Disrupted Glial Fibrillary Acidic Protein Network in Astrocytes from Vimentin Knockout Mice

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Abstract. Glial fibrillary acidic protein (GFAP) is an intermediate filament protein expressed predominantly in astrocytes. The study of its expression in the astrocyte lineage during development and in reactive astrocytes has revealed an intricate relationship with the expression of vimentin, another intermediate filament protein widely expressed in embryonic development. These findings suggested that vimentin could be implicated in the organization of the GFAP network. To address this question, we have examined GFAP expression and network formation in the recently generated vimentin knockout (Vim-) mice. We show that the GFAP network is disrupted in astrocytes that normally coexpress vimentin and GFAP, e.g., those of the corpus callosum or the Bergmann glia of cerebellum. Furthermore, Western blot analysis of GFAP protein content in the cerebellum suggests that posttranslational mechanisms are implicated in the disturbance of GFAP network formation. The role of vimentin in this process was further suggested by transfection of Vim- cultured astrocytes with a vimentin cDNA, which resulted in the normal assembly of the GFAP network. Finally, we examined GFAP expression after stab wound-induced astrogliosis. We demonstrate that in Vim- mice, reactive astrocytes that normally express both GFAP and vimentin do not exhibit GFAP immunoreactivity, whereas those that normally express GFAP only retain GFAP immunoreactivity. Taken together, these results show that in astrocytes, where vimentin is normally expressed with GFAP, the GFAP fails to assemble into a filamentous network in the absence of vimentin. In these cells, therefore, vimentin appears necessary to stabilize GFAP filaments and consequently the network formation.
Reactive astrocytes are characterized by an increased expression of GFAP which polymerizes in numerous IFs (Reier et al., 1983; Miller et al., 1986), by nuclear hypertrophy (Nathaniel, 1981), and sometimes by cell hyperplasia (Latov et al., 1979; Janeczko, 1993). During gliosis, astrocytes recover the capacity to express vimentin that disappeared during normal development (Takamiya et al., 1988). The simultaneous increase in GFAP and vimentin expression occurs only in cells that are in the vicinity of the lesion (Takamiya et al., 1988). In this region, 60% of the astrocytes coexpress both vimentin and GFAP, while the remainder are positive only for GFAP (Calvo et al., 1991).

When astrocytes are placed in culture, the IF network undergoes reorganization. Like other mammalian cells grown in vitro, they acquire vimentin expression (Franke et al., 1979) that remains at a high level, even in well-differentiated cells (Schnitzer et al., 1981). With respect to the expression of IF proteins, the differentiation program of a subpopulation of cultured astrocytes from a newborn mouse brain closely follows that of astrocytes in the developing brain, suggesting that changes in IF expression are not simply adaptive responses to culture conditions (Fedoroff et al., 1983).

Thus, ex vivo and in vivo experiments point to a tightly scheduled expression in the astrogial lineage of vimentin and GFAP during development: the vimentin containing IFs seem to be assembled first in astrocyte precursor cells, followed by the coexpression of vimentin and GFAP, and finally the presence of two populations of differentiated astrocytes—one expressing GFAP only, the other also expressing vimentin—in the adult. Similarly, in pathological conditions, after CNS injury, some astrocytes elicit upregulation of GFAP and vimentin while others upregulate GFAP alone.

To address the question of a possible link between vimentin and GFAP expression and network formation, we took advantage of the existence of mice devoid of vimentin (Vim−) that we had previously generated (Colucci-Guyon et al., 1994) and examined network formation in these mice in normal development and after CNS injury. The results indicate that in Vim− mice, the GFAP network is disrupted in those astrocytes that normally coexpress vimentin and GFAP, e.g., the Bergmann glia of the cerebellum and an astrocyte subpopulation of the corpus callosum. Furthermore, examination of reactive astrocytes induced upon brain injury showed that in Vim− mice, astrocytes in the immediate vicinity of the wound did not elicit GFAP immunoreactivity (IR) in contrast to Vim+ mice, where these astrocytes exhibit a mixed GFAP/vimentin network. Conversely, in the distal region, where no vimentin overexpression occurs in normal mice, upregulation of GFAP was consistently observed in Vim− mice. Finally, to further delineate the possible implication of vimentin in GFAP network formation, we performed primary cultures of astrocytes prepared from the brains of Vim− mice. In these astrocytes, the GFAP network also appeared disorganized and, most significantly, we could show that reexpression of vimentin by transfection of a human vimentin cDNA restored GFAP assembly into a normal network. Collectively, these results indicate that in the absence of vimentin, GFAP network formation is abnormal in vivo and that this effect seems limited to the cells that normally coexpress vimentin and GFAP, suggesting that in these cells, the presence of vimentin is necessary for the organization and/or the stabilization of the GFAP IF system.

