Intermediate Filament Protein Partnership in Astrocytes*

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Intermediate filaments are general constituents of the cytoskeleton. The function of these structures and the requirement for different types of intermediate filament proteins by individual cells are only partly understood. Here we have addressed the role of specific intermediate filament protein partnerships in the formation of intermediate filaments in astrocytes. Astrocytes may express three types of intermediate filament proteins: glial fibrillary acidic protein (GFAP), vimentin, and nestin. We used mice with targeted mutations in the GFAP or vimentin genes, or both, to study the impact of loss of either or both of these proteins on intermediate filament formation in cultured astrocytes and in normal or reactive astrocytes in vivo. We report that nestin cannot form intermediate filaments on its own, that vimentin may form intermediate filaments with either nestin or GFAP as obligatory partners, and that GFAP is the only intermediate filament protein of the three that may form filaments on its own. However, such filaments show abnormal organization. Aberrant intermediate filament formation is linked to diseases affecting epithelial, neuronal, and muscle cells. Here we present models by which the normal and pathogenic functions of intermediate filaments may be elucidated in astrocytes.

Intermediate filaments (IFs)* represent the least understood part of the cytoskeleton. These 10-nm thin structures form an interconnected scaffolding-like network within the cell cytoplasm. IFs are composed of different IF proteins, depending on the cell type, the developmental stage, and in some cases also on the functional status of a given cell (1). This can be well demonstrated in astrocytes, in which three IF proteins may be found: nestin, vimentin, and glial fibrillary acidic protein (GFAP). Nestin and vimentin are the main IF proteins in immature astroglial cells, whereas maturing and adult astrocytes contain vimentin and GFAP. Nestin production is resumed, and vimentin and GFAP expression is up-regulated in activated astrocytes in reactive gliosis accompanying, e.g., trauma, tumor growth, or neurodegenerative diseases affecting the CNS (2, 3).

The reasons for the existing diversity of IF proteins remain an enigma as much as the functions of IFs themselves. Analyses of animal and human mutations affecting IFs have in the recent years brought some insight into general and specific functions of IFs, at least in some cell types. Keratin IFs in the epithelial cells constituting the epidermis have been shown to confer mechanical resistance to the skin, and a number of mutations affecting keratin genes and preventing proper assembly of IFs have been shown to be associated with human skin blistering diseases. Thus, mutations affecting K5 or K14, the keratin pair expressed in the basal layer of the epidermis, lead to epidermolysis bullosa simplex, and mutations in K1 or K10, the keratins in the adjacent spinous layers, result in epidermolytic hyperkeratosis (1, 4–6). Neurofilaments, the IFs of neuronal cells, seem to modulate axonal caliber (7–12). Desmin, which forms IFs in muscle cells, has been shown to be crucial for long term structural maintenance of all three types of muscles (13–17). The functions of GFAP remain incompletely understood even though recent findings suggest GFAP involvement in the long term maintenance of the brain architecture (18), proper function of blood-brain barrier (18, 19), and modulation of some neuronal functions by astrocytes (20, 21). We also propose that GFAP and vimentin are required for the proper formation of a glial scar following trauma to the central nervous system (22). Generation of transgenic mouse models lacking one or several IF proteins has proven to be a powerful way to learn more about the functions of IFs in different cell types, and these models have clearly broadened the experimental possibilities available.

It has been shown that IFs are often composed of two or more IF proteins, which co-polymerize with each other. For example, IF formation in epithelial cells requires the co-polymerization of two types of keratins, one basic and one acidic (5). Similarly, experiments performed with non-neuronal IF-free cell lines transfected with expression vectors for individual neurofilament proteins (NF-L, NF-M, or NF-H), or experiment with transgenic mice have demonstrated the need for specific partnerships in the assembly of IFs; NF-L/NF-M or NF-L/NF-H co-expression both led to IF formation, while homopolymeric assembly of any single neurofilament protein or NF-M/NF-H co-assembly did not result in IF formation (23, 24). It remains to be established whether a co-polymerization of different IF proteins is a more general prerequisite in IF formation.

By crossing GFAP-deficient mice (25) with vimentin-defi-
cient mice (26), we have now generated mice that lack both GFAP and vimentin. Using these mice, we have studied the impact of the absence of GFAP and/or vimentin on the production of IFs in normal or reactive astrocytes in vivo and in astrocyte cultures and determined the obligatory partnership between GFAP, vimentin, and nestin.

EXPERIMENTAL PROCEDURES

Preparation of Astrocyte-enriched Cultures from Whole Brain

All mice used were hybrids between C57BL/6 and 129 mouse strains and they were generated within the same litters to equalize genetic variation between the groups. Mice at postnatal days 1–2 were killed by decapitation, and primary astrocyte-enriched cultures were prepared and maintained as described previously (27).

Histology and Immunostaining

Mouse brains were fixed in Bouin’s fixative (75 ml of saturated picric acid, 5 ml of glacial acetic acid, 25 ml of 40% formaldehyde), paraffin-embedded, and serially sectioned in the frontal plane. Selected sections were stained with hematoxylin and erythrosin or processed for immunohistochemistry. Pretreatment of the sections and immunohistochemical staining for GFAP and vimentin was performed as described elsewhere (25). For nestin immunostaining, slides were incubated with nestin rabbit antiserum (a kind gift from Dr. U. Lendahl, Karolinska Institute, Stockholm, Sweden), then with horseradish peroxidase-conjugated goat antiserum against rabbit immunoglobulins (Dako A/S), and processed further for 3′-diaminobenzidine tetrahydrochloride staining as described previously (25). For immunocytochemical detection of IF proteins, sparse astrocyte cultures 5 days following plating grown on 30-mm glass discs were used. Immunostaining for GFAP and vimentin with 3′-diaminobenzidine tetrahydrochloride detection was performed as described in Ref. 27; for nestin immunodetection, polyclonal rabbit antibody (nestin antiserum 6, described below) was used with the consecutive steps performed as described in Ref. 27.

Electron Microscopy

For the Ultrastructural Studies in Vivo — For the ultrastructural studies in vivo, 2 wild type, 2 GFAP−/−, 2 vimentin−/−, and 2 GFAP−/−vimentin−/− mice were used. The mice were anesthetized with Avertin intraperitoneally. After a vascular rinse with 100 ml of Tyrode’s solution through the left ventricle, the mice were perfused with a mixture of 2% paraformaldehyde and 2% glutaraldehyde dissolved in isotonic phosphate buffer. Each animal received 250 ml of the fixative infused over a period of 20 min. The brains were removed, and 500-μm-thick slices were cut from the corpus callosum, hippocampus, or cervical spinal cord. The slices were postfixed overnight at 6 °C, osmicated for 4 h, dehydrated in a graded series of acetone, and embedded in Vestopal W (Fluka Chemie, Liechtenstein). Sections (80 μm thick) were cut on an LKB Ultrotome, picked up on Formvar-coated one-hole copper grids, contrasted first with uranyl acetate and then with lead citrate, and examined in a Philips EM 400 electron microscope.
Fig. 2. Electron micrographs of astrocytes in situ. a, b, and c, corpus callosum; c, d, and f, hippocampus, concave aspect of dentate gyrus. In wild type mice (a and c), the astrocytic cytoplasm (A) contains distinct bundles of IFs (asterisks). In GFAP−/− mice (b and d), the astrocytic cytoplasm is devoid of IFs. In vimentin−/− mice (e and f), the astrocytic cytoplasm contains densely packed IFs (asterisks). Spinal cord, segment C6–7, the dorsal funiculus (g–j). Loose bundles of IFs in wild type mice (g) contrast with more compacted bundles of IFs in vimentin−/− mice (i). No IFs were detected in GFAP−/− or GFAP−/−vim−/− mice (h and j). The micrographs were chosen as representative of 40 or more astrocytic cell bodies examined for each of the genotypes and anatomical locations. A, astrocytic cytoplasm; M, myelinated nerve fiber; N, astrocytic nucleus; C, capillary; P, cellular processes of the surrounding neuropile; e, endoplasmic reticulum; g, Golgi apparatus; x, myelinoid body; arrows, mitochondria; A1 and A2, two bordering astrocytic profiles.

For the Ultrastructural Evaluation of IFs in Vivo —For the ultrastructural evaluation of IFs in vitro, 10-day-old primary astrocytic cultures were used. Medium was removed, and the cultures in 24-well Falcon plates were rinsed three times with isotonic phosphate buffer. The wells were filled with the fixative consisting of a mixture of 2% paraformaldehyde and 2% glutaraldehyde dissolved in an isotonic phosphate buffer. The cultures were kept in the fixative overnight at 6 °C, osmicated for 2 h, treated en bloc for 2 h with 2% uranyl acetate dissolved in 50% ethanol in water, dehydrated in a graded series of ethanol, and embedded, still in the wells, in Epon. After polymerization, the specimens were freed from the wells and sectioned as described above.

Quantitative Evaluation of the Density of IFs—The quantitative evaluation of the density of IFs was performed on electron micrographs (magnification, ×100,000). An IF bundle was defined as more than three IFs running in parallel with a distance of less than 40 nm between them. The number of IFs in transversally sectioned IF bundles was determined in wild type and vimentin−/− astrocytes in vivo by counting the IFs inside randomly placed circles (radius of 1 or 2 cm corresponding to 100–200 nm). The distance between individual IFs in longitudinally sectioned IF bundles was determined in wild type and vimentin−/− astrocytes both in vivo and in vitro. The distance was calculated by subtracting the thickness of IFs within the bundle (a × 10 nm) from the width of the bundle and dividing this number by n − 1 (the number of IFs within the bundle diminished by 1), i.e. the formula was: the distance between IFs = (bundle width − 10n)/n − 1.

Brain Injury Experiments

Anesthetized animals were wounded by the introduction of a 27-gauge fine needle through the skull and the frontal brain cortex as described previously (25). The animals were killed 3, 4, 7, or 14 days after the injury; brains were carefully dissected out of the skull, fixed in Bouin’s fixative, and processed for histology and immunohistochemistry.

SDS-PAGE and Immunoblotting

Primary astrocyte-enriched cultures were prepared from wild type (3 mice), GFAP−/−vim+/+ (2 mice), GFAP−/−vim+/− (3 mice), vim−/−GFAP−/− (2 mice), and GFAP−/−vim−/− (3 mice) as described above. The cultures were used 10 days following plating on 10-cm Falcon plate, and they were in the exponential phase of their growth as determined by cell count in parallel cultures. Whole cell extracts were prepared by lysing cells in SDS-lysis buffer (20 mM Tris-HCl, pH 7.2, 5 mM EGTA, 5 mM EDTA, 10 mM sodium pyrophosphate, 1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml antipain, 10 mg/ml leupeptin, and 10 mg/ml pepstatin). Cells were harvested by scraping, boiled for
2–5 min, and sonicated for 15 s with a probe sonicator. Proteins were separated on SDS-polyacrylamide gels (SDS-PAGE; Ref. 28). Gels were stained with Coomassie Brilliant Blue to control for equal loading. For quantitative Western immunoblotting, protein samples were transferred from gels to nitrocellulose membranes (Schleicher & Schuell) using a Bio-Rad semidy transfer apparatus. Cytoskeletal proteins were detected using monoclonal antibodies against actin (N350, Amersham Pharmacia Biotech) and GFAP (691102, ICN). For vimentin detection a polyclonal rabbit vimentin antibody 264 (29) was used. Nestin was detected using a polyclonal rabbit antibody (nestin antiserum 6) raised against a nestin fusion protein according to a previously described protocol (30). When tested with Western blotting, nestin antiserum 6 recognized specifically the nestin fusion protein and the 220-kDa nestin band in whole cell extract of ST15A cells. The nestin immunoreactivity pattern was identical with that observed with the previously described nestin antiserum 130 (30). Secondary antibodies used for immunoblotting were peroxidase-labeled sheep anti-mouse immunoglobulins (NA 931, Amersham Pharmacia Biotech) and peroxidase-labeled donkey anti-rabbit immunoglobulins (W401B, Promega). The binding of antibodies to proteins was visualized by enhanced chemiluminescence using an ECL Western blotting detection kit (Amersham Pharmacia Biotech). The relative intensities of the protein bands on immunoblots were analyzed by computer-assisted video-based densitometry. Statistical analysis of the relative intensities was performed by one-way analysis of variance, followed by Newman-Keuls multiple comparison test by using the Prism program package (GraphPad Software, Inc.).

**[35S]Methionine Labeling, [32P] Labeling, and Two-dimensional Protein Electrophoresis of Cultured Cells**

**[35S]Methionine Labeling**—Primary astrocyte cultures grown in 25-cm² flasks were labeled for 14 h with [35S]methionine in 2 ml of modified Eagle’s medium lacking methionine and containing 2% dialyzed (against 0.95% NaCl) fetal calf serum and 250 μCi of [35S]methionine (SJ 204, Amersham Pharmacia Biotech). At the end of the labeling period, the monolayers were resuspended in 0.4 ml of lysis solution (31) and 20 μl applied to the first dimension gel.

**[32P] Labeling**—Cultures of astrocytes or human ear fibroblasts were labeled with [32P]orthophosphate as described previously (32).

**Cytoskeleton Preparation and Two-dimensional Protein Electrophoresis**—Following [35S]methionine labeling, the cultures were washed with Hanks’ buffered saline and treated for 90 s with 0.1% Triton X-100 in the same buffer. After washing with Hanks’ solution, the cytoskeletal fraction was resuspended in the lysis buffer (31). Two-dimensional PAGE was carried out according to Ref. 31.
Northern Blot Analyses

Total RNA was prepared from wild type, GFAP−/−, vimentin−/−, and GFAP−/−vimentin−/− primary astrocyte cultures using the LiCl/urea method (33) and processed on formaldehyde gels according to standard protocols. The murine nestin cDNA (a gift from Dr. Martha Marvin, National Institutes of Health, Bethesda, MD) was labeled using [32P]dCTP and the Megaprime labeling kit (Amersham Pharmacia Biotech). The nestin mRNA was detected and quantified using a Storm 820 PhosphorImager (Molecular Dynamics).

RESULTS

Vimentin Cannot Form IFs in the Absence of GFAP in Astrocytes in the Normal Adult Mouse Brain—Using two different vimentin antibodies, immunohistochemical examination of the presence of GFAP and vimentin was performed on brain sections covering two areas that normally contain astrocytes strongly positive for these IF proteins: corpus callosum and hippocampus. In the corpus callosum of wild type mice, three cell types could be distinguished that were vimentin-positive: astrocytes, endothelial cells of brain capillaries, and ependymal cells (Fig. 1a). Vimentin immunoreactivity (IR) was lost in astrocytes of the corpus callosum in GFAP−/− mice, while both endothelial and ependymal cells remained vimentin-positive (Fig. 1c). We have previously shown that the hippocampus of GFAP−/− mice contains a normal number of astrocytes (25). Thus, the loss of vimentin-positive cells in this region does not reflect a loss of astrocytes. Ultrastructural examination of astrocytes showed complete...
lack of IFs in corpus callosum and hippocampus in GFAP\(-/-\) mice (Fig. 2, b and d), while IFs were readily identified in astrocytes in the same locations in wild type mice (Fig. 2, a and c).

**GFAP Forms Abnormal IF Bundles in the Absence of Vimentin in Astrocytes in Normal Mouse CNS**—In contrast to the loss of vimentin-IR in GFAP\(-/-\) astrocytes, GFAP-IR was retained at normal intensity and distribution in vimentin\(-/-\) astrocytes in corpus callosum and the hippocampus (Fig. 1, d and h versus b and f). Ultrastructurally, however, vimentin\(-/-\) astrocytes at these locations exhibited bundles of IFs that were consistently more condensed than in the wild type mice (Fig. 2, e and f). This compact appearance of the bundles of IFs was also observed in the astrocytes of the white matter of the spinal cord of vimentin\(-/-\) mice (compare with normal IFs in wild type mice and with the absence of IFs in GFAP\(-/-\) or GFAP\(-/-\)vim\(-/-\) mice; Fig. 2, g-j). Quantitative comparison between wild type and vimentin\(-/-\) astrocytes performed in the white matter of the spinal cord revealed that in vimentin\(-/-\) astrocytes the number of IFs per area within a bundle was increased on average to 155% with the distance between individual IFs being reduced by 58% (Fig. 3, Table I).

**Vimentin and Nestin Protein Levels and Vimentin Phosphorylation Are Not Influenced by GFAP Absence in Astrocytes in Culture**—Primary astrocytes in culture produce GFAP, vimentin and nestin. Since vimentin-IR was lost in GFAP\(-/-\) astrocytes, we used primary cultured cells to address the issue of IF protein levels. We have previously reported that such cultures of astrocytes prepared from wild type and GFAP\(-/-\) mice exhibit normal patterns of immunoreactivity using vimentin or nestin antibodies, although, at the ultrastructural level, GFAP\(-/-\)astrocytes contained reduced numbers of IFs (27). To find out whether vimentin or nestin production were influenced quantitatively by the absence of GFAP, we compared the levels of the two IF proteins in primary astrocyte-enriched cultures prepared from wild type or GFAP\(-/-\) mice. Western blot analysis from exponentially growing cultures showed that...
neither vimentin nor nestin levels were altered in the absence of GFAP (Fig. 4).

Phosphorylation is the major mechanism regulating the assembly and disassembly of IFs. To compare vimentin phosphorylation in wild type and GFAP−/− astrocytes, we have performed 32P labeling of primary cultures followed by two-dimensional gel electrophoresis. As shown in Fig. 5, no differences were detected in vimentin phosphorylation between wild type and GFAP−/− astrocytes (Fig. 5, A and B). As expected, no phosphorylated vimentin was found in vimentin−/− or GFAP−/−vim−/− astrocytes (Fig. 5, C and D). A comparison between mouse astrocytes and human fibroblasts showed that the pools of phosphorylated and non-phosphorylated vimentin in two cell types are of a comparable size (Fig. 6, A–D).

Abnormal IF Bundles in Vimentin−/− Astrocytes and Lack of IFs in GFAP−/−vim−/− Astrocytes in Culture—To further address the effects of depletion of GFAP and/or vimentin on the production and assembly of the remaining IF proteins, we studied astrocyte-enriched cultures from wild type, GFAP−/−, vimentin−/−, and GFAP−/−vim−/− mice. Immunocytochemical staining of nestin in wild type or GFAP−/− astrocytes showed typical arrangement of IF bundles (Fig. 7, a and b), while astrocytes from either vimentin−/− or GFAP−/−vim−/− astrocytes exhibited only a diffuse pattern of weak

![FIG. 5. Isoelectric focusing two-dimensional PAGE of [32P]orthophosphate-labeled proteins from astrocytes in vitro. Comparable amount of phosphovimentin was detected in wild type (A) and GFAP−/− (B) astrocytes. Phosphovimentin is absent in vimentin−/− (C) and GFAP−/−vim−/− (D) astrocytes.](image)

![FIG. 6. Isoelectric focusing two-dimensional PAGE of [35S]methionine-labeled proteins from mouse astrocytes and human fibroblasts. A and B, whole protein extracts from wild type mouse astrocytes (A) and human fibroblasts (B). C and D, Triton X-100-extracted cytoskeletal fractions from wild type mouse astrocytes (C) and human fibroblasts (D). Comparable amounts of vimentin and phosphovimentin were present in the two cell types. The positions of vimentin, phosphovimentin, GFAP, β-tubulin, and actin are indicated.](image)

![FIG. 7. Immunolocalization of nestin or vimentin in wild type, GFAP−/−, vimentin−/−, and GFAP−/−vim−/− astrocytes in vitro. Nestin immunoreactivity reveals distinct bundles of IFs in wild type or GFAP−/− astrocytes (a and b) in contrast to vimentin−/− or GFAP−/−vim−/− astrocytes, which exhibit only a very weak and diffuse nestin immunoreactivity (c and d). GFAP immunostaining of wild type and vimentin−/− astrocytes reveals similar distinctly filamentous staining patterns (e and f). Arrowheads in c and d depict cell borders. Bar, 10 μm.](image)
nestin-IR, lacking any bundle arrangement (Fig. 7, c and d). GFAP-IR on vimentin−/− astrocytes exhibited a pattern similar to the wild type (Fig. 7, e and f). At the ultrastructural level, distinct IFs were present in wild type and in GFAP−/− astrocytes; in the latter case, their density was reduced in accordance with previous findings (Fig. 8, a and b; Ref. 27). In vimentin−/− astrocytes, IFs were present in highly compacted bundles (Fig. 8c). The average distance between individual IFs in vimentin−/− astrocytes was reduced more than 3-fold (Table I). In vimentin−/− or GFAP−/− vim−/− mice by Western blotting showed decreased amount of nestin to 10–20% of the levels in wild type cultures (Fig. 4). In contrast, nestin mRNA levels in vimentin−/− or GFAP−/− vim−/− astrocytes were up-regulated more than 3-fold compared with the wild type or GFAP−/− astrocytes (Fig. 9). The lower nestin protein level in the absence of vimentin is therefore a posttranscriptional phenomenon, and it is conceivable that this reflects more rapid degradation of unpolymerized nestin. Moreover, the increased levels of nestin mRNA in vimentin-deficient cells suggest the existence of a feedback that increases the nestin transcription levels when nestin cannot polymerize into IFs.

**Nestin Cannot Form IFs in GFAP−/− vim−/− Reactive Astrocytes in Vivo**—Fine needle injury in the frontal cortex of wild type, GFAP−/−, vimentin−/−, or GFAP−/− vim−/− mice followed by nestin immunohistochemical detection 3 days later, revealed distinct nestin-IR in cortical astrocytes surrounding the defect in wild type or GFAP−/− mice (Fig. 10, a and b). However, in the injured cortex of vimentin−/− or GFAP−/− vim−/− mice, normal nestin-IR was absent and was replaced by a weak and diffuse signal (Fig. 10, c and d) similar to the nestin-IR observed in vimentin−/− or GFAP−/− vim−/− astrocytes in vitro (Fig. 7, c and d). These results imply that also
following a CNS trauma in vivo, nestin IFs cannot form in vimentin−/− or GFAP−/−/vim−/− mice. No difference was detected in GFAP-IR of cortical astrocytes in the injured area in wild type and vimentin−/− mice (Fig. 10, e and f). This is in agreement with the normal GFAP-IR observed in vimentin−/− astrocytes in vitro (Fig. 7, e and f).

Vimentin-IR Depends on Temporary Expression of Nestin in Reactive GFAP−/−/Astrocytes in Vico—Reactive astrocytes in vivo, similar to primary astrocytes in culture, produce GFAP, vimentin, and nestin. In some regions of the CNS, nestin-IR persists for months following mechanical injury (3). In contrast, we found that, in reactive wild type cortical astrocytes, nestin-IR was transient and became undetectable 2 weeks following injury, while both GFAP-IR and vimentin-IR were still readily detected at this time point (data not shown). Since these “late” reactive astrocytes seem to retain expression of vimentin and GFAP only, we asked whether vimentin-IR persists also in the GFAP−/− mice at time points when nestin-IR has disappeared. To address this, we performed fine needle injury of the brain in wild type and GFAP−/− mice, followed by GFAP, vimentin, and nestin immunohistochemical staining 3, 4, 7, or 14 days later. We detected vimentin and nestin-IR in astrocytes in the injured cortex of both wild type and GFAP−/− mice at 3, 4, and 7 days following the injury (data not shown), but at 14 days nestin-IR was virtually undetectable in both wild type and GFAP−/− mice (Fig. 10, g and h). Immunostaining with vimentin antibodies performed 14 days after the injury revealed positive reaction in both astrocytes and endothelial cells of the forming glial scar in the cortex of wild type mice (Fig. 10b). However, in the GFAP−/− mice, no distinctly stained astrocytes were seen, although both vimentin-positive endothelial cells and additional diffuse immunostaining were detectable (Fig. 10, compare j with i). These results show that vimentin-IR in reactive astrocytes can be detected as long as nestin is present. When the transient nestin expression declines in reactive GFAP−/− astrocytes, these cells lose their normal vimentin staining pattern, probably because vimentin under these conditions is incapable of forming IFs on its own.

**DISCUSSION**

Using mice deficient for GFAP and/or vimentin as a model system, we found that a particular combination of IF proteins was required for normal IF production in astrocytes. The data presented here enable us to propose specific prerequisites for IF partnerships in astrocytes. These are outlined in Fig. 11 and discussed below.

**Possible Partnerships**—Vimentin-GFAP and vimentin-GFAP-nestin partnerships result in the productive formation of IFs, and these partnerships represent the usual situations in unchallenged astrocytes in the adult and in reactive astrocytes in vivo (or in astrocytes in vitro), respectively. As shown in GFAP−/− primary astroglial cultures (this study and Ref. 27) and in reactive gliosis in vivo in GFAP−/− mice (this study), the **vimentin-nestin** partnership is also possible. This finding is not surprising since such a partnership must probably occurs during astrocyte maturation, prior to the onset of the GFAP expression.

**An Abnormal Partnership**—Data from vimentin−/− astrocytes in vivo and in vitro show that GFAP can form IFs on its own. However, the arrangement of these, apparently intact, GFAP-GFAP filaments is abnormal; instead of the usual loose bundles, compact bundles are formed with 60–70% less space between individual filaments. We speculate that the presence of these abnormal bundles of IFs in vimentin−/− mice may lead to altered mechanical properties of the vimentin−/− astrocytes (e.g. increased cell rigidity) or have other negative effects. The concept that an aberrant IF system is more detrimental to a cell than the complete absence of IFs has been proposed. Skin blistering appeared milder in those cases of epidermolyis bullosa simplex, both in humans and in mouse models, where null rather than dominant mutations in keratin 14 gene were present (1). Abnormal accumulation and disorganization of neurofilaments are present in a number of motor neuron diseases, such as sporadic amyotrophic lateral sclerosis, infantile spinal muscular atrophy, and hereditary sensory motor neuropathy as well as in transgenic mice overexpressing neurofilament proteins (1, 34). Recently, mice overexpressing human GFAP were generated, and they exhibit hypertrophic astrocytes with inclusion bodies resembling Rosenthal fibers of human neuropathology and consisting of IFs and other aggregated proteins. The severe phenotype of these mice is reminiscent of Alexander’s disease (35).

Our finding of normal GFAP-IR but abnormally bundling GFAP IFs in vimentin−/− astrocytes contradicts that of Galou and co-workers (36), who report absence of GFAP-IR in astrocytes in some areas of the cerebrum and cerebellum, and in wound-induced reactive gliosis in vimentin−/− mice. Curiously, the ultrastructural analysis of cerebellum revealed variable presence of IFs; while the authors failed to identify IFs in some astrocytes, they saw abundant IFs in other astrocytes (36). Moreover, Galou et al. reported that the cytoskeletal fraction of vimentin−/− astrocytes contained a normal level of GFAP, which seems inconsistent with the loss of GFAP IFs. We consistently detected astrocytic IFs in vimentin−/− mice in all areas of the CNS examined, i.e. corpus callosum, hippocampus, and white matter of the spinal cord. We also show that the IFs in vimentin−/− astrocytes consistently formed compacted bundles, both in the intact CNS and in astrocytes in culture. Like Galou et al., we find that the levels of GFAP protein are not decreased in vimentin−/− astrocytes. Taken together, therefore, biochemical and ultrastructural analysis favor our interpretation that GFAP may assemble into IF networks in the absence of vimentin.

**Impossible Partnerships**—As shown both in the unchallenged brain in GFAP−/− mice and in later stages of reactive gliosis in the brain cortex, vimentin-vimentin partnership does not result in the production of IFs (Fig. 7). Instead, to form normal IFs, vimentin needs a partner, which may be either
nestin or GFAP. If such a concept is generally applicable to astrocytes throughout the CNS, it would imply that, to have IFs, astrocytes or their precursors would have to express either nestin (astroglial precursors) or GFAP (mature astrocytes) at any given vimentin-positive stage of their development. In the light of the common view that vimentin may form IFs on its own at least in selected cell types, it is possible that either the astrocyte environment has a major effect on IF assembly, or that in some cell types, other IF-associated proteins such as synemin and paranemin (37) can enable vimentin self-polymerization either by stabilizing the IF network or by acting directly as IF proteins (38). Vimentin phosphorylation, which could

![Diagram of astrocyte partnerships](image)

**Possible**
- vimentin-nestin
  - GFAP−/− cell cultures
  - GFAP−/− mice - reactive gliosis (injured brain 3, 4 and 7 days)
- vimentin-GFAP
  - wild type uninjured brain
- vimentin-nestin-GFAP
  - wild type cell cultures
  - wild type mice - reactive gliosis (injured brain 3, 4 and 7 days)

**Abnormal**
- GFAP-GFAP
  - vim−/− cell cultures (EM)
  - vim−/− mice in vivo

**Not possible**
- vimentin-vimentin
  - GFAP−/− mice in vivo
  - GFAP−/− mice - reactive gliosis (injured brain 2 weeks)
- nestin-nestin
  - GFAP−/− cell cultures
  - GFAP−/− mice - reactive gliosis (injured brain)
- nestin-GFAP
  - vim−/− cell cultures
  - vim−/− mice - reactive gliosis (injured brain)

**FIG. 10. Reactive astrocytes in cortical lesions in GFAP−/−, vimentin−/−, or GFAP−/−vimentin−/− mice.** Astrocytes in cortical lesions 3 days following the injury (a−f). Nestin immunostaining reveals distinct reactive astrocytes in wild type or GFAP−/− mice (a and b). In the corresponding areas in vimentin−/− or GFAP−/−vimentin−/− mice, only weak and diffuse astrocytic profiles are depicted as nestin-immunoreactive; the situation closely resembling nestin immunoreactivity in cultured astrocytes of the same genotype (Fig. 7, c and d) and indicating absence of IFs. GFAP immunostaining reveals distinct reactive astrocytes in the injured cortex of both wild type and vimentin−/− mice (e and f). Vimentin and nestin immunoreactivity 2 weeks following the injury (g−j). In cortical lesions in both wild type and GFAP−/− mice, nestin immunostaining becomes almost undetectable 2 weeks after the injury (g and h). While normal vimentin immunostaining persists at the site of injury in wild type mice and permits an easy identification of individual reactive astrocytes (i), in GFAP−/− mice, vimentin immunostaining is limited to the central zone of the injury and has a diffuse character; endothelial cells are clearly stained but astrocytic profiles cannot be distinguished. The sections in g and h were counterstained with 50-fold diluted hematoxylin and erythrosin. Arrows, astrocytes; arrowheads, endothelial cells. Bar, 10 μm (a−f) and 20 μm (g−j).

**FIG. 11. Partnerships of IF proteins in astrocytes; present examples.**
potentially be responsible for cell-type specific differences in the assembly of IFs, was found to be comparable in wild type and GFAP+/− astrocytes as well as in normal human fibroblasts. It is therefore unlikely that the degree of vimentin phosphorylation in astrocytes depends on GFAP or differs substantially from other cell types.

Similarly, nestin-nestin or nestin-GFAP partnerships are non-productive as far as the formation of IFs is concerned. As shown by the data from reactive gliosis in vimentin+/− or GFAP+/−− mice or from primary astrocyte cultures, nestin cannot form IFs on its own or with GFAP. This is a surprising finding; while no cell type at any developmental or activation stage has been known to co-express GFAP and nestin in the absence of other IF proteins, it has not previously been ruled out that at least some cells of neuroectodermal origin are exclusively nestin-positive during the early developmental stages. It remains to be shown whether the inability of nestin to form IFs on its own lies substantially from other cell types.

The surprising finding; while no cell type at any developmental or activation stage has been known to co-express GFAP and nestin in the absence of other IF proteins, it has not previously been ruled out that at least some cells of neuroectodermal origin are exclusively nestin-positive during the early developmental stages. It remains to be shown whether the inability of nestin to form IFs on its own lies substantially from other cell types.

importance of IF up-regulation in different conditions that trigger reactive gliosis since these mice fail to produce astrocytic IFs.

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