JSHC
Japan Society of Histochemistry and Cytochemistry
Established 1959

Acta Histochem. Cytochem. 42 (1): 9–14, 2009
doi:10.1267/ahc.08038

Localization of Reversion-Induced LIM Protein (RIL) in the Rat Central Nervous System

Yuko Iida[1], Toshiyuki Matsuzaki[2], Tetsuro Morishima[1], Hiroshi Sasano[1], Kiyofumi Asai[3], Kazuya Sobue[1] and Kuniaki Takata[2]

[1]Department of Anesthesiology and Medical Crisis Management, Nagoya City University Graduate School of Medical Sciences, Mizuho, Nagoya, Nagoya 467–8601, Japan, [2]Department of Anatomy and Cell Biology, Gunma University Graduate School of Medicine, Maebashi, Gunma 371–8511, Japan and [3]Department of Molecular Neurobiology, Nagoya City University Graduate School of Medical Sciences, Mizuho, Nagoya, Nagoya 467–8601, Japan

Received December 15, 2008; accepted January 7, 2009; published online February 25, 2009

Reversion-induced LIM protein (RIL) is a member of the ALP (actinin-associated LIM protein) subfamily of the PDZ/LIM protein family. RIL serves as an adaptor protein and seems to regulate cytoskeletons. Immunoblotting suggested that RIL is concentrated in the astrocytes in the central nervous system. We then examined the expression and localization of RIL in the rat central nervous system and compared it with that of water channel aquaporin 4 (AQP4). RIL was concentrated in the cells of ependyma lining the ventricles in the brain and the central canal in the spinal cord. In most parts of the central nervous system, RIL was expressed in the astrocytes that expressed AQP4. Double-labeling studies showed that RIL was concentrated in the cytoplasm of astrocytes where glial fibrillary acidic protein was enriched as well as in the AQP4-enriched regions such as the endfeet or glia limitans. RIL was also present in some neurons such as Purkinje cells in the cerebellum and some neurons in the brain stem. Differential expression of RIL suggests that it may be involved in the regulation of the central nervous system.

Key words: rat, brain, RIL, ependyma, astrocyte

I. Introduction

Reversion-induced LIM protein (RIL) is a member of the ALP (actinin-associated LIM protein) subfamily of the PDZ/LIM protein family, and has a PDZ domain in the N-terminus and a LIM domain in the C-terminus [for review see 15]. RIL was identified as one of LIM-domain proteins whose expression was down-regulated in transformed cells, and hence was considered to play a role in the control of cellular proliferation as a negative growth regulator [6]. In fact, expression of RIL was found down-regulated in prostate cancer [18]. Silencing or low level of expression of RIL in cancer cells was attributed to deletion or promoter methylation [2].

Through PDZ and LIM domains, RIL is considered to interact with many proteins. By binding to alpha-actinin through its PDZ domain, RIL interacts with actin cytoskeleton, and regulates stress fiber turnover [17]. RIL serves as an adaptor protein by binding to the AMPA glutamate receptor subunit GluR-A C-terminal peptide through its LIM domain and alpha-actinin via its PDZ domain [11]. By linking AMPA receptors to the actin cytoskeleton, RIL seems to regulate the dendritic transport of AMPA receptors in neuronal cells. RIL also serves in the anchoring of signaling molecules such as protein tyrosine phosphatase through its LIM domain [3] and C-terminus [19].

AQP4 is a member of water channel protein, aquaporin (AQPs) [13]. In the central nervous system, AQP4 is mainly expressed in astrocytes. AQP4 is concentrated in the

Correspondence to: Kuniaki Takata, Department of Anatomy and Cell Biology, Gunma University Graduate School of Medicine, Maebashi, Gunma 371–8511, Japan. E-mail: takata@med.gunma-u.ac.jp

Present address: (Toshiyuki Matsuzaki) Department of Anatomy and Neurobiology, Nippon Medical School, Graduate School of Medicine, Sendagi, Bunkyo-ku, Tokyo 113–8602, Japan.

perivascular endfeet surrounding blood vessels, and regulates the water transfer in the blood-brain barrier [13]. Concentration to the endfeet seems to be due to the binding to the PDZ domain of a scaffolding adaptor protein alpha-syntrophin [8]. AQP4 in astrocytes was also shown to be closely associated with the F-actin cytoskeleton [9, 10].