Materials and Methods

Animals

All the experiments were performed on the Vim− knockout mice. This line contains the lacZ gene inserted in frame in the first exon of the vimentin gene, and therefore expresses β-galactosidase in place of vimentin (Colucci-Guyon et al., 1994).

Surgery

The mice were deeply anesthetized with 0.3 ml i.p. of avertine (7 mM 2,2,2-tribromoethanol in 2-methyl-2-butanol). Stab wound lesions were performed in the right cerebral hemisphere of adult mice as previously described (Galou et al., 1994).

Histological Procedures

3-4-mo-old transgenic mice were deeply anesthetized with 10 mg/kg i.p. sodium pentobarbitaI. After an intracardiac injection of 2,500 Ul/ml heparin and 1% sodium nitrate, the animals were perfused intracardially with 10 ml of PBS buffer followed by 100 ml of a fixative solution containing 4% paraformaldehyde (PFA) in PBS for light microscopy or 4% PFA with 0.5% glutaraldehyde for EM. Brains were removed, sectioned, and postfixed for 1 h in 4% PFA in PBS. Sections (40-100-μm) were performed with a vibratome. Floating sections were placed in a solution of 20% sucrose in PBS for 30 min at room temperature. The β-galactosidase activity was revealed with an X-gal staining solution (GIBCO BRL, Gaithersburg, MD) using overnight incubation at 31°C, as previously described (Galou et al., 1994). Sections were then rinsed in PBS and immunostained with the peroxidase technique, as described elsewhere (Dupouey et al., 1985), using a rabbit anti-GFAP (Dakopatts, Dako A/S, Glostrup, Denmark) or rabbit antivimentin (a kind gift from Dr. A.M. Hill, Université Paris Sud, France) as the primary antibody. The specificity of anti-GFAP serum used in our study was ascertained by testing it on cultured cells that expressed only vimentin and on adult CNS sections. This antiserum stained astrocytes, Bergmann cells, tanyctyes, and radial glia. As expected, it did not stain any ependymal cells, which in the adult contain only vimentin.

Electron Microscopy

Selected sections were postfixed in 2% PFA-2% glutaraldehyde overnight at 4°C and then rinsed in phosphate buffer (0.12 M). After a rapid rinse in bidistilled water, they were postfixed for 1 h in 1% osmium tetroxide containing 1.5% potassium ferricyanide (Sigma Immunocnmuicals, St. Louis, MO) in phosphate buffer. After dehydration in graded ethanol, sections were embedded in Araldite (Teuazart et Matignon S.A., Vitry sur Seine, France). Serial ultrathin sections (100-11m thickness) were made with an RMC-MT17 ultramicrotome (Microtom France, Venisieux, France). They were collected on copper grids (300 mesh) and observed at 80 kV with an electron microscope (model 900; Carl Zeiss, Inc., Thornwood, NY).

Astrocytes Primary Cultures Preparation and Transfection

Primary cultures of mouse astrocytes were prepared from cerebral hemispheres of E18 mouse embryos, as previously described (Booher and Senseabrenner, 1972; Chiu and Goldman, 1984). Transient transfection of primary astrocytes was performed at ~60% confluence with an expression vector, pcDNA A3, containing the human vimentin cDNA under the control of the cytomegalovirus promoter (Chen, J.H., Z. Li and D. Paulin, manuscript in preparation). 1 μg of pcDNA A3 mixed with 5 μl of lipofectin reagent (GIBCO BRL) in 0.8 ml of culture medium without serum was left at room temperature for 15 min. This mixture was added dropwise to the astrocyte primary culture and incubated at 37°C for 6 h under 5% CO2. GFAP and vimentin expression was checked 48 h after transfection. Plates were washed, fixed in 4% paraformaldehyde for 5 min at 4°C, and incubated with rabbit anti-GFAP (dilution = 1:3,000; Dako A/S) or rabbit antivimentin (a gift from A.M. Hill; dilution = 1:1,000) or mouse anti-
vimentin for double labeling or preimmune serum for 24 h. After several
washes, plates were incubated with FITC-conjugated anti-rabbit IgG (di-
lution = 1:500; Biosis, Compiegne, France) or rhodamine-conjugated
anti-mouse IgG (dilution = 1:500; Biosis).

