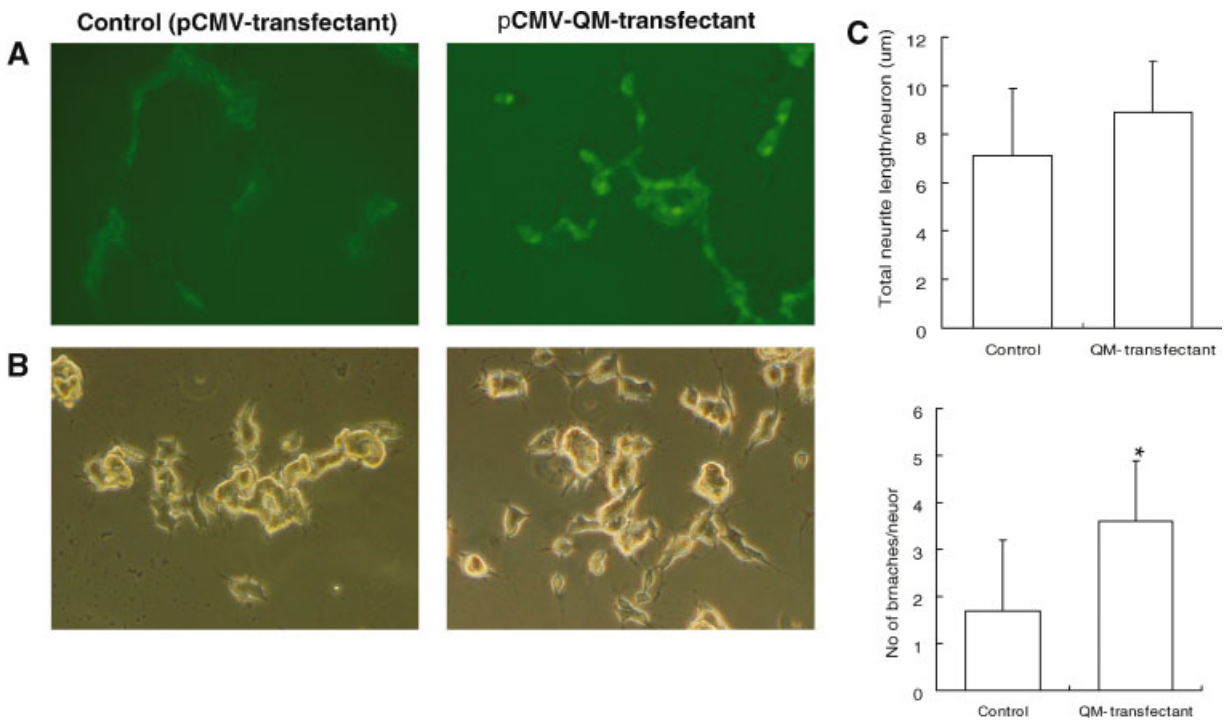

Fig. 6. Increase in axon branching caused by exogenous RP-L10 expression. **A**: Immunofluorescence detection of RP-L10 expression in SH-SY-5y cells. The cells were transfected with pCMV(control) or pCMV-QM and examined by fluorescence microscopy 1 day later. **B**: The morphological changes in neurite growth were examined by optical microscopy 48 h after transfection. **C**: Total neurite length and number of branches were measured as described in the Materials and Methods section. Data represent the mean ± SD of triplicate determinations, Asterisks (*) indicate $P < 0.05$ compared to the control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

