Localization and Characterization of Gelsolin in Nervous Tissues: Gelsolin Is Specifically Enriched in Myelin-forming Cells

Junya Tanaka and Kenji Sobue
Department of Neurochemistry and Neuropharmacology, Biomedical Research Center, Osaka University Medical School, Osaka 565, Japan

Gelsolin is a Ca**+-sensitive actin filament–severing protein. To elucidate the role of gelsolin in nervous tissues, we have investigated localization and expression of gelsolin in rat CNS and PNS using biochemical and morphological methods with a polyclonal antibody against the COOH-terminal fragment of plasma gelsolin. Immunohistochemical study showed that gelsolin was specifically enriched in oligodendrocytes and Schwann cells, and was also detected in myelin sheath, especially around the Ranvier’s nodes. The immunohistochemical stainings using indirect immunofluorescence, avidin-biotin-peroxidase complex, and immunogold methods were carefully confirmed by immunoblotting against the tissue homogenates. The expression changes of gelsolin in developing brain were investigated. The protein was detectable in newborn rat brain; however, it began to increase at 8–10 d after birth and reached maximal at 20–30 d when myelinogenesis actively occurred. After this period, the protein decreased gradually, although myelin basic protein was detectable in newborn rat brain; however, it began to increase at 8–10 d after birth and reached maximal at 20–30 d when myelinogenesis actively occurred. After this period, the protein decreased gradually, although myelin basic protein was increasing until 6 months after birth. The immunostaining of gelsolin in Schwann cells was enhanced upon regeneration of injured sciatic nerves by freezing. Immunoelectron microscopy revealed that gelsolin was present not only in the cytoplasm but also in compact myelin. Following solubilization by detergents, gelsolin in the myelin fraction could be purified using anion exchange and blue Sepharose column chromatographies. The purified protein possessed a Ca**+-dependent severing activity against actin filaments similar to that of cytoplasmic and plasma gelsolin. These data strongly suggest that gelsolin in nervous tissues might be involved in lamellipodial movement to wrap axons of myelin-forming cells by modulating actin polymerization.

[Key words: gelsolin, oligodendrocyte, Schwann cell, myelination, remyelination, actin, cell motility]

Although mechanochemical enzymes such as myosins participate in many cellular motile events, the actomyosin system might not be the only molecular base of motile force for cell locomotion. For example, cells in which gene coding myosin II was disrupted are mobile (De Lozanne and Spudich, 1987; Knech and Loomis, 1987) and immunocytochemical studies have revealed that the moving lamellipodia do not contain myosin II (Symons and Mitchison, 1991). Instead, cell movement might be dependent on cycles of assembly and disassembly of actin filaments (Bray and White, 1988) regulated by many kinds of actin-modulating proteins (Stossel, 1989). Among these proteins, gelsolin was originally isolated from macrophages in rabbit lungs (Yin and Stossel, 1979). It severs and caps actin filaments in a Ca**+-dependent manner, and is involved in cell motility (Cunningham et al., 1991). Besides Ca**+, phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP2) act as regulatory factors to dissociate the Ca**+-insensitive or EGTA-resistant binding between monomeric actin and gelsolin (Janmey and Stossel, 1987; Janmey et al., 1987). Judging from some reports on its localization in actively moving cells, gelsolin could be involved in many types of cell movement. In platelets, gelsolin might be involved in their shape changes during activation by thrombin (Hartwig et al., 1988; Hartwig, 1992). In cells transformed by Rous sarcoma virus, gelsolin concentrates in rosette contacts or podosomes (Gavazzi et al., 1989), which actively move on a time scale of minutes (Chen, 1989). Ruffling membrane, which also actively moves at the cell periphery, contains gelsolin (Cooper et al., 1988). These and other observations suggest gelsolin to be an essential factor for fast movement of cells.

In nervous tissue, active movement of many cell types is observed during synaptogenesis and myelogenesis of developing brain, or in inflammatory reactions in the damaged brain. The filopodia of neuronal growth cones are well known to move rapidly in a Ca**+-dependent manner (Sobue and Kanda, 1989). Microglial cells might contain gelsolin, since they are regarded as the macrophages of the CNS. It is likely that gelsolin is involved in these neural cell activities, taking into account the above findings in non-neural cells. The presence of a gelsolin-like molecule with an M, of about 90 kDa in brain in 1:1 complex form with actin has been reported (Nishida et al., 1981; Yin et al., 1981). Petruci et al. (1983) have purified the gelsolin-like protein from nervous tissue possessing Ca**+-dependent severing activity against actin filaments. They also demonstrated the presence of this protein in cultured neurons. However, a second report on the neural gelsolin-like protein has denied its actin filament–severing activity and named it “Cap 90” (Isenberg et al., 1983). Since both authors used very severe conditions to dissociate the gelsolin-like protein from actin, the separated protein might have been denatured. Other investigators have reported that the 1:1 complex prepared from bovine brain severed tropomyosin-bounded actin filaments (Verkhovsky et al., 1984) or stress fibers of cultured fibroblasts (Verkhovsky et al.,...
Conflicting data on the localization of the gelsolin-like protein in nervous tissues have also been presented. It was not detected in neurons (except transient immunostaining of Purkinje cells) but in oligodendrocytes of immature rabbit brain (Legrand et al., 1986). Recently, the latter group has identified the protein in many cell types of developing rabbit retina including ganglion cells, Müller cells, and photoreceptors (Legrand et al., 1991). In addition, a gelsolin-like protein from adrenal medulla has been purified, but with a smaller molecular weight than that of macrophage gelsolin (Ashino et al., 1987; Rodriguez del Castillo et al., 1990; Sakurai et al., 1990).

In the present study, we have investigated the localization and characterization of the gelsolin-like protein in nervous tissues to resolve the aforementioned conflicting results and questions, such as which cells contain the gelsolin-like protein in nervous tissues, whether the protein severs actin filaments in the presence of Ca2+, and in which neural function the protein is involved. To achieve enhanced immunohistochemical staining, we prepared a specific antibody against the COOH-terminal fragment of plasma gelsolin, containing the Ca2+-sensitive domain. The antibody specifically immunostained oligodendrocytes in brain and Schwann cells in peripheral nerves. Immunoreactivity was the strongest in rat brain at the period of 20–30 d after birth when myelogenesis actively occurred. This morphological study was carefully confirmed by biochemical methods. The gelsolin-like protein was purified from a myelin-enriched fraction. The purified protein had a severing activity against actin filaments similar to that of plasma gelsolin. We conclude that the protein is similar to plasma or macrophage gelsolin and hypothesize that it regulates movement of lamelipodia of the myelin-forming cells to wrap around nerve fibers by regulating actin assembly.

Materials and Methods

Purification of human plasma gelsolin and its fragment. Human plasma gelsolin was purified by a combination of two methods as previously reported. One method was based on the Ca2+-dependent affinity of gelsolin to a DEAE-cellulose column (Kurokawa et al., 1990). Gelsolin was specifically eluted from the column in a Ca2+-dependent manner. Another one was the specific elution of gelsolin from a blue Sepharose column with ATP (Yamamoto et al., 1989). Briefly, both methods were employed as follows. Human plasma separated from fresh blood (1000 ml) was fractionated between 35% and 50% ammonium sulfate saturation. After removing ammonium sulfate by dialysis, the fraction was applied to a DEAE-cellulose (DE-52, Whatman, Maidstone, England) column in the absence of Ca2+. Gelsolin was eluted with 1 mM Ca2+. Following dialysis to remove Ca2+, the eluted fraction was applied to a blue Sepharose (Pharmacia, Uppsala, Sweden) column. Gelsolin was eluted with 1 mM ATP (Sigma, St. Louis, MO). About 25 mg of plasma gelsolin was obtained by this procedure.

To prepare specific antibody against the COOH-terminal fragment of plasma gelsolin, a fast and easy method to separate the fragment from α-chymotrypsin (Sigma)-digested gelsolin was developed. Proteolytic digestion was performed as previously reported (Kwiatkowski et al., 1985), and the three fragments (NH2-terminal, central, and COOH-terminal fragments) were obtained. These digested fragments were applied to a blue Sepharose column equilibrated with Tris-EGTA buffer (10 mM Tris-HCl, pH 8.0, 20 mM KCl, 1 mM EGTA, 0.5 mM dithiothreitol, 10 μM para-aminodiphenylmethanesulfonyl fluoride hydrochloride (p-APMSF, Wako, Osaka, Japan)). The NH2-terminal fragments were specifically eluted with Tris-Ca2+ buffer (Tris-EGTA buffer containing 1 mM CaCl2 instead of 20 mM KCl). Preparation of antibodies against human plasma gelsolin. Anti-human plasma gelsolin antiserum was raised in New Zealand White rabbits. The antiserum was purified by an affinity column coupled with parent gelsolin, the COOH-terminal fragment, or NH2-terminal fragment. The procedure for affinity purification of antibody has been described elsewhere (Sakurai et al., 1988).

Immunoblotting to detect gelsolin in nervous tissues. For investigation on the distribution of gelsolin and its expression change during development of rat nervous tissues, electrophoretic samples of tissue homogenates were prepared. Before taking out brains or sciatic nerves, rats anesthetized with ether were perfused with chilled saline through the heart. Blood was completely washed out to avoid contamination of plasma gelsolin into the homogenates. After removing the brains, the brains were cut into several regions and weighed. Connective tissues around the sciatic nerves were carefully removed using a stereoscopic microscope. The tissues were minced and mixed with 10 or 20 vol of homogenizing buffer (20 mM Tris-HCl, pH 8.0, 40 mM NaCl, 50 μM p-APMSF, 10 μg/ml leupeptine) and homogenized using Polytron. In the study comparing the gelsolin content in the gray matter with that in the white, both tissues were punched out using glass capillaries. The homogenates were quickly dissolved in Laemmli’s sample solution (Laemmli, 1970). The protein concentration was determined with BCA protein assay kit (Pierce, Rockford, IL), and the serial samples for electrophoresis were adjusted to have equal protein concentrations. The samples were electrophoresed in the buffer system of Laemmli with SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting was performed as described elsewhere (Tanaka et al., 1993). For the developmental study, immunostained bands were quantitated by densitometry.

Immunohistochemical staining. Morphological studies with the anti-gelsolin antibody (antibody against the COOH-terminal fragment of gelsolin) were performed at both the light and electron microscopic level. In general, rats anesthetized with ether were fixed by transcardial perfusion for 30 min at room temperature with a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). For immunoelectron microscopy, 0.1% glutaraldehyde was included in the fixative. For the developmental study, the fixative contained 0.2% picric acid and 0.1% glutaraldehyde for better fixation of the tissue structure brain and the tissue was embedded (20 μm thick) using a microlicer (DTK-1000, Dousaka E.M., Kyoto, Japan), and washed in Tris-buffered saline (TBS, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl). For immunoelectron microscopy with colloidal gold–labeled antibody, sections 5–10 μm thick were prepared for better penetration of the antibody. Sciatic nerves were thin sectioned to 8 μm thick using a cryo-cryostat. In the immunohistochemical study, we employed indirect immunofluorescence, avidin-biotin-peroxidase complex (Elite ABC, Vector, Burlingame, CA) (Hsu et al., 1981), and immunogold methods. Although accumulation of biotin in oligodendrocytes has been reported (LeVine and Macklin, 1988), the avidin-biotin complex used in this study could not stain the cells unless both the anti-gelsolin antibody and the biotin-labeled secondary antibody were used (see Fig. 7G). Before immunoreaction, the sections were incubated in TBS containing 1% bovine serum albumin (BSA; crystallized and gelatin-free; Sigma). Following permeabilization with Triton X-100 (0.02–0.2%) for 6–24 hr at 4°C or at room temperature, the sections were incubated in TBS containing the primary antibody and BSA (1%) for 12–16 hr at room temperature. After extensive washing, the sections were incubated in TBS containing fluorescein isothiocyanate (FITC)–labeled anti-rabbit IgG (2%) (Sigma), rhodamine-labeled anti-mouse IgG (2%) (Sigma), or colloidal gold–labeled anti-rabbit IgG (10%) (E-Y Lab), or biotin-labeled anti-rabbit IgG (0.3%) (Vector). When the ABC method was employed, the sections were incubated in H2O2–methanol solution to destroy endogenous peroxidase activity, and then incubated in ABC solution (1%). The immunoreaction was visualized using 3,3-diaminobenzidine (0.02%) (DAB; Dojin, Kumamoto, Japan) and hydrogen peroxide (0.005%). Methods for double immunolabeling by indirect immunofluorescence have been described in detail elsewhere (Tanaka et al., 1993). Zeiss axiophot was used for light microscopic observation. For electron microscopy, the immunostained samples using the ABC or immunogold methods were postfixed with 1% OsO4, dehydrated in graded ethanol, flat embedded in Luveak 812 (Nakarai, Kyoto, Japan), and ultrathin sectioned. The sections were stained with lead citrate and uranyl acetate in a JEM 100CX electron microscope (JEOL, Tokyo, Japan) operated at 100 kV.

Nerve injury and regeneration. To investigate the role of gelsolin in peripheral nerves, the regenerating experiment was performed according to the method of Tashiro and Komiya (1991). After anesthesia with...
Figure 1. Distribution of gelsolin in nervous tissues of 8-week-old male rats was examined by immunoblotting with the anti-gelsolin antibody. The homogenates of the endbrain, the interbrain, the midbrain, the pons, the cerebellum, the medulla oblongata, and the spinal cord were applied on lanes a–g, respectively, of A. Fourteen micrograms of protein were loaded on each lane. Gelsolin predominantly distributed in inferior regions of the CNS. The contents of gelsolin in brain were compared between the gray and the white matter (B). Lanes a–d indicate the cerebral cortex, the corpus callosum, the hippocampus, and the middle cerebellar peduncle, respectively. The homogenate of the sciatic nerves was loaded on lane e of B. Note that gelsolin was predominantly detected in the white matter (lanes b and d). The protein was also present in the peripheral nerves. A 9% polyacrylamide gel was used for the myelin fraction, which was prepared according to the method of Norton and Poduslo (1973). Figure 1C shows that the myelin fraction prepared from 30-d- and 13-week-old rat brains, respectively. C shows the Coomassie brilliant blue-stained gel (15%), and the four arrowheads indicate MBP that was confirmed by immunoblotting using the anti-MBP antibody (D). E (9% gel) shows immunoblotting by the anti-gelsolin antibody. Numbers indicate M, kDa.

ether and pentobarbital, the sciatic nerves of rats (8 weeks old) were frozen by pressing with a stainless steel stick precooled in liquid nitrogen. At 5 d after the operation, the injured nerves were taken out and fixed in phosphate buffer containing 4% paraformaldehyde for 3 d at 4°C. After fixation, the nerves were thin sectioned by cryostat and immunostained using the ABC method as described above.

Preparation of myelin fraction and solubilization of brain gelsolin. The myelin fraction was prepared as described by Norton and Poduslo (1973), with slight modification. In solubilization experiments of gelsolin from the myelin fraction, we referred to a report on solubilizing myelin membrane (Alvadehdo et al., 1991) and tried Triton X-100, Lubrol PX (Sigma), n-octyl-@D-thioglucoside (Dojin), CHAPS (Dojin), and deoxycholic acid. Among the detergent, 1% Lubrol PX and deoxycholic acid solubilized most of gelsolin in the myelin fraction (data not shown). Brain gelsolin was also solubilized from the myelin fraction or the crude myelinated axon fraction prepared by the method of Lien et al. (1978).

Falling ball viscometry and electron microscopy on negatively stained specimens. To characterize the purified brain gelsolin, its effect on skeletal muscle actin was evaluated by falling ball viscometry and electron microscopic observation. The methods were described elsewhere (Ashino et al., 1987). The final assay conditions for both methods were as follows: brain gelsolin (4 pg/ml); actin filaments (1.2 mg/ml) or monomeric actin (0.7 mg/ml); in 20 mM PIPES, pH 7.0, 100 mM KCl, 2 mM MgCl2, 1 mM dithiothreitol, 1 mM EGTA, 1 mM ATP, and 10 mM p-APMSF. When the effects of gelsolin in the presence of Ca2+ were observed, 1.2 mM CaCl2 was included in the assay mixture. Following incubation at 23°C for 30 min, the apparent viscosity of the mixtures was measured. The mixtures were also negatively stained with 2% uranyl acetate solution and viewed by JEOL 100CX electron microscopy.

Other materials. Antibodies against myelin basic proteins (polyclonal) (MBPs), glial fibrillary acidic protein (GFAP) (polyclonal), and neurofilament 68 kDa protein (monoclonal) were purchased from Zymed (San Francisco, CA), Biomedia (Foster City, CA), and BioMakor (Rehovot, Israel), respectively. Rhodamine-labeled phalloidin for fluorescent staining of actin filaments was obtained from Sigma. Normal rabbit IgG for the control study was from Jackson Immunoresearch Lab (West Grove, PA). Actin was purified from rabbit skeletal muscle (Pardee and Spudich, 1982). Molecular weight markers were obtained from Bio-Rad (Richmond, CA).

Results
Preparation of antibody against the COOH-terminal fragment of plasma gelsolin
We first purified an antibody against the parent molecule of plasma gelsolin using the affinity column coupled with the purified protein. This antibody, however, cross-reacted with some proteins other than the 90 kDa band, or gelsolin in the rat brain homogenate (data not shown). Plasma gelsolin has an additional sequence in the NH2-terminus that is absent in the cytoplasmic gelsolin (Kwiatkowski et al., 1986). The COOH-terminal fragment possesses Ca2+-dependent actin-binding domain (Kwiatkowski et al., 1985), and is common between plasma and cytoplasmic gelsolins (Kwiatkowski et al., 1986). Therefore, we prepared the anti-COOH-terminal fragment–specific antibody from anti-gelsolin antiserum and the antibody was specifically cross-reacted with gelsolin in nervous tissues, as shown in Figure 1. By contrast, the anti-NH2-terminal fragment antibody preferentially cross-reacted with other proteins (data not shown). In the following studies, we used the antibody against the COOH-terminal fragment of gelsolin as anti-gelsolin antibody.

Distribution of gelsolin in nervous tissues—immunoblotting study
The distribution of the protein in nervous tissues was investigated by immunoblotting using the anti-gelsolin antibody. Although gelsolin is distributed throughout the CNS, the protein was more enriched in the inferior regions of the brain (the pons, the medulla oblongata, and the spinal cord) than in the cerebrum and the cerebellum (Fig. 1A). Figure 1B shows that gelsolin was predominantly present in the white matter as opposed to the gray matter (compare the immunoreaction between lanes a, c and b, d; the former are the gray matter, and the latter, the white). Gelsolin was also detected in the homogenate of rat sciatic nerves (Fig. 1B, lane e). We further examined its localization in the myelin fraction, which was prepared according to the method of Norton and Poduslo (1973). Figure 1C shows the Coomassie brilliant blue-stained gel (15%), and the four arrowheads indicate MBP that was confirmed by immunoblotting using the anti-MBP antibody (D). E (9% gel) shows immunoblotting by the anti-gelsolin antibody. Numbers indicate M, kDa.
Immunohistochemical staining of gelsolin in oligodendrocytes

To identify gelsolin-containing cells in rat brain, indirect immunofluorescence method was used with the anti-gelsolin antibody. Figure 2 shows the immunoreactive cells in the pons from 15-d-old rats. The cells have the characteristic morphology of oligodendrocytes, having few processes without any branching and a small cell body (about 20 µm). Note the enriched staining of myelin sheath, especially of Ranvier's nodes (arrows in Fig. 2A, D). Thick myelinated axons from the primary motoneurons in the facial nerve nucleus (Fig. 2A) and thin axons of the middle cerebellar peduncle (Fig. 2C) were both well stained. Ring-like staining could be seen in the transverse section of myelinated neurites (Fig. 2E). In the cell body, the cytoplasm staining was patchy (Fig. 2, arrowheads). The highly stained regions corresponded to Golgi complex, which was identified by immunoelectron microscopy (see Fig. 10).

Normal rabbit IgG did not stain any specific structures (Fig. 2B). Figure 3, A and B, shows the same field in the pons of the 21-d-old rat. The location of neuronal cell bodies and neurites was identified using monoclonal antibody against neurofilament 68 kDa protein (Fig. 3A). The positions of the neurons identified in Figure 3A are indicated in Figure 3B by asterisks. These micrographs show that gelsolin was below the detectable level in neurons. Next, we identified the location of astrocytes using anti-GFAP antibody. In Figure 3, C and D are the same field of the cerebellar white matter. Since both the anti-GFAP and the anti-gelsolin antibodies had been produced in rabbits, we performed double labeling using the combination of the ABC and indirect immunofluorescence methods. The ABC (Elite-ABC) method is 100 times more sensitive than indirect immunofluorescence. Therefore, after immunostaining by the ABC method with a very low concentration of the anti-gelsolin antibody, the brain section can be immediately immunostained with the anti-GFAP antibody.
antibody by the immunofluorescence method without washing out the former antibody. By comparing Figure 3, C and D, the absence of detectable gelsolin in astrocytes is clear. The specific enrichment of gelsolin in oligodendrocytes is compatible with the results of the immunoblotting study shown in Figure 1.

Figure 3, E and F, shows the same field of the pons stained by rhodamine-labeled phalloidin, to detect actin filaments (E), and by the anti-gelsolin antibody (F). Note the lack of rhodamine fluorescence in the cell bodies of oligodendrocyte (E, arrows), which are stained with the anti-gelsolin antibody (F). Alternatively, the processes and the myelin sheath from oligodendrocytes seemed to contain actin filaments. Such distribution of actin filaments is compatible with the previous observations on cultured oligodendrocytes (Kachar et al., 1986). The cells indicated by asterisks in Figure 3, E and F, might be astrocytes, which did not contain detectable actin filaments or gelsolin.

Localization of gelsolin in peripheral nerves
Gelsolin was localized in thin cytoplasm of Schwann cells in rat sciatic nerves with the enhanced ABC method using nickel ion (Fig. 4A). Significant immunostaining was not observed when normal rabbit IgG was used instead of the anti-gelsolin antibody (Fig. 4B). As seen in the brain, regions near Ranvier's nodes were densely immunostained by anti-gelsolin antibody (Fig. 4A, arrowheads). In addition, the antibody also immunostained
bubble-like structures (Fig. 4A, arrows). These structures are “neurokeratins,” which are composed of proteins in the myelin sheath of peripheral nerves (Adams et al., 1971). MBP was specifically localized in the neurokeratin (Fig. 4C). Therefore, gelsolin was localized in the myelin sheath of both CNS and PNS. Figure 4, D and E, indicates the same field of the sciatic nerve double stained with rhodamine-labeled phalloidin (D) and the anti-gelsolin antibody (E). In Figure 4E, the neurokeratin was not seen, probably due to a lower sensitivity of the indirect immunofluorescence method. As in the CNS, actin filaments were scarce in the cytoplasm of Schwann cells, although they were accumulated in the regions near the nodes (Fig. 4D, arrowheads).

Changes of gelsolin in developing brain

Changes in gelsolin content in developing rat brain were examined in the forebrain (the end and the interbrain) and the brainstem (the midbrain, thepons, and the medulla oblongata). Twelve brain homogenates were prepared from rats 1 d to 29 weeks old, of which protein concentrations were adjusted to be equal. The same volumes of homogenates were electrophoresed and immunoblotted by both the anti-gelsolin and the anti-MBP antibodies. Figure 5A shows the immunoblots by the anti-gelsolin antibody. Immunoreacted bands with 90 kDa were quantitated by densitometry, as shown in Figure 5B. Gelsolin was slightly detectable in 1-d-old rat brain, and began to increase at 8 d after birth in the brainstem and 10 d in the forebrain. The amount of gelsolin always altered earlier in the brainstem than in the forebrain. When gelsolin content began to increase, MBP simultaneously became detectable. The expression of gelsolin reached maximal 24 d after birth in the brainstem and 28 d in the forebrain, after which gelsolin content decreased gradually.

In contrast to the expresional changes of gelsolin, MBP increased at nearly a constant rate until 29 weeks after birth. These expresional changes of gelsolin and MBP were morphologically investigated. Forebrain sections were prepared from rats aged 3, 8, 15, and 21 d and 4, 8, 16, and 24 weeks, and immunostained by the anti-gelsolin and the anti-MBP antibodies (Fig. 6). Gelsolin-positive oligodendrocytes were clearly observed in the pons of 8-d-old rat brain (data not shown), but only small numbers of the gelsolin-positive cells were identified in the endbrain. Numbers of oligodendrocytes reacting to the anti-gelsolin antibody in the forebrain increased dramatically at 15 d after birth and were maximal at 4 weeks after birth. Thereafter, the gelsolin immunoreactivity decreased, though MBP was easily detectable. These immunohistochemical results are compatible with those of the immunoblotting study shown in Figure 5. Figure 7 also shows the gelsolin immunostaining of oligodendrocytes in developing brains. The immunoreaction at the myelin sheath was most evident in the 15-d-old rat brain (Fig. 7A), and the cell bodies were stained the strongest at 21-28 d after birth (Fig. 7B,C). Although the size of oligodendrocytes decreases with age in rats (Raine, 1989), they could be identified using the anti-gelsolin antibody even in the brain of a 6-month-old rat (Fig. 7F). Normal rabbit IgG did not significantly stain any structures (Fig. 7G).

Alteration of gelsolin immunoreactivity in Schwann cells accompanying nerve regeneration

The changes in gelsolin immunoreactivity of injured rat sciatic nerves were investigated. Damaged sciatic nerves were immunostained with the anti-gelsolin antibody using the ABC method. The upper panel of Figure 8 shows regenerating sciatic nerve. The arrow indicates where the nerve was damaged. Proximal
Figure 5. The developmental changes of gelsolin in the forebrain (the end- and interbrain) and the brainstem (the midbrain, the pons, and the medulla oblongata). Following preparation of 12 brain homogenates, the homogenates were immunoblotted with antibodies against gelsolin and MBP. The results of immunoblotting for gelsolin are shown in A. The numbers over the blots indicate the ages (day or week) of rats. The numbers on the left show $M_r$ (kDa). Slight cross-reaction with another band is detected in the early stage of forebrain development. The gelsolin bands were densitometred and the values are plotted along the y-axis against the rat age (day: the x-axis) (B). Only a small amount of gelsolin was detected in newborn rat brain, but the amounts dramatically increased 8–10 d after birth. MBP began to be detectable at the same period and continued to increase until six months. By contrast, the amount of gelsolin reached maximal at 20–30 d and decreased thereafter.
to the damage (left side of panel), the gelsolin immunoreaction was moderate. Immunoreaction increased to maximum around the damage point, and again became weaker distal to this point. In Figure 8D, slightly enhanced immunoreaction of gelsolin and some gelsolin-positive macrophages (arrowheads) are also observed. Figure 8E shows mostly short Schwann cells, which appear during nerve regeneration. The anti-gelsolin antibody stained these Schwann cells darker than normal cells. In the distal region, the destroyed myelinated axons predominate and the immunoreaction for gelsolin was weak (Fig. 8F). In the regenerating region, actin staining by rhodamine-phalloidin was also enhanced (data not shown). Normal rabbit IgG did not stain Schwann cells (Control).

Localization of gelsolin in oligodendrocytes at electron microscopic level

The localization of gelsolin in oligodendrocytes was further investigated by immunoelectron microscopy. Brains (pons) from 21-d-old rats were thin sectioned and immunostained using the ABC method before embedding. Figure 9A shows an interfascicular cell, a type of oligodendrocyte actively forming myelin sheath (Raine, 1989), surrounded by many myelinated axons. Besides dense staining of the cell body, the myelin sheaths were also gelsolin positive, which confirms the results shown in Figure 1C-E. Significant staining was not observed in the control experiment (Fig. 9B). Figure 9C shows two perineuronal satellite cells attaching to neurons (labeled “N”). The satellite cell is another type of oligodendrocyte, which presumably supports the neuronal metabolism (Raine, 1989). Although the satellite cells were also gelsolin positive, as a rule the immunoreaction was not as strong as that of interfascicular cells. The positive cells in Figure 9 do not possess the features of microglial cells. Although glutaraldehyde-containing fixatives caused considerable nuclear staining as shown here, oligodendrocyte nuclei were not stained in the brain fixed only with paraformaldehyde (see Fig. 2).
Figure 7. Oligodendrocytes in the developing forebrains immunostained with the anti-gelsolin antibody. A-F correspond to the bottom area of the right parietal lobes of brains from rats 15, 21, and 28 d and 8, 16, and 24 weeks old, respectively. Normal rabbit IgG does not stain oligodendrocytes (G) in 28-d-old rat brain. The immunoreaction in myelin sheath was the most evident in 15-d-old rat brain, after which the immunoreaction grows weaker with age. By contrast, oligodendrocyte cell bodies were immunostained, though they become smaller with age. Scale bar, 100 μm.

Purification and characterization of brain gelsolin

We have purified and partially characterized the gelsolin in the myelin fraction to elucidate whether it has the severing and capping activity against actin similar to that of cytoplasmic or plasma gelsolin. At first, the solubility of myelin gelsolin was examined using various detergents. The lower concentration of the detergents such as 0.1% Triton X-100 or deoxycholic acid solubilized only a small portion of gelsolin and MBP (data not shown). The higher concentration (1%) of deoxycholic acid, however, could solubilize most of the gelsolin in the myelin fraction.

Gelsolin was purified from the myelin fraction prepared by the axonal flotation method (Fig. 12). The crude fraction of myelinated axons prepared from 30-d-old rat brains (42 gm) was washed in water several times to remove the contents of the axons. The myelin fraction is shown in lane a of Figure 12A (silver-stained gel), and Figure 12B is immunoblotting for gelsolin. Proteins solubilized by deoxycholic acid are shown in lane b. After removing the detergent by dialysis, many insoluble substances appeared, but gelsolin was not precipitated by centrifugation (lane c). The dialyzed fraction was then applied to DE-52 anion-exchange column chromatography according to the method of Kurokawa et al. (1990). Polypeptides shown in lane d were not adsorbed to the column. The materials eluted from the DE-52 column with 1 mM Ca2+ are shown in lane e. Although plasma and cytoplasmic gelsolin have been reported to elute from the column in the presence of 1 mM Ca2+ (Kurokawa et al., 1990), the brain gelsolin was not eluted. By eluting the column with a buffer containing 1 M NaCl, the myelin gelsolin was recovered. Following dialysis to remove the salt, the eluted fraction (lane f) was applied to blue Sepharose column...
chromatography. A major part of gelsolin, however, was not adsorbed to the column (lane g), in contrast to plasma and cytoplasmic gelsolin. Adsorbed gelsolin was eluted by 1 mM ATP (lane h). The eluted fraction (2 ml) was mainly composed of gelsolin. Actin was not a contaminant of the purified gelsolin fraction, confirmed by dot immunoblotting using anti-actin polyclonal antibody (data not shown).

The severing and capping activity of the purified gelsolin was investigated (Figs. 13, 14). Monomeric or polymerized rabbit skeletal muscle actin was mixed with the gelsolin in the presence or absence of Ca\(^{2+}\). Following incubation, the apparent viscosity of the mixtures was measured. The gelsolin reduced viscosity of actin filaments only in the presence of Ca\(^{2+}\). When monomeric actin was polymerized in the presence of the gelsolin and Ca\(^{2+}\), viscosity was also reduced (Fig. 14). Under the same experimental condition, the human plasma gelsolin showed similar effects on actin (data not shown). Electron microscopy revealed that the myelin gelsolin fragmented actin filaments only in the presence of Ca\(^{2+}\) (Fig. 14).

**Discussion**

In the present study, we have investigated the gelsolin localization in the CNS and PNS employing immunohistochemical staining as well as biochemical procedure. The results indicate...
that gelsolin in the nervous system is specifically accumulated in the myelin-forming cells, which are oligodendrocytes and Schwann cells. No detectable immunostaining was observed in neurons and other classes of glial cells. The enrichment of gelsolin in oligodendrocytes in the CNS suggests that gelsolin can be used as a marker for the cells. Although many markers have been reported for myelin-forming cells such as galactocerebroside, cyclic nucleotide phosphohydrolase, and MBPs, a cytoskeletal marker has not been well characterized. When using the anti-gelsolin antibody, it is advantageous to stain the whole body of oligodendrocytes in immature rat brain, including their wrapping processes around the neurites.

A small amount of gelsolin was present in the cerebral cortex at embryonic day 18 (data not shown), before oligodendrocytes differentiate from the oligodendrocyte-type 2 astrocyte (O-2A) progenitor cells (Miller et al., 1985). Moreover, gelsolin was
hardly detected in oligodendrocytes even in postnatal brain before postnatal day 8-15 (Fig. 6). This suggests that a small amount of gelsolin is derived from other cell types. Recently, we have identified gelsolin localization in growth cones of dorsal root ganglion neurons and PC12 cells differentiated with NGF, although the content is not high (Tanaka et al., unpublished observations). Therefore, some gelsolin in pre- and perinatal rat brain might be originated in neurons. As gelsolin localization in fibroblasts has been reported (Cooper et al., 1988), gelsolin might be present in non-neural cells. Their content of gelsolin, however, seems far lower than that in the myelin-forming cells. When the cells in the primary culture system of rat dorsal root ganglion are immunostained with anti-gelsolin antibody, the staining of fibroblasts is far weaker than that of Schwann cells (Tanaka et al., unpublished observation).

The localization of gelsolin in compact myelin was evidenced in the immunohistochemical and the subcellular fractionation study. Low concentration of detergents such as 0.1% Triton X-100 could not solubilize gelsolin in the myelin fraction (data not shown), also suggesting that not only the soluble fraction around the Ranvier's nodes but myelin membrane itself contains gelsolin. This is an unusual distribution for gelsolin, considering the observation in non-neural cells that gelsolin near plasma membrane usually exists with actin filaments (Gavazzi et al., 1989; Hartwig et al., 1989). Gelsolin localization in compact myelin, however, might be reasonable, since myelin contains abundant polyphosphoinositide and enzymes related to phosphatidylinositol turnover (Saltiel et al., 1987), and gelsolin binds to polyphosphoinositide (Janney et al., 1992). Indeed, the relationship between polyphosphoinositide and myelogenesis has been suggested in developmental studies. In rat brain, the PIP kinase activity was elevated from day 13 after birth, when myelogenesis began (Salway et al., 1968). Shaikh and Palmer (1976, 1977) reported that deposition of PIP, correlated closely with myelogenesis in chick brain and sciatic nerves. The polyphosphoinositide in the myelin membrane might regulate gelsolin function.

Gelsolin-like protein has been detected in nervous tissues...
Figure II. The localization of gelsolin in compact myelin is shown using the immunogold method (A). Normal rabbit IgG does not stain myelin membrane (B). The sections shown here were stained with both lead citrate and uranyl acetate. The brain sections were prepared from the pons of a 21-d-old male rat. Scale bar, 200 nm.

(Nishida et al., 1981; Yin et al., 1981; Verkhovsky et al., 1984). Complete purification, however, was difficult because the protein was tightly linked to monomeric actin. Since SDS was used to separate the two proteins (Petrucci et al., 1983), full characterization of brain gelsolin has been difficult. In the present study, however, the protein was easily purified from the myelin fraction using two column chromatographies without any contamination of actin. This might be related to the abundance of polyphosphoinositide in the myelin fraction; that is, the EGTA-resistant actin–gelsolin complex might be dissociated by PIP or PIP₂ (Janmey et al., 1987).

The myelin gelsolin is not only a capping protein, as has been suggested (Isenberg et al., 1983), but also it can sever actin filament. Although the Ca²⁺-dependent severing activity was similar to that of cytoplasmic or plasma gelsolin, it has not been elucidated whether the protein is the same as other gelsolin. The molecular weight of the brain gelsolin was not distinguishable from that of gelsolin in other tissues such as lung or stomach (data not shown). The behavior of the gelsolin against the column chromatographies, however, was different. In particular, it seems interesting that the protein could not be eluted from the DE-52 column by the Ca²⁺ buffer, although the plasma and cytoplasmic gelsolin were eluted under the same condition (Kurokawa et al., 1990). The remarkable accumulation in the myelin membrane might suggest its difference from gelsolin in nonneural cells, considering that gelsolin has been purified from soluble fraction of macrophages. The primary structure should be determined to elucidate whether the brain gelsolin is the same as the cytoplasmic gelsolin. Since the brain gelsolin can be purified from the myelin fraction of bovine spinal cord using a method similar to the one described here (data not shown), we are going to compare the properties of the protein with those of bovine plasma gelsolin.

The brain gelsolin might be involved in myelinogenesis, in view of several present findings, including its enrichment in myelin-forming cells and its elevated expression during myelinogenesis and remyelination. The severing, capping, and nucleating activity of gelsolin might be important for protrusive movements of cells (Oster and Perelson, 1987), because the protein can alter the intracellular osmotic pressure by remodeling the actin networks with gelation factors such as actin-binding protein (ABP or filamin) (Ito et al., 1992). During myel...
Figure 13. Effects of the brain gelsolin on actin were evaluated by falling ball viscometry. The apparent viscosity of actin filaments (F-Actin) incubated with or without gelsolin and Ca\(^{2+}\), and the viscosity of the mixture are shown in the three columns to the left. Monomeric actin (G-Actin) was polymerized in the presence of Ca\(^{2+}\) with or without gelsolin and viscosity of the mixture is shown in the two columns to the right. The apparent viscosity is expressed along the y-axis (centipoise, c.p.) with averaged value and standard error bar calculated from the data of six experiments. The assay conditions are described in Materials and Methods.

Figure 14. Severing activity of the brain gelsolin was examined by electron microscopy. The gelsolin and actin filaments with or without Ca\(^{2+}\) were mixed and incubated. The mixtures were then negatively stained and viewed by electron microscopy. A shows the fragmented actin filaments in the presence of Ca\(^{2+}\). Without Ca\(^{2+}\), actin filaments seem intact in the presence of gelsolin (B). Scale bar, 200 nm.

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