Northern blot analysis showed that RIL is expressed in a variety of tissues in the rat [6]. *In situ* hybridization in mouse tissues revealed that RIL expression was evident in the brain and certain epithelia. Preliminary immunofluorescence examination showed localization of RIL in epithelial cells in the mouse lung, stomach, and skin [3]. However, precise immunohistochemical localization of RIL protein has yet to be documented. In this work, we show the localization of RIL in the rat central nervous system and compared it with that of AQP4 [13].

II. Materials and Methods

Antibodies

Primary antibodies used were as follows: affinity-purified rabbit anti-AQP4 antibody [7]; goat anti-RIL antibody raised against C-terminal amino acids of rat RIL (Abcam, Cambridge, UK); mouse anti-glial fibrillary acidic protein (GFAP) monoclonal antibody (Millipore, Billerica, MA). Secondary antibodies used were as follows: Alexa 488-labeled donkey anti-goat IgG (Invitrogen, Carlsbad, CA, USA); Rhodamine Red X-labeled donkey anti-rabbit IgG (Jackson Immunoresearch); Rhodamine Red X-labeled donkey anti-mouse IgG (Jackson Immunoresearch).

Animals and tissue preparation

Male Wistar rats, 4 weeks of age, were used except otherwise stated. The protocol followed in this study was approved by the Animal Care and Experimentation Committee, Gunma University. For immunofluorescence histochemistry, rats were anesthetized by an intraperitoneal injection of sodium pentobarbital. They were perfused from the left ventricle with phosphate-buffered saline (PBS) followed by 3% paraformaldehyde in PBS. Specimens of the brain and spinal cord were removed, cut into slices of 3–5 mm thick, and further fixed in the same fixative overnight at 4°C. They were washed with PBS and immersed in a graded series of sucrose in PBS up to 20%. Samples were embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), rapidly frozen with liquid nitrogen, and stored at –80°C until use. Cryostat sections, 8–10-μm thick, were cut with a Leica CM 1900 cryostat (Vienna, Austria), mounted on MAS-coated glass slides (Matsunami, Osaka, Japan), air-dried, and used for immunofluorescent labeling.

Immunoblotting

Cortical astrocytes were prepared from postnatal cerebral cortices of Wistar rats (P1) and cultured as described previously [5]. Homogenate from the primary culture of astrocytes and the brain from adult female Wistar rats were homogenized and processed for immunoblotting according to the standard procedure as described [5].

Immunofluorescent staining

Immunofluorescent staining was carried out basically as previously described [1, 10]. Sections were washed three times in PBS for 15 min, and were then incubated with 2% bovine serum albumin (BSA) in PBS for 1 hr. Sections were first incubated with goat anti-RIL antibody overnight at 4°C, washed with PBS, and incubated with Alexa 488-labeled donkey anti-goat IgG for 1 hr at room temperature. For AQP4 labeling, specimens were further incubated with rabbit anti-AQP4 antibody for 1 hr at room temperature and Rhodamine Red X-labeled donkey anti-rabbit IgG. For astrocyte labeling, specimens were incubated with mouse anti-GFAP antibody for 1 hr at room temperature followed by incubation with Rhodamine Red X-labeled donkey anti-mouse IgG. Nuclear staining was performed by adding DAPI (4',6 diamidino-2-phenylindole) (Roche Diagnostics, Basel, Switzerland) [12]. After immunolabeling, sections were mounted as described [1]. They were examined with an Olympus BX-60 epifluorescence microscope (Tokyo, Japan) and images were recorded with a CoolSNAP K4 cooled CCD camera (Nippon Roper, Tokyo, Japan).

