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Expression of Beta-catenin and Integrin-linked Kinase in the Mouse Sciatic Nerve

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In the present study, the expressions of beta-catenin and integrin-linked kinase (ILK) in the adult mouse peripheral nerve were investigated by means of immunoblotting analyses and electron microscopy using the immunogold pre -embedding method.

As a result, beta-catenin and ILK were shown to be expressed both in the axon and the Schwann cell of the myelinated and unmyelinated fibers. By electron microscopy, some molecules of beta-catenin and ILK tended to concentrate under the axolemma in the unmyelinated fibers, while these molecules were distributed in a scattered form throughout the axoplasm in the myelinated fibers. Concerning the cytoplasm of the Schwann cells, the loop region was too slender to detect whether beta-catenin and ILK were associated with the plasmalemma; however, beta-catenin and ILK were distributed diffusely without any relationship in regard to the plasmalemma or the cell organelles around the nucleus region. The density of beta-catenin and ILK around the nucleus region was greater than that within the nucleus region.

From these results, some molecules of beta-catenin mediate the axon-Schwann cell adhesion of the unmyelinated fibers, while other molecules are thought to be separated from the cadherin-catenin complex on the plasmalemma. Accordingly, it is hypothesized that the cell-cell adhesion property in the peripheral nerve is not strong but dynamic, and this adhesion is possibly regulated by ILK.

Axon-Schwann cell attachment in the peripheral nerve is mediated by various adhesion molecules including the cadherin superfamily and the immunoglobulin superfamily [e.g. neural cell adhesion molecule (N-CAM) and L1], whereas axon-basal lamina contact is for the most part arbitrated by integrins and laminin (2, 10, 12). Cadherin, a transmembrane glycoprotein, constitutes a superfamily of calcium-dependent intercellular adhesion molecules (28). At present, we have identified the localization of N- and R-cadherin in the normal unmyelinated and regenerating chick sciatic nerve (24, 26). The distal portion of the cytoplasmic tail of cadherin binds alpha-catenin *via* beta-catenin, and these molecules link as a complex to the actin cytoskeleton, thus influencing the firm cell-cell contact (22, 30, 31). Alpha E- and alpha N-catenin have been identified as subtypes of alpha-catenin (7,8,11,17), and both have been also shown to be expressed in the nerve fibers of the peripheral nervous system (PNS) (15,25), whereas these alpha-catenins are localized not under the plasmalemma but throughout the cytoplasm. This scattered distribution of alpha-catenins suggests that the cadherin-catenin complex in the PNS is not sufficient to mediate strong

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cell-cell adhesion. On the other hand, integrin is a family of heterodimeric receptors for cell-extracellular matrix adhesion (9). The expressions of integrin alpha-6/beta-4 and integrin alpha-1/beta-1 in the peripheral nerves have been already revealed (18,27). Integrin-linked kinase (ILK), a multidomain protein of a serine/threonine protein kinase, is localized in focal adhesion and interacts with the cytoplasmic domains of beta-1 or beta-3 integrin. When cells are forced to artificially overexpress ILK, then the expression of E-cadherin and beta-catenin in the intercellular adherence junction is suppressed (33), while the nuclear accumulation of beta-catenin increases (20). Under these circumstances, beta-catenin acts as the penultimate downstream component of the Wnt signaling pathway, which is regulated by signaling through ILK (20). Accordingly, it is hypothesized that ILK is expressed in the peripheral nerve and causes the separation of catenins from cadherin-catenin complex, however, there has so far been no report regarding the expression of ILK and the ultrastructural localization of beta-catenin in the PNS. Consequently, we investigated the expression and the ultrastructural localization of ILK and beta-catenin in the adult mouse peripheral nerve by means of immunoblotting analyses and electron microscopy using the immunogold pre -embedding method.

MATERIALS AND METHODS

Animals

Mouse (BALB/C) (8 weeks of age) was used in the present study. All animal experiments were conducted in accordance with the Guidelines for Animal Experimentation at Kobe University School of Medicine.

Immunoblotting analyses

The immunoblotting analyses were performed in a similar manner as previously reported (13, 19). Fresh nerve segments dissected from the mouse sciatic nerve were homogenized with an ultrasonic disruptor, and solubilized with lysis buffer [50 mM Tris-HCl (pH7.4), 0.5% (v/v) NP-40, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethyl sulphonyl fluoride (PMSF), 10 μg/ml leupeptin and 10 μg/ml aprotinin]. As a positive control, homogenates of mouse brain were used. The cell lysates were prepared by centrifugation at 12,000 rpm for 15 min to remove insoluble materials. The supernatants were eluted with Laemmli sample buffer. The whole cell lysates were then separated by SDS-PAGE, and transferred to polyvinylidene fluoride (PVDF) membrane filters (Immobilon, Millipore). The membranes were immunoblotted with rabbit anti-beta-catenin polyclonal antibodies (catalogue no. sc -7199) (Santa Cruz Biotechnology, CA), rabbit anti-beta-catenin monoclonal antibodies(catalogue no. 1247-S)(EPITOMICS,CA), goat anti-ILK polyclonal antibodies (catalogue no. sc -7516) (Santa Cruz Biotechnology, CA), rabbit anti-ILK monoclonal antibodies(catalogue no. 1979-S)(EPITOMICS,CA) using chemiluminescence reagent (Renaissance, NEN).

Immunocytochemical staining with the immunogold method

The animals were anesthetized by an intraperitoneal injection of Nembutal (sodium pentobarbiturate 50 mg/kg body weight) and fixed by transcardiac perfusion with a fixative containing 4% paraformaldehyde in 0.15 M NaCl in 100 mM phosphate-buffered saline (PBS) at pH7.5, supplemented with 1 mM $CaCl_2$ and 8% sucrose. The nerve segments were excised from the sciatic nerve and immersed in the same fixative as described above for 4 h at 4^{o}C. Fixed nerve segments were cryoprotected through a series of increasing sucrose concentrations (10%, 15%, 20%, and 25%) in 0.2 M NaCl in 50 mM Tris-buffered saline (TBS) at pH7.5, embedded in OCT compound (SAKURA, USA), quick-frozen, and sectioned at 8 μm thickness on a cryostat. These frozen sections were then mounted on slides,

washed in TBS supplemented with 1 mM $CaCl_2$ (TBS-Ca) and incubated with blocking solution (DAKO Protein Block Serum-Free, DAKO) for 30 min. Thereafter, the sections were further incubated for 24 h with anti-beta-catenin antibody diluted 1:30, or with anti-ILK antibody diluted 1:100 with PBS containing 0.005% blocking solution. As a control for nonspecific labeling, some sections were incubated with rabbit serum instead of anti-beta-catenin antibody, and with goat serum instead of anti-ILK antibody. After washing 3 times with PBS, all of the sections were incubated for 24 h with a goat anti-rabbit IgG Fab' fragment labeled with 1.4 nm gold particles (catalogue no.2004) (Nanogold, Nanoprobes, Inc.), or with a rabbit anti-goat IgG Fab' fragment labeled with 1.4 nm gold particles (catalogue no.2006) (Nanogold, Nanoprobes, Inc.) at a final dilution of 1:40. After washing, the tissue-bound gold particles were enhanced by incubation with a silver developer (HQ silver, Nanoprobes, Inc.) for 13 min in the dark. The sections were examined by light microscopy and were postfixed at 4°C in 1.0% OsO_4 in 0.1 M sodium cacodylate buffer for 90 min. After washing in 0.1 M sodium cacodylate buffer, the sections were dehydrated in a graded series of ethanol, followed by n-butyl glycidyl ether (QY-1), and embedded in Epon812. Ultrathin sections were then cut on an LKB Ultratome, stained with lead citrate, and examined with a HITACHI H-7100 electron microscope.

RESULTS

1)Immunoblotting analyses

On the immunoblots of the homogenates from the mouse sciatic nerve, as well as the brain homogenates used as a positive control, the anti-beta-catenin antibodies (sc-7199 and 1247-S) detected a band of 88 kD and the anti-ILK antibodies (sc -7516 and 1979-S) detected a band of 59 kD (Fig.1).

2)Immunocytochemical staining

***Control**

In the sections prepared as a control for nonspecific labeling, no immunoreactive labeling could be observed on the myelinated or unmyelinated fibers by light microscopy and electron microscopy.

***Light microscopy (Figs. 2a, b)**

In the immunocytochemical staining with beta-catenin, normal sciatic nerves showed immunoreactive stripes on a negative background by light microscopy. The immunoreactive stripes of beta-catenin and ILK were similar in appearance to each other. Among these stripes, thin stripes were observed to give an outline of the myelinated fibers characterized by the nodes of Ranvier, including the faint thick immunoreactive stripe of the myelinated axons. On the other hand, the faded immunoreactive stripes between the myelinated fibers were assumed to be unmyelinated fibers because of the electron microscopic findings as described below.