**Western Blot Analysis**

Cytoskeletal proteins were extracted from the cerebellum and analyzed
by Western blot (Laemmli, 1970). Tissue (20 mg) was rapidly homoge-
nized in 0.5 ml of 50 mM Tris-HCl, pH 6.8, 2% SDS, 1 mM EDTA, 1 mM
PMSF. Undissolved material was pelleted by centrifugation at 13,000 g for
5 min at 4°C. The pellet was then reextracted as before in the same buffer
including 30% sucrose to remove myelin. The insoluble material remain-
ing (pellet) was the crude cytoskeletal fraction, and the first supernatant
was designated the soluble fraction. For one-dimensional PAGE, the pel-
let was dissolved in 6% SDS, 10% β-mercaptoethanol buffer. Equal
amounts (60 μg) of total protein from each sample were loaded onto 12%
polyacrylamide mini-slab gels and electrophoresed at 190 V for 1 h. Pro-

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**Figure 1.** GFAP immunostaining of brain coronal sections of Vim⁺ (A and C) and Vim⁻ (B and D) mice. Sections (40-μm) were per-
formed at two levels of the corpus callosum (arrow), near the lateral ventricle (A and B) and near the third ventricle (C and D). GFAP
is revealed by fluorescent immunostaining with a polyclonal rabbit anti-GFAP antibody. The corpus callosum of Vim⁻ mice shows a
dramatic decrease of GFAP-positive astrocytes (B and D) compared to the corpus callosum of control mice (A and C). Bar, 25 μm.
Results

Total cellular RNA was extracted from primary culture cells according to RNA Preparation and Reverse Transcription PCR. For hybridization with oligonucleotide probes, filters were prehybridized in the same buffer with a [γ-32P]ATP labeled probe at melting temperature. Absence of genomic DNA contamination was performed with peroxidase-conjugated goat anti-rabbit Fabs (dilution = 1:1,000; Biosis) or peroxidase-conjugated goat anti-mouse antibodies (dilution = 1:1,000; Biosis). Peroxidase activity was revealed using the ECL method (Amersham).

RNA Preparation and Reverse Transcription PCR

Total cellular RNA was extracted from primary culture cells according to the method of Chomczynski and Sacchi (1987), and RNA concentration was measured by OD. First-strand cDNA was synthesized (in a final volume of 20 μl) at 42°C for 1 h using 1 μg of total cellular RNA, superscript reverse transcriptase (GIBCO BRL), and reverse primer 562 R, chosen in Gfap gene exon 3 (5’CTTGCTAGCTACATCGAGAAGGTCCGCT3’). Second-strand synthesis and amplification were carried out by PCR using 1/20 of the single-stranded cDNA reaction product in 50 μl final volume with 400 ng of each primer, 562R and 2glF in exon 1 (5’CGAAq’CAAC-CTTTCTCTCCAAATCCACACG3’), used as a forward primer. The parameters of amplification were a first denaturation at 94°C for 8 min, then for each cycle a denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and synthesis at 72°C for 3 min. After 25 cycles, the samples were heated at 72°C for 5 min. Absence of genomic DNA contamination was verified using a primer located in the third intron, which allowed distinction of genomic DNA from mRNA. If a contamination occurred, the length of the amplified product would be 1,300 bp, while a 281-bp long fragment should be obtained upon amplification of the GFAP mRNA. For hybridization with oligonucleotide probes, filters were prehybridized for 2 h with 0.5 M buffer phosphate, pH 7, 7% SDS, and then hybridized in the same buffer with a γ[32P]ATP labeled probe at melting temperature –5°C. Filters were washed with 5x SSC, 0.1% SDS at room temperature twice for 15 min.

Results

Lack of GFAP Immunoreactivity in Some Astroglial Cells of Vimentin-deficient Mice

A comparative study was performed on coronal sections of brains from 3 mo-old Vim- and wild-type mice. The analysis of the coronal sections stained with a polyclonal antibody raised against GFAP revealed a decrease in the number of GFAP-positive cells in the brains of Vim- mice. This marked decrease varied depending on the area. In the corpus callosum of knockout mice, the number of GFAP-positive astrocytes was clearly lower than that of the controls (Fig. 1, A vs B). In the cerebral cortex, the few remaining GFAP-positive astrocytes exhibited more slender and highly branched-out processes in vimentin-deficient mice than in the control mice (data not shown).