III. Results

Homogenates from the rat brain and cultured astrocytes were subjected to immunoblotting with antibodies to RIL. A 37-kDa-band, which is within the range of the predicted molecular weight of RIL (35.5 kDa) [11], was detected in both specimens, showing that RIL protein is expressed in these specimens (Fig. 1). In addition, a strong band in cultured astrocytes suggested that RIL might be concentrated in the astrocytes. These results demonstrated the specificity of the anti-RIL antibody and the presence of RIL protein in the brain.

We next examined the localization of RIL in the rat central nervous system by immunofluorescent labeling. In positive cells, labeling for RIL was mostly confined to the cytoplasm. In the cerebrum, RIL was concentrated along the ependyma (Fig. 2a). RIL was also rich in star-shaped cells. Double-labeling with astrocyte marker GFAP revealed that RIL was colocalized with GFAP, showing that RIL is expressed in the astrocytes (Fig. 2a, b). AQP4 is concentrated in the endfeet surrounding the blood vessels and the glia limitans of astrocytes [13]. Double-labeling studies showed that RIL was concentrated in the cytoplasm of astrocytes where GFAP was enriched as well as in the AQP4-enriched regions such as the endfeet or glia limitans (Fig. 2c). In the diencephalon, RIL was enriched in the epedymal cells lining the third ventricular wall as in other parts of the brain (Fig. 3). Double-labeling with AQP4 revealed that RIL was rich in blood vessels in the side wall of the third ventricle, but was scarce in those in the roof region (Fig. 3).

In the cerebellum, RIL was found in Purkinje cells and cells in the granular layer (Fig. 4). Molecular layers were weakly labeled, but no labeling in the medulla, showing that

oligodendrocytes did not express RIL. In the brain stem, RIL was highly concentrated in the epedymal cells lining the fourth ventricle (Fig. 5a). RIL was also found in the blood vessels. Some of neurons were strongly positive for RIL (Fig. 5b). The astrocytes were rarely labeled for RIL in this region. In the spinal cord, RIL was also highly concentrated in the epedymal cells lining the central canal (Fig. 6b). In addition, the astrocytes were positive for RIL (Fig. 6a). The expression of RIL in the central nervous system is summarized in Table 1.

IV. Discussion

We show in this work that the RIL protein is present in the rat brain by immunoblotting. This observation is in accord with the results of the Northern blot analysis that RIL is expressed in a variety of organs including the brain [6]. Some of the Purkinje cells in the cerebellum were positive for RIL. In situ hybridization revealed that mRNA for RIL was detected in subgroups of neurons including Purkinje cells in the rat brain [6]. Although all of the Purkinje cells were shown to have similar levels of RIL mRNA [6], we found the level of RIL protein varied from one Purkinje cell to another. The regulatory mechanism in the translation of

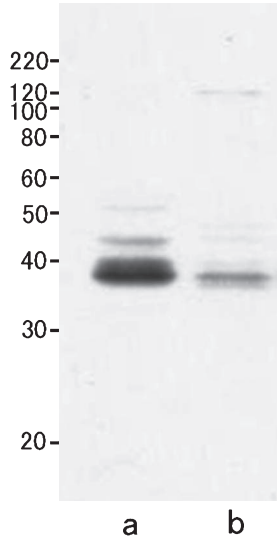

Fig. 1. Immunoblotting of astrocytes and brain with antibodies to RIL. Twenty micrograms of cultured astrocytes (**a**) and rat brain (**b**) homogenates were subjected to immunoblotting. Positions of molecular size markers (kDa) are shown to the left of the blot.