***Electron microscopy**

- **Beta-catenin (Figs. 3-5)**

According to findings of electron microscopy, the immunoreactivities for beta-catenin were generally detected in the cytoplasm of Schwann cells and the axoplasm of both myelinated and unmyelinated fibers.

In the myelinated fibers, positive immunoreactivities were observed in the axon and the outer loop or the lateral loop of the Schwann cells (Figs. 3, 4). These Schwann cell loops were slender in shape; therefore, it was difficult to detect whether the immunoreactivities were associated with the plasmalemma, or not. In contrast, around the nucleus region of the Schwann cells, the immunoreactivities were scattered throughout the cytoplasm without any

relationship with cell organelles, such as the Golgi apparatus, rough ER or vesicle. The density of the immunoreactivities within the nucleus was rather thin in comparison to that in the surrounding cytoplasm (Fig. 4b). The inner loop of the Schwann cells could not be distinguished from the axoplasm in this study; therefore, it was unclear whether the immunoreactivities existed in the inner loop or not. The immunoreactivities of the axon were distributed throughout the axoplasm. They were not concentrated under the axolemma, including the nodal and paranodal regions (Figs. 4a).

In the unmyelinated fibers, the immunoreactivities tended to accumulate under the axolemma, while some immunoreactivities were scattered throughout the axoplasm (Figs. 3a, 5a, b). The process of the Schwann cells which surrounded the unmyelinated axon was too slim to detect whether the immunoreactivities were associated or not with the plasmalemma. Around the nucleus region of the Schwann cells, the immunoreactivities were distributed throughout the cytoplasm without any relationship with cell organelles, such as the Golgi apparatus, rough ER or vesicle. The density of the immunoreactivities within the nucleus were rather scattered in comparison to that in the surrounding cytoplasm (Fig. 5b).

- **ILK (Fig. 6a, b)**

In the myelinated fibers, positive immunoreactivities for ILK were seen in the outer loop of the Schwann cell and the axoplasm (Fig. 6a). It was difficult to determine whether the immunoreactivities of the outer loops were associated with the area under the plasmalemma component or not, because the width of the loops was rather narrow. The inner loop of the Schwann cell was indistinguishable from the axoplasm in this study. There were no immunoreactivities in the myelin sheath. On the other hand, the immunoreactivities within the axon were not concentrated under the axolemma, but were located throughout the axoplasm.

In the unmyelinated fibers, the immunoreactivities tended to concentrate under the plasmalemma of the axon and the Schwann cell, whereas some immunoreactivities were scattered throughout the axoplasm (Fig. 6b).

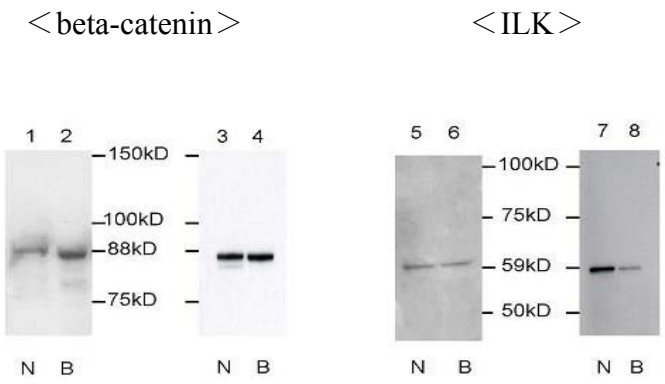

Fig.1.
Immunoblotting findings for the homogenates of the mouse sciatic nerve in lanes 1 and 3 and 5 and 7, and of brain as a positive control in lanes 2 and 4 and 6 and 8. The anti-beta-catenin antibodies detect a single band of 88kD on the lanes 1 and 2 and 3 and 4. The anti-ILK antibodies detect a single band of 59kD on the lanes 5 and 6 and 7 and 8.