EM Shows an In Vivo-disrupted GFAP Network in Astroglial Cells of Vimentin-deficient Mice

To learn whether the GFAP network was disrupted, we performed an ultrastructural analysis of astroglial cells. We chose to analyze the astrocyte population of the cerebellum because of the dramatic decrease of GFAP IR in Vim- mice compared to control mice. As expected, in control mice, cerebellar astrocytes contained the typical bundles of IF in their pericaryon (Fig. 3 A) and in their processes (Fig. 3 B), whether they were located in the white or grey matter of the cerebellum. In vimentin-deficient mice, however, grey matter astrocytes appear totally devoid of IF. The processes appear electron lucent, often dilated (Fig. 3 C). The pericaryon of Bergmann astrocytes, as well as that of velate astrocytes of the granular layer (Fig. 4 A), is also clear, with a paucity of organelles and a total absence of glial IF. Conversely, white matter astrocytes appear almost normal, with a denser cytoplasm and the presence of characteristic bundles of glial IF in the perinuclear cytoplasm and in the processes (Fig. 4 B).

The Soluble Pool of GFAP Polypeptides Is Increased in Vimentin-deficient Mice Relative to Control Mice

To monitor the presence of GFAP subunits, cytoskeletal and soluble fractions of proteic extracts from the cerebellum were prepared from Vim+ and Vim- mice. 60 μg of protein from cytoskeletal and soluble fractions were sub-
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Figure 3. Ultrastructure of cerebellum astrocytes from 3-mo-old control (A and B) and vimentin-deficient (C) mice. (A) Subpial astrocyte of the molecular layer of a control animal. Note the characteristic end-foot and thin processes. Bar, 1 μm. (B) High magnification of a subpial end-foot of a control mouse illustrating the characteristic bundles of glial filaments (arrowheads). Bar, 0.5 μm. (C) Astrocyte vascular end-foot in the molecular layer of the cerebellum of a Vim− mouse. These processes are dilated, electron lucent, and do not contain glial filaments. Bar, 0.5 μm.

Disorganized GFAP Network in Cultured Vimentin-deficient Astrocytes

To further analyze the possible role of vimentin in the formation of the IF network, we performed primary cultures of astrocytes from E18 embryo brains. After 2 wk in culture, primary astrocytes from wild-type embryos elicited a strong vimentin and GFAP staining, manifested by immunodetection (Fig. 6, A and B) as a clear and fine network of thin glial filaments. In contrast, in astrocyte cultures of the Vim− embryos, the GFAP staining was completely abnormal. First, the proportion of cells negative for GFAP IR increased largely (Fig. 6, B and D). Second, those cells that remained positive exhibited a poorly defined GFAP network; some positive cells seemed to yield short, somewhat irregular filaments that did not have the parallel arrays of an organized GFAP network (Fig. 6, C and D). In view of the diminished and abnormal GFAP IR, we set out to assess the levels of GFAP mRNA in the astrocyte cultures. To that end, reverse transcription (RT)-PCR was performed on total RNA obtained from Vim− and Vim+ astrocytes. Total RNA was prepared from mutant and normal cells, and 1 μg of each preparation was used for RT-PCR, with the appropriate GFAP gene–specific oligonucleotides as primers (see Materials and Methods). In both preparations, a fragment of the expected length (281 bp) was obtained (Fig. 7 A), the specificity of which was further assessed by hybridization with a GFAP-specific probe (Fig. 7 B). Thus, GFAP mRNA was detected in both Vim− and Vim+ astrocytes; however, analysis of the GFAP mRNA levels in the two types of astrocytes by Masterscan (Scanalytics, CSPL, Billerica, MA) indicated that there was a slight but consistent decrease of GFAP mRNA in Vim− astrocytes as compared to the Vim+. Similar results were obtained in a total of five independent experiments using three different RNA preparations.

Transfection of Vimentin Restored the GFAP Network Organization in Vimentin-deficient Astrocytes

GFAP IR and network formation appear abnormal in cultures of Vim− astrocytes: all the astrocytes derived from Vim− embryos failed to elicit a defined GFAP network (see above). This suggests that vimentin could play a role in the proper organization and deployment of the GFAP network. To address this question, we attempted to express the vimentin protein in Vim− astrocytes. To achieve this goal, we performed transient transfection using an expression vector consisting of the human vimentin cDNA driven by a cytomegalovirus promoter. A total of 14 3-mm plates, each containing ~10^3 cells, were used in two separate experiments. 24-48 h after transfection, the appearance of a vimentin network was assessed by immunofluorescence detection using specific mouse antibodies and secondary antibodies conjugated with rhodamine. Around 5% of the cells were found to express vimentin, as demonstrated by the decoration of a well-elaborated vimentin network throughout the cytoplasm without any obvious sign of filament disruption such as formation of nonfilamentous aggregates (Fig. 8 A). Strikingly, these cells were double labeled with anti-GFAP antibodies and sec-
Figure 4. Ultrastructure of cerebellum astrocytes from 3-mo-old vimentin-deficient mice. (A) Astrocyte of the granular layer of the cerebellum of a Vim− mouse. Note the electron lucent cytoplasm and the absence of IFs. (B) Astrocyte of the white matter of the cerebellum of a Vim− mouse. This cell exhibits a denser cytoplasm than that of A, as well as characteristic bundles of IF in the perinuclear cytoplasm and in the processes (arrowheads). Bars, 1 μm.