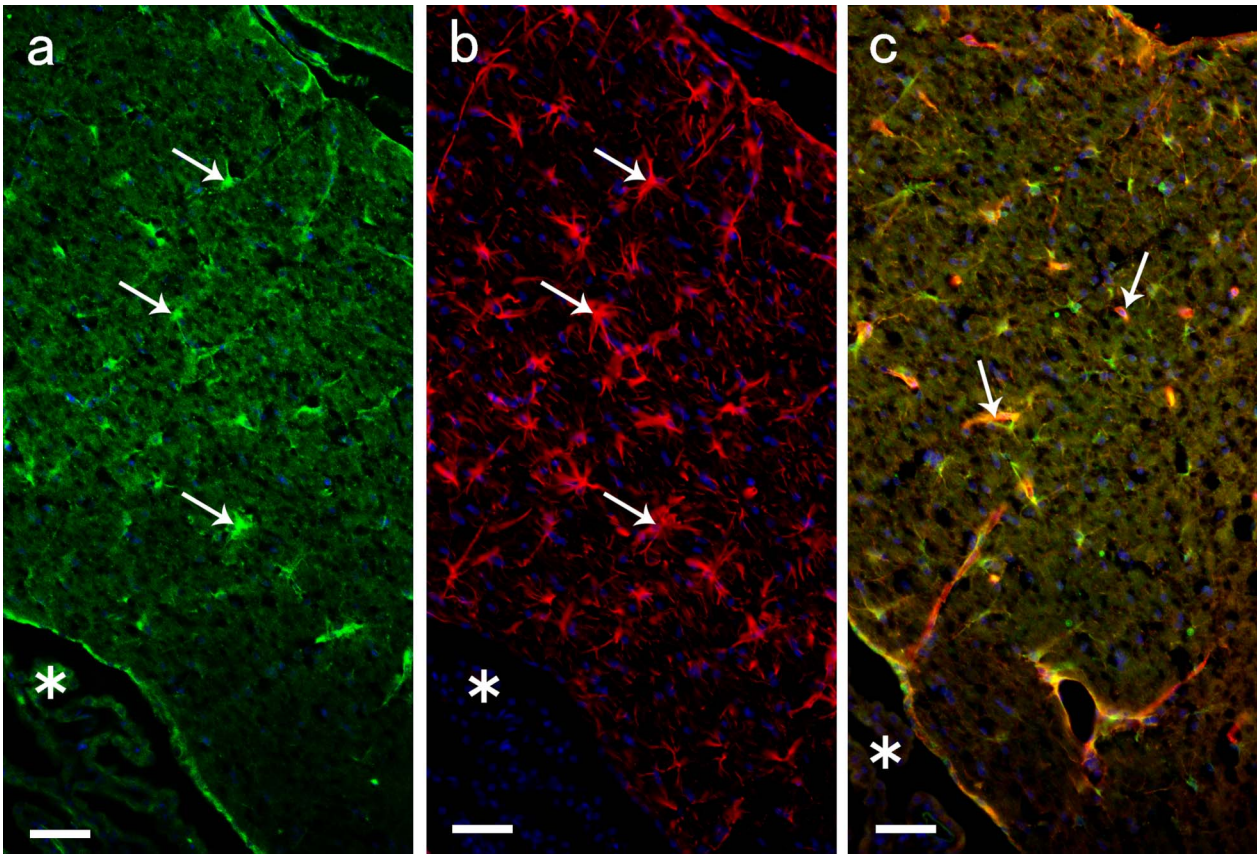

Fig. 2. Immunofluorescence localization of RIL, GFAP, and AQP4 in the fimbria of hippocampus. Nuclei were stained with DAPI (blue). *: choroid plexus in the lateral ventricle. (**a, b**) Double-labeling for RIL (**a**) and GFAP (**b**) shows that RIL is concentrated in astrocytes marked with GFAP labeling (arrows). (**c**) Double-labeling for RIL (green) and AQP4 (red) shows that RIL is present in blood vessels as well (arrows). Bars=50 μm.