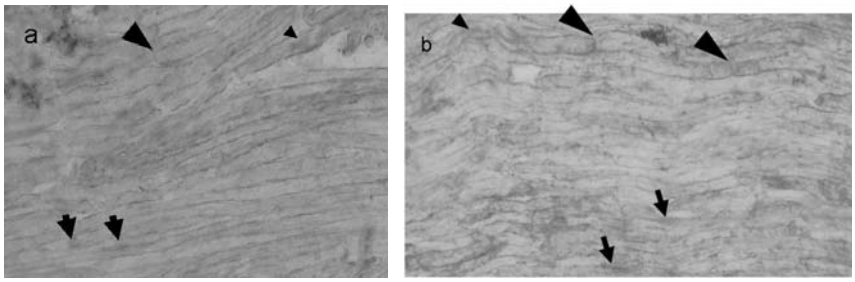

Fig.2.
A light micrograph showing a longitudinal section of a mouse sciatic nerve.
a) Immunostained with anti-beta-catenin antibody (×100).
The immunoreactivities appear to be various stripes in a negative background. Thin stripes giving an outline of the node of Ranvier (large arrowhead) are assumed to be the outer loop of the Schwann cells of the myelinated fibers, which include a faint thick stripe of the myelinated axon (small arrowhead). On the other hand, faint stripes, without any enclosure of thin stripes, are assumed to be the unmyelinated fibers (arrows), based on the electron microscopic findings.

b) Immunostained with anti-ILK antibody (×100).
This light micrograph reveals a normal sciatic nerve to be seen to have immunoreactive stripes in a negative background. The thin stripes correspond to the outlines of the myelinated fibers characterized by the nodes of Ranvier (large arrowheads) which contain a faint thick stripe of axon (small arrowhead), while the faded stripes between the myelinated fibers are assumed to be unmyelinated fibers (arrows), based on the electron microscopic findings.

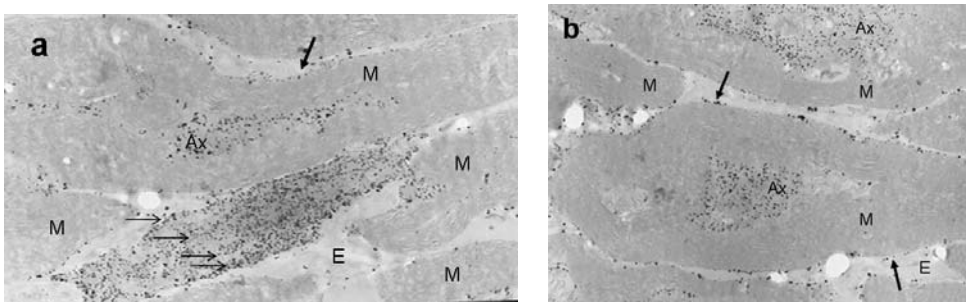

Fig.3.
Electron micrographs immunostained with anti-beta-catenin antibody.
a) A longitudinal section of the myelinated fibers and the unmyelinated fibers (×4000).
b) A cross section of the myelinated fibers (×3500).
The immunoreactivities are observed in the cytoplasm of the Schwann cells and the axons, while they are not detected in the myelin sheath (M) and the extracellular matrix (E). In the myelinated fibers, the immunoreactivities are shown in the outer loop of the Schwann cells (thick arrows) and the axoplasm (Ax). It is difficult to distinguish the inner loop of the Schwann cell from the axoplasm. The immunoreactivities in the myelinated axons are not concentrated under the axolemma, but are distributed diffusely throughout the axoplasm (Ax). In contrast, the immunoreactivities in the unmyelinated fibers tend to be concentrated under the plasmalemma of the axons and the Schwann cells (thin arrows).

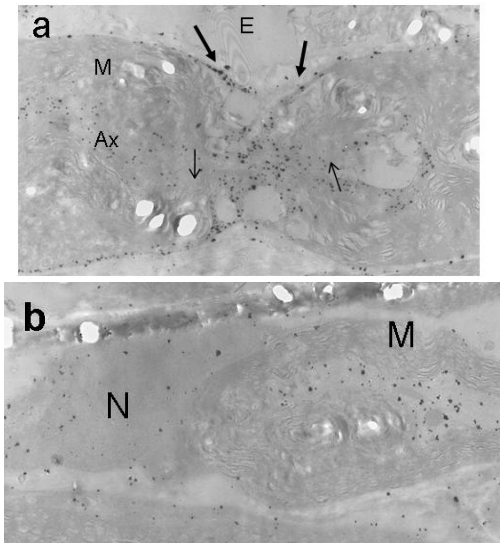

Fig.4.
Electron micrographs of the myelinated fibers immunostained with anti-beta-catenin antibody.
a) A longitudinal section of the node of Ranvier (×6000).
The diffuse beta-catenin immunoreactivities are found in the axoplasm (Ax) and the lateral loop of the Schwann cell (thick arrows). There is no concentrated distribution of the immunoreactivities at the paranodal axolemma (thin arrows) where the axon and lateral loop of the Schwann cells come in contact with each other.
b) A nucleus region (×6000).
A fewer scattered beta-catenin immunoreactivities are found in the nucleus region in comparison to the cytoplasm of the axons and the Schwann cells.
(M, myelin sheath; E, extracellular matrix)

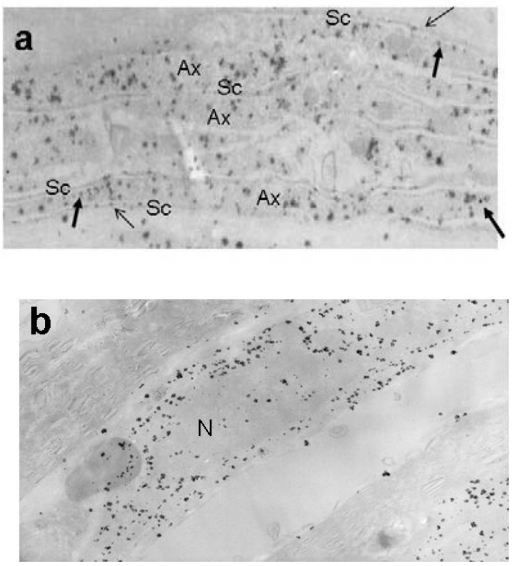

Fig.5.
Electron micrographs of the unmyelinated fibers immunostained with anti-beta-catenin antibody.
a) A longitudinal section (×8000).
The beta-catenin immunoreactivities are localized in the cytoplasm of the Schwann cells (thin arrows) and axons. In the axoplasm (Ax), there are diffuse beta-catenin immunoreactivities, while some immunoreactivities are located under the axolemma (thick arrows). (Sc, Schwann cell)
b) A nucleus region (×20000).
Beta-catenin immunoreactivities are sparser in the nucleus region (N) than in the surrounding cytoplasm of the Schwann cell.

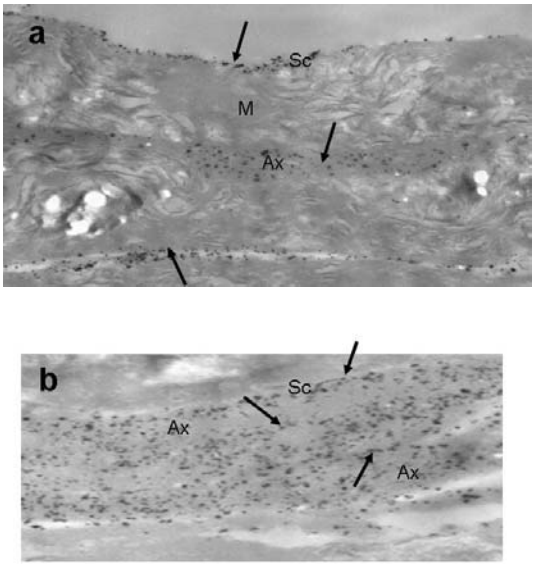

Fig.6.
Electron micrographs immunostained with anti-ILK antibody.
a) A longitudinal section of myelinated fibers (×9000).
The immunoreactivities are detected in the cytoplasm of the Schwann cell (Sc) and axoplasm (Ax), which are not concentrated under the plasmalemma, but they are distributed diffusely (arrows). The distribution of the gold particles is uniform and diffuse. No immunoreaction is detected in the myelin sheath (M).
b) A longitudinal section of unmyelinated fibers (×8000).
The immunoreactivities are detected in the cytoplasm of the Schwann cells (Sc) and axoplasm (Ax). Most of the gold particles are distributed uniformly and diffusely, while some particles are concentrated under the plasmalemma where the axon-Schwann cell contact occurs (arrows).

DISCUSSION

In this study, the expression of beta-catenin and ILK in adult mouse sciatic nerve was demonstrated by means of immunoblotting analyses, and the ultrastructural localization of beta-catenin and ILK in the cytoplasm of the axon and the Schwann cell was confirmed by electron microscopy using the immunogold pre-embedding method.

In normal epithelial cells, beta-catenin is assembled under the plasmalemma at the cell-cell contact, and binds cadherins to actin filaments to mediate strong cell-cell adhesion (1, 16, 21). On the other hand, the present study showed the diffuse beta-catenin distribution throughout the axoplasm in the myelinated fibers. The axolemma of the myelinated fibers

has been demonstrated to lack the expression of N-cadherin and R-cadherin (24, 26); therefore, beta-catenin is thought to separate from the axolemma because there is nothing to serve as an anchor on the axolemma. On the other hand, beta-catenin tended to become concentrated under the plasmalemma of the axon and the Schwann cell of the unmyelinated fibers. In addition, the axolemma of the unmyelinated fibers has been shown to express N-cadherin and R-cadherin (24,26); therefore, some beta-catenins are thus considered to come in contact with those cadherins. However, other beta-catenins are thought to be localized diffusely in the axoplasm of the unmyelinated fibers in this study. This diffuse distribution is similar to that of alpha N-catenin in our previous studies (15, 25). A dissociation of beta-catenin from the cadherin-catenin complex leads to obstruction in the cell-cell adhesion, because the cell-cell adhesive function is dependent on the beta-catenin interaction with the cytoplasmic domain of cadherins (1, 14). Accordingly, it is hypothesized that the axon-Schwann cell adhesion of the unmyelinated fibers is not strong, but it instead indicates a weak and a dynamic adhesive property. There have been some reports describing how beta-catenin separates from the cadherin-catenin complex. Tyrosine phosphorylation of beta-catenin causes a dissociation of beta-catenin from the cadherin-catenin complex (23), and the transcriptional activator of heterotrimeric G proteins of the G12 subfamily, comprised of Galpha12 and Galpha13, interact with the cytoplasmic domain of cadherins which induce the release of beta-catenin from the cadherin-catenin complex (14). Of course, it is unclear how beta-catenin actually separates from the plasmalemma of the unmyelinated fibers in the present study.

As for skin damage, there is a switch process that involves epithelial-mesenchymal transition (EMT) or mesenchymal-epithelial transition (MET), in which dynamic local position changes of adhesion molecules occur to switch the cell movement ability. In EMT, the activity of the cell-cell adhesion modality by cadherin is down regulated, while the activity of the cell-extracellular matrix modality by integrin is up regulated, and the reverse regulation arises in MET. In other words, if either cadherin or integrin functions, the other is down regulated and is distributed throughout the cytoplasm (5, 6). Integrin-linked kinase (ILK), a multidomain protein of a serine/threonine protein kinase, is thought to mediate those reversible regulations of cadherins and integrins. It is known that ILK, which becomes localized in the focal adhesion, interacts with the cytoplasmic domains of beta-1 or beta-3 of integrin; therefore, it is possible that the peripheral nerves are also regulated by ILK, because the expression of integrin alpha-1/beta-1 has already been revealed in the peripheral nerves (27).

By the overexpression of ILK or the amplification of the Wnt signaling pathway, beta-catenin shifts further into the nucleus. Under these circumstances, beta-catenin is thought to function as one of the transcriptional regulators (20). In this study, beta-catenin was found not to be accumulated, but to be scattered in the nucleus of Schwann cells. It is unclear, however, whether beta-catenin functions as the transcriptional regulator in the nucleus of the Schwann cells.

ILK accumulates within the intercellular adhesive site in the differentiated keratinocytes, while in undifferentiated keratinocytes, ILK is localized diffusely in both focal-adhesion and throughout the cytoplasm (32). In the early stage after birth (P day1-5), ILK is concentrated specifically in the cytoplasmic processes of the Schwann cells at the nodes of Ranvier in the peripheral nervous system during early myelination, whereas ILK in the oligodendrocytes (P day2) of the central nervous system is distributed throughout the cell body and the neural processes (4). If ILK is localized throughout the cytoplasm, then it is possible that ILK also plays an important role as the signal transmitter in cell proliferation, differentiation,

construction and reconstruction of the cytoskeleton, and control of the gene expression (3). In this study, ILK was localized throughout the axoplasm of the myelinated nerves. It is unclear why ILK is not concentrated under the axolemma, or whether ILK functions as the signal transmitter. The present study suggested dynamic local position change of ILK in the peripheral nerve; however, further study is needed for conclusive proof.

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