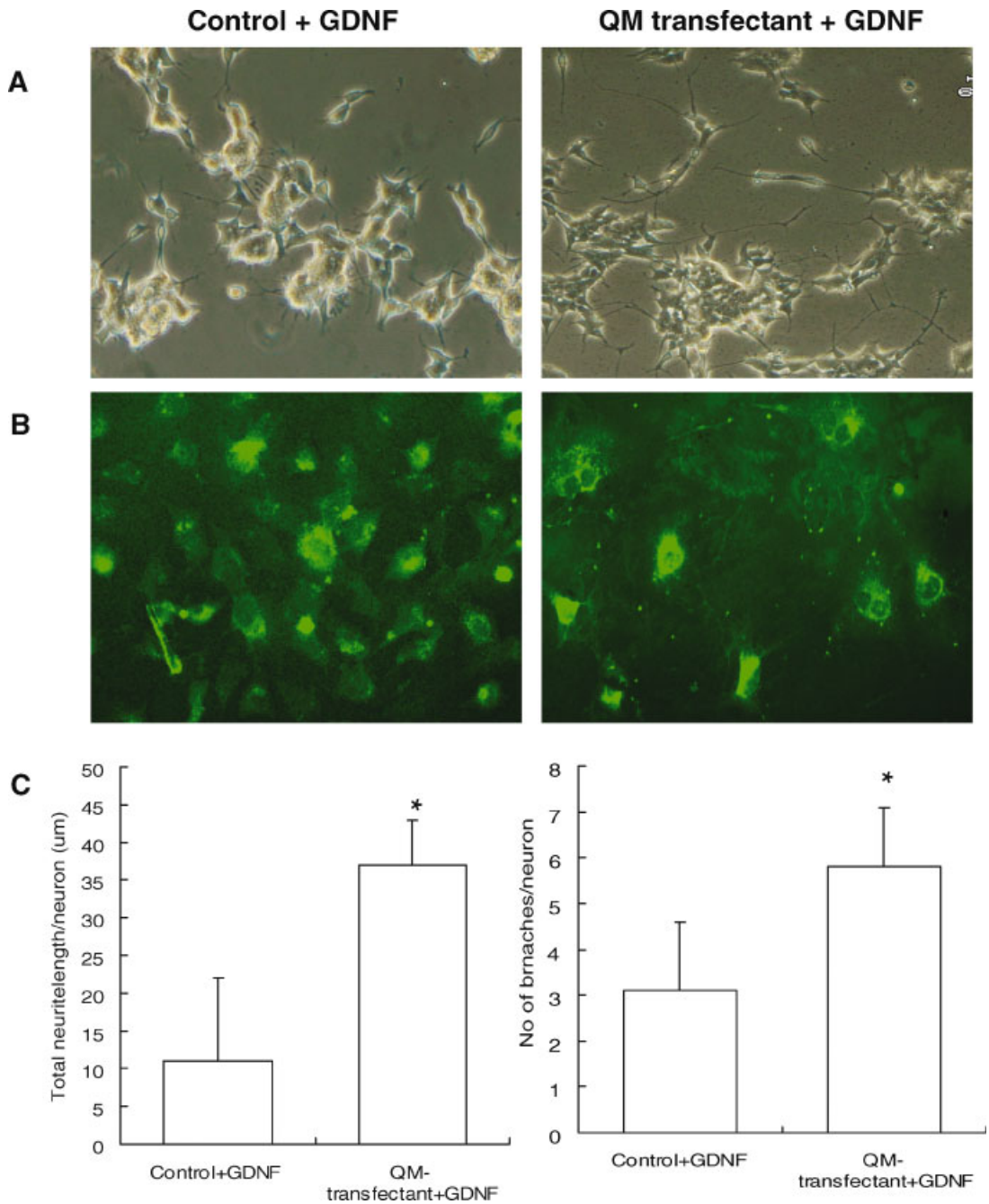

Fig. 7. The effect of ectopic RP-L10 expression on glial cell line-derived neurotrophic factor (GDNF)-induced neurite growth and vesicle formation. The cells were transfected with pCMV (control) or pCMV-QM and treated with 50 ng/ml GDNF. **A**: Morphological changes in neurite growth. GDNF was applied to the cultures 12 h after transfection (for periods of 12 h during 48 h), and the cultures were examined by optical microscopy. **B**: Immunofluorescence detection of RP-L10 expression in SH-SY-5y. The cells were examined by fluorescence microscopy followed by FM-1–43 staining to detect the intracellular vesicles. **C**: The total neurite length and number of branches were measured as described in the Materials and Methods section. Data represent the mean ± SD of triplicate determinations, Asterisks (*, **) indicate $P < 0.05$ and $P < 0.01$ compared to the control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

treated with GDNF averaged (11 ± 6) μm (n = 182) showing an increase of 70% (Fig. 7B). Approximately 67% of the GDNF-treated QM-transfectant axons had three or more branches compared with 11% for the GDNF-treated control, and 8% had five or more branches compared with 1% for control axons. Thus the increase in length and amount of branching induced by GDNF was greater in the QM-transfectant than the control. We also

found a difference in the endocytosis vesicle distribution (Fig. 7C), such that an increase in the total distribution of the vesicles induced by GDNF was observed in the QM-transfected axons. Thus, the overexpression of endogenous RP-L10 evidently increased the axon length and amount of branching induced by GDNF, while the effect of RP-L10 overexpression was observed in the form of branching activation in the absence of GDNF.

Overexpression of RP-L10 Affects Actin Polymerization

To determine whether endogenous RP-L10 was capable, in principle, of affecting actin polymerization, we examined the nucleation of F-actin in SH-SY-5y neuron cells transfected with the QM gene. Figure 8 shows that RP-L10 overexpression activated the nucleation of F-actin, induced by GDNF. Phalloidin staining revealed that the overall abundance and distribution of F-actin appeared to be dominant in the RP-L10 transfectants, indicating that RP-L10 also plays a role in the regulation of actin polymerization in neuronal cells. The above data suggest that RP-L10 could participate in a common regulatory pathway affecting F-actin dynamics.

Overexpression of RP-L10 Modulates Actin-Polymerizing Signal

Although many signaling proteins have been identified to be involved in the regulation of actin networks, we focused on a terminal signaling component, Nck1 in linking to the organization of actin cytoskeleton. It has been reported that Nck1 bound with the WASP and WIP, which are regulators of the actin cytoskeleton [Ramesh et al., 1999; Zigmond, 2000]. WASP promotes F-actin assembly by activating the ARP2/3 (actin-related protein) complex [Rohatgi et al., 1999]. To identify whether RP-L10 affects Nck1 binding with skeletal proteins such as WASP and WIP, co-immunoprecipitation was performed using anti-Nck1 antibody and QM transfectant. As shown in Figure 9, RP-L10 augmented the binding of Nck1 with both WASP and WIP.

DISCUSSION

Immunoprecipitation and the GST pull-down assay showed that RP-L10 binds to Src-SH3. To identify the regulatory role of RP-L10, the N- and C-terminal deletion mutants and several QM subclones were used. From the computer analysis (Compute pI/Mw, EXPASy) of RP-L10, it was predicted that the pI value of RP-L10 is greater than 11 and that there are four α-helices. The N- and C-terminal-deleted subclones were designed to have at least one α-helix (pEBG-QM2, pEBG-QM3, pEBG-QM4, pEBG-QM3c, pEBG-QM2c, and pEBG-QM1c). All these RP- L10 fragments and pEBP-QM1 were associated with Src-SH3. This indicates that there is more than one binding site in RP-L10.

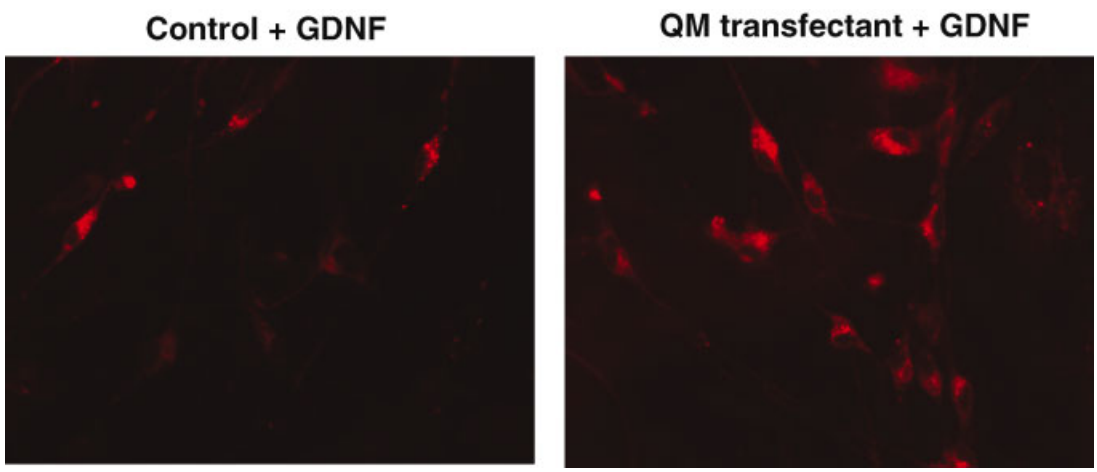

Fig. 8. The effect of RP-L10 overexpression on GDNF-induced actin polymerization. The cells were transfected with pCMV (control) or pCMV-QM and treated with 50 ng/ml GDNF. GDNF was applied to the cultures 12 h after transfection for the periods of 12 h during 48 h, and the cultures were stained for actin with phalloidin-Alexa 568 as described in the Materials and Methods section. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

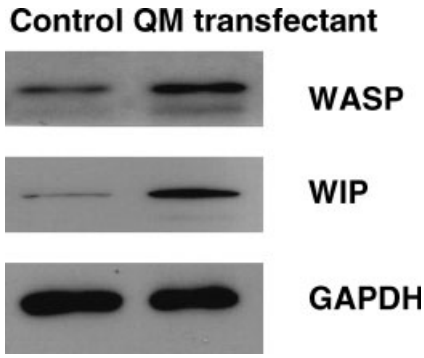

Fig. 9. The effect of RP-L10 overexpression on the binding of Nck1 with Wiskott-Aldrich Syndrome Protein (WASP) or WIP. The cells were transfected with pCMV (control) or pCMV-QM and lysed as described in the Materials and Methods section. The lysates were co-immunoprecipitated with anti-Nck1 antibody and analyzed by Western blot analysis.

To characterize the functional relevance of RP-L10 to cytoskeletal scaffolding during neuronal cell differentiation, herein we investigated the effect of ectopic RP-L10 expression. RP-L10 was found to positively regulate neurite branching, since RP-L10 transfectant exhibited increased branch formation. In addition, the overexpression of RP-L10 in SH-SY-5y cells activated GDNF-induced neurite outgrowth, indicating that RP-L10 has a stimulatory effect on GDNF-receptor Ret-dependent signaling. Overall, it is likely that RP-L10 interferes with different signaling pathways encompassing a number of SH3 partner proteins. In the past few years, a number of ligands for the SH3 domains of SFKs have been documented, including Dok, Gab1, Grb2, Rho, and RasGAP [Brown and Cooper, 1996].

Cytoskeletal growth in organisms, is controlled by the Rho GTPasee, including RhoA, Rac, and Cdc42 [Hall, 1998]. Activated Rho proteins can activate members of the WASP family [Mullins, 2000; Takenawa and Mike, 2001; Miyamoto et al., 2004]. Nck1 can bind directly to WASP, and these act cooperatively to stimulate WASP-Arp2/3-mediated actin polymerization. WASP and its homologuegs, N-WASP and the Wave/Scar proteins, promote F-actin assembly by activating the ARP2/3 (actin-related protein) complex [Rohatgi et al., 1999]. This regulation is essential for the formation of lamellipodia during axonal and dentritic growth in mammalian neurons [Banzai et al., 2000; Irie and Yamaguchi, 2002]. A previous study also suggested that Nck coordinates the assembly of an actin nucleation complex at the viral surface in the actin-based motility of Vaccinia virus [Frischknecht et al., 1999]. Recently, it was shown that the Nck SH3 domains stimulate the rate of actin nucleation by purified N-WASP in the presence of Arp2/3 [Rohatgi et al., 2001]. Therefore, to determine whether endogenous RP-L10 was capable, in principle, of affecting actin polymerization, we examined the nucleation of F-actin in SH-SY-5y neuron cells transfected with the QM gene. Our result shows that RP-L10 overexpression activated the nucleation of F-actin, induced by GDNF in Figure 8. These data suggest that RP-L10 could intricate a regulatory pathway affecting F-actin dynamics although further investigation to explore specific signaling components needs.

To identify whether RP-L10 affects Nck1 binding with skeletal proteins such as WASP and WIP, co-immunoprecipitation was performed using anti-Nck1 antibody and QM transfectant. As shown in Figure 9, RP-L10 augmented the binding of Nck1 with both WASP and WIP. However, when the GST-pull down assay was performed using pEBG-QM, neither the F-actin nor G-actin proteins were detected (data not shown). Thus, RP-L10 did not directly interact with actin assembly. These observations indicate that the binding of RP-L10 with Src-SH3 may contribute to the recruitment and/or activation of WASP and WIP so as to stimulate actin-based motility.

Thus, we suggest a possibility that the RP-L10 acts as a novel regulator protein which binds the SH3 containing Src and positively regulates neurite growth. This result provides a cellular function of RP-L10 in neuronal cells.

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