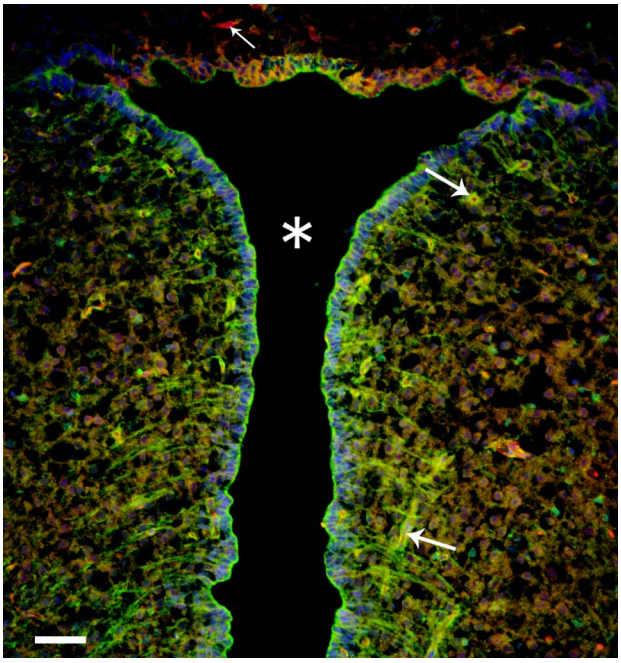

Fig. 3. Immunofluorescence localization of RIL (green) and AQP4 (red) in the third ventricle (*). Large arrows show blood vessels positive for RIL and AQP4. A small arrow indicates an AQP4-positive and RIL-negative blood vessel. Nuclei were stained with DAPI (blue). Bar=50 μm.

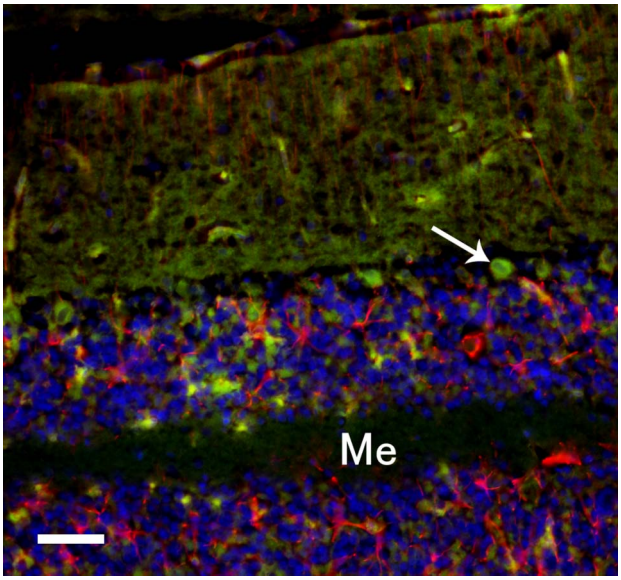

Fig. 4. Immunofluorescence localization of RIL (green) and AQP4 (red) in the cerebellum. Nuclei were stained with DAPI (blue). RIL is present in some of Purkinje cells (arrow). Me: medulla. Bar=50 μm.

RIL mRNA and/or RIL protein turnover may be responsible for the difference between the mRNA level and protein level. In the brain stem, some of the neurons were strongly positive for RIL as well. Differential expression of RIL might contribute to the differential regulation of neurons.

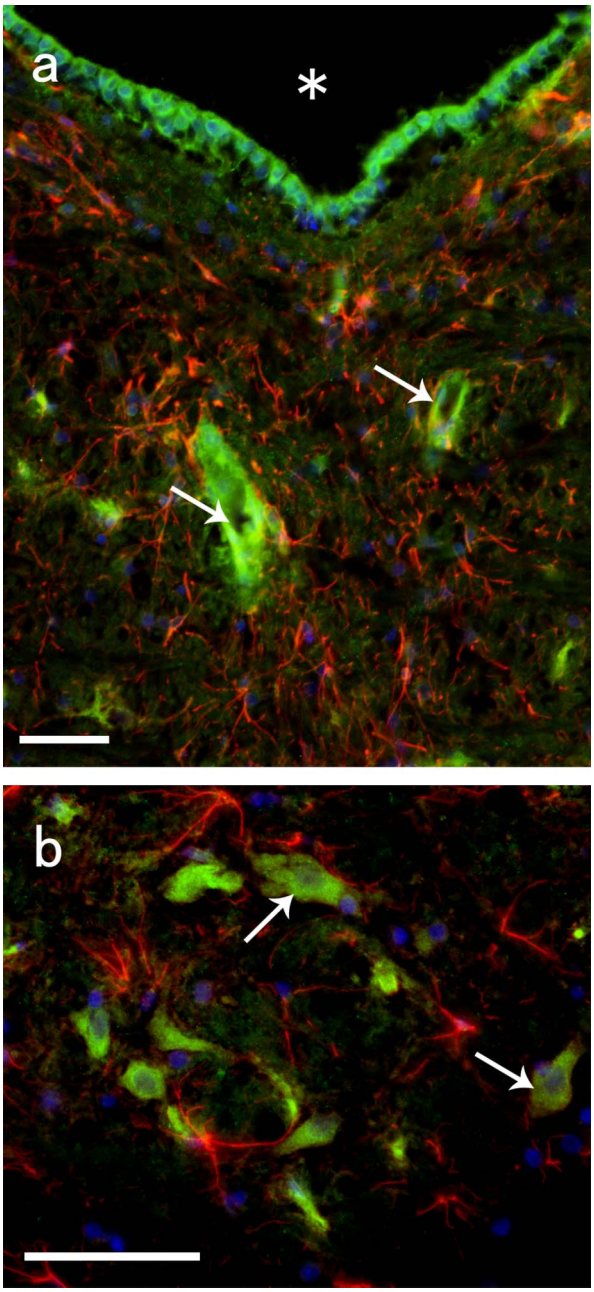

Fig. 5. Immunofluorescence localization of RIL (green) and GFAP (red) in the brain stem. Nuclei were stained with DAPI (blue). (**a**) RIL is concentrated in the ependyma and blood vessels (arrows). *: fourth ventricle. (**b**) RIL is abundant in some of neurons in the brain stem (arrows). Bars=50 μm.

Abundant RIL was detected in the primary culture of rat astrocytes. Double immunofluorescence microscopy with GFAP revealed the expression of RIL in the astrocytes in the brain and spinal cord. Double-labeling with AQP4 also confirmed the expression of RIL in astrocytes. Expression level of RIL was not uniform in the central nervous system as in neurons. RIL was not detected in astrocytes in the brain stem. The significance of the differential expression of

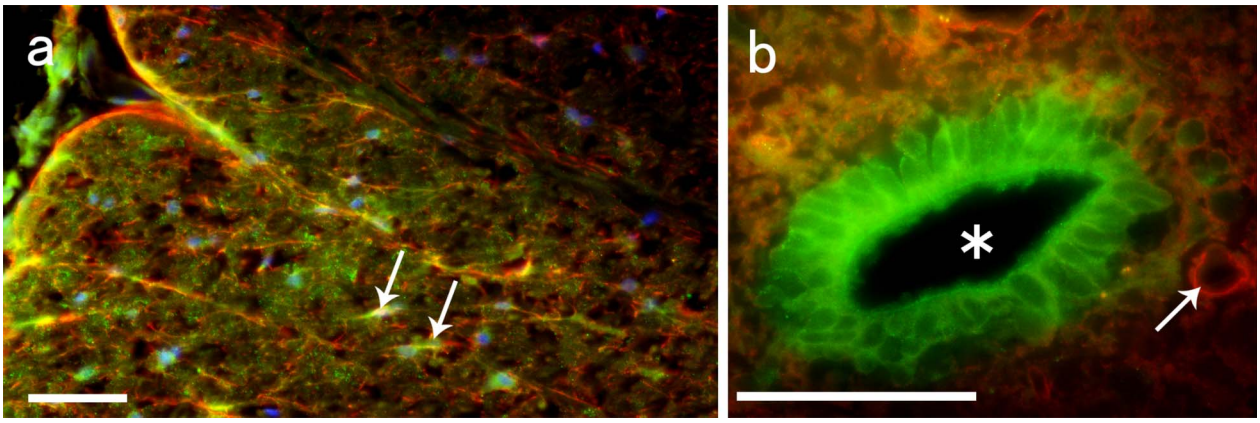

Fig. 6. Immunofluorescence localization of RIL, GFAP, and AQP4 in the spinal cord. (**a**) Double-labeling for RIL (green) and GFAP (red). RIL is present in astrocytes marked with GFAP (arrows). Nuclei were stained with DAPI (blue). (**b**) Double-labeling with RIL (green) and AQP4 (red). RIL is abundant in the epedyma. Blood vessel wall is positive for AQP4 (arrow). *: central canal. Bars=50 μm.

Table 1. *Expression of RIL in the central nervous system*

	Immunoreactivity to RIL	Remarks
Glia limitans	+++	
Blood vessel	±/++	strongly positive in the brain stem
Astrocyte	–/++	intensity varies; negative in the brain stem
Neuron	–/++	positive in Purkinje cells of the cerebellum and in neurons of the brain stem
Ependyma	+++	
Choroid plexus	–	

Immunoreactivity was scored from +++ (strongly positive) to – (negative).

RIL in astrocytes remains to be clarified.

In adult mouse tissues, RIL mRNA was detected in various epithelia, such as in the uterus and lung by *in situ* hybridization [6]. RIL protein was also found in the epithelia of the lung, stomach, and skin [3]. In the central nervous system, we found that the ependymal cells lining the ventricles in the brain and central canal in the spinal cord were positive for RIL by immunofluorescence microscopy, with stronger expression in the spinal cord and the brain stem. RIL in these epithelia may contribute to the shaping and organization of polarized epithelial cells by modulating actin cytoskeleton with its PDZ domain [3].

Since RIL binds to the cytoskeletal elements via its PDZ domain through alpha-actinin, RIL may be involved in the regulation of cytoskeletal organization in the central nervous system. RIL was proposed to play an important role in the transport of AMPA receptors in neurons [12]. In astrocytes, concentration of AQP4 to glia limitans and perivascular process is modulated by actin cytoskeleton [10]. Since actin cytoskeleton is important in regulating the localization and trafficking of water channels AQP2 in renal collecting duct cells [14], RIL may play a role in the localization of AQP4 in astrocytes by regulating their actin cytoskeleton.

CLP-36 (C-terminal LIM domain protein 36), another member of PDZ/LIM protein family, is expressed in nervous tissues in addition to RIL [16]. CLP-36 was also suggested to serve as adaptors to the cytoskeleton via alpha-actinin in non-muscle cells [16]. We used polyclonal antibodies raised against C-terminal 14 peptides of rat RIL in the present study. Although C-terminal amino acids of rat CLP-36 is distinct from that of rat RIL, it has an identical 3-amino-acid sequence. Therefore, we cannot exclude the possibility that we detected CLP-36 in addition to RIL. Further study is needed to clarify this point.

Although the exact function of RIL remains to be clarified, RIL seems to be involved in cellular growth and differentiation [4, 6, 13]. Differential expression levels in neurons, astrocytes, and blood vessel walls (Table 1) suggest that RIL might reflect the levels of differentiation and growth among them.

V. Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

VI. References

1. Ablimit, A., Matsuzaki, T., Tajika, Y., Aoki, T., Hagiwara, H. and Takata, K. (2006) Immunolocalization of water channel aquaporins in the nasal olfactory mucosa. *Arch. Histol. Cytol.* 69; 1–12.
2. Boumber, Y. A., Kondo, Y., Chen, X., Shen, L., Gharibyan, V., Konishi, K., Estey, E., Kantarjian, H., Garcia-Manero, G. and Issa, J. P. (2007) RIL, a LIM gene on 5q31, is silenced by methylation in cancer and sensitizes cancer cells to apoptosis. *Cancer Res.* 67; 1997–2005.
3. Cuppen, E., Gerrits, H., Pepers, B., Wieringa, B. and Hendriks, W. (1998) PDZ motifs in PTP-BL and RIL bind to internal pro-

tein segments in the LIM domain protein RIL. *Mol. Biol. Cell.* 9; 671–683.
4. Fu, S. L., Waha, A. and Vogt, P. K. (2000) Identification and characterization of genes upregulated in cells transformed by v-Jun. *Oncogene* 19; 3537–3545.
5. Fujita, Y., Yamamoto, N., Sobue, K., Inagaki, M., Ito, H., Arima, H., Morishima, T., Takeuchi, A., Tsuda, T., Katsuya, H. and Asai, K. (2003) Effect of mild hypothermia on the expression of aquaporin family in cultured rat astrocytes under hypoxic condition. *Neurosci. Res.* 47; 437–444.
6. Kiess, M., Scharm, B., Aguzzi, A., Hajnal, A., Klemenz, R., Schwarte-Waldhoff, I. and Schäfer, R. (1995) Expression of ril, a novel LIM domain gene, is down-regulated in Hras-transformed cells and restored in phenotypic revertants. *Oncogene* 10; 61–68.
7. Matsuzaki, T., Tajika, Y., Tserentsoodol, N., Suzuki, T., Aoki, T., Hagiwara, H. and Takata, K. (2002) Aquaporins: a water channel family. *Anat. Sci. Int.* 77; 85–93.
8. Neely, J. D., Amiry-Moghaddam, M., Ottersen, O. P., Froehner, S. C., Agre, P. and Adams, M. E. (2001) Syntrophin-dependent expression and localization of Aquaporin-4 water channel protein. *Proc. Natl. Acad. Sci. U S A* 98; 14108–14113.
9. Nicchia, G. P., Srinivas, M., Li, W., Brosnan, C. F., Frigeri, A. and Spray, D. C. (2005) New possible roles for aquaporin-4 in astrocytes: cell cytoskeleton and functional relationship with connexin43. *FASEB J.* 19; 1674–1676.
10. Nicchia, G. P., Rossi, A., Mola, M. G., Procino, G., Frigeri, A. and Svelto, M. (2008) Actin cytoskeleton remodeling governs aquaporin-4 localization in astrocytes. *Glia* 56; 1755–1766.
11. Schulz, T. W., Nakagawa, T., Licznerski, P., Pawlak, V., Kolleker, A., Rozov, A., Kim, J., Dittgen, T., Köhr, G., Sheng, M., Seeburg, P. H. and Osten, P. (2004) Actin/alpha-actinin-dependent transport of AMPA receptors in dendritic spines: role of the PDZ-LIM protein RIL. *J. Neurosci.* 24; 8584–8594.
12. Suzuki, T., Matsuzaki, T., Hagiwara, H., Aoki, T. and Takata, K. (2007) Recent advances in fluorescent labeling techniques for fluorescence microscopy. *Acta Histochem. Cytochem.* 40; 131–137.
13. Takata, K., Matsuzaki, T. and Tajika, Y. (2004) Aquaporins: water channel proteins of the cell membrane. *Prog. Histochem. Cytochem.* 39; 1–83.
14. Takata, K., Matsuzaki, T., Tajika, Y., Ablimit, A. and Hasegawa, T. (2008) Localization and trafficking of aquaporin 2 in the kidney. *Histochem. Cell Biol.* 130; 197–209.
15. te Velthuis, A. J. and Bagowski, C. P. (2007) PDZ and LIM domain-encoding genes: molecular interactions and their role in development. *Scientific World Journal* 7; 1470–1492.
16. Vallenius, T., Luukko, K. and Mäkelä, T. P. (2000) CLP-36 PDZ-LIM protein associates with nonmuscle alpha-actinin-1 and alpha-actinin-4. *J. Biol. Chem.* 275; 11100–11105.
17. Vallenius, T., Scharm, B., Vesikansa, A., Luukko, K., Schäfer, R. and Mäkelä, T. P. (2004) The PDZ-LIM protein RIL modulates actin stress fiber turnover and enhances the association of alpha-actinin with F-actin. *Exp. Cell Res.* 293; 117–128.
18. Vanaja, D. K., Ballman, K. V., Morlan, B. W., Cheville, J. C., Neumann, R. M., Lieber, M. M., Tindall, D. J. and Young, C. Y. (2006) PDLIM4 repression by hypermethylation as a potential biomarker for prostate cancer. *Clin. Cancer Res.* 12; 1128–1136.
19. van den Berk, L. C., van Ham, M. A., te Lindert, M. M., Walma, T., Aelen, J., Vuister, G. W. and Hendriks, W. J. (2004) The interaction of PTP-BL PDZ domains with RIL: an enigmatic role for the RIL LIM domain. *Mol. Biol. Rep.* 31; 203–215.