Secondary antibodies conjugated with fluoresceine, a well-defined GFAP network superimposable to that of vimentin was consistently observed (Fig. 8, A vs B). Thus, it appears that reexpression of vimentin protein in Vim− astrocytes restores their ability to assemble a GFAP network.

Absence of GFAP IFs in a Population of Reactive Astrocytes in the Vimentin-deficient Mice after Stab Wound Injury

We have examined GFAP expression in adult vimentin knockout and wild-type mice after a stab wound injury.
1–15 d after the lesion, GFAP and vimentin immunodetection was performed on brain floating sections. As expected, in wild-type mice, the presence of reactive astrocytes was manifested by GFAP upregulation, as revealed by a strong GFAP IR, both in the vicinity of the wound and distal to it (Fig. 9 A); vimentin was also upregulated in astrocytes in the vicinity of the wound (Fig. 9 C). In contrast, in the Vim − mice, the astrocytes distal to the wound still elicited upregulation of GFAP, whereas no GFAP IR could be demonstrated in the vicinity of the wound (Fig. 9 B). In fact, the space negative for GFAP IR observed in vimentin knockout mice could be perfectly superimposed on the area filled by reactive astrocytes in control mice that reexpressed vimentin (Fig. 9, B vs C). In summary, it appears that reactive astrocytes that elicit a mixed GFAP/vimentin network in wild-type mice, i.e., in the vicinity of the wound, fail to do so in Vim − mice. Interestingly, because the Vim − mice used in this study express β-galactosidase in place of vimentin (Colucci-Guyon et al., 1994), we could show that the process of vimentin gene upregulation still took place in these mice: indeed, when we incubated sections in X-gal staining solution to reveal β-galactosidase activity, cells with blue nuclei were demonstrated in the area where reactive astrocytes are found to express vimentin in wild-type mice (Fig. 9 D). Taken together, these experiments show that despite the absence of vimentin and the defective organization of GFAP filaments in Vim − mice, the reactive gliosis after brain injury takes place normally.

Discussion

We had previously generated, by targeted mutagenesis, mice in which the synthesis of vimentin was completely abolished (Colucci-Guyon et al., 1994). In this report, we have taken advantage of the existence of these mice to analyze the formation of filaments by another IF protein, GFAP, which is mainly expressed in astrocytes, and the expression of which bears intricate relations with that of vimentin, both in the normal astrocyte lineage during development and in reactive astrocytes after brain injury. We show that in astrocytes where vimentin is normally coexpressed with GFAP, the former appears necessary for the network formation of the latter. In astrocytes where GFAP is the only IF protein expressed, however, it can organize into a normal network. These results therefore point to two different subpopulations of astrocytes regarding their ability to assemble an IF network.

Astrocytes are a broad cellular category subdivided into fibrous and protoplasmic according to their morphology and their localization. In the adult brain, the white matter in the large myelinated tract of the corpus callosum contains an astrocyte population that is exclusively GFAP positive, as well as an astrocyte population where vimentin and GFAP coexist (Pixley and de Vellis, 1984). In vimentin-null mice, the corpus callosum showed a significant decrease in GFAP-positive cells. GFAP IFs were disrupted in those astrocytes, where both vimentin and GFAP should have been present. Furthermore, the Bergmann glia, which constitute a population of astrocytes normally coexpressing vimentin and GFAP, were devoid of GFAP IR in Vim − mice. In the remaining astroglial population, which does not express vimentin in normal mice, GFAP IFs displayed normal features. This population included some fibrous astrocytes of the white matter and some astrocytes of the hippocampal grey matter. These observations suggest the existence of two astrocyte cell populations: those that are able to form GFAP IFs independently of vimentin expression, and those that are not able to build GFAP IFs when vimentin is lacking. In this context, it is interesting to note that we could extend our observation of GFAP network formation depending on vimentin to another completely different cell lineage, the epithelial cells of the lens: whereas GFAP and vimentin IR can be demonstrated in these cells in wild-type mice (Boyer et al., 1990; Hatfield et al., 1984), GFAP IR was abolished in Vim − mice (our unpublished results), suggesting that in lens epithelial cells, the presence of vimentin is also necessary for GFAP network formation.

Previous studies using a double-labeling immunofluorescence technique with antibodies to vimentin and GFAP have shown that both GFAP and vimentin are colocalized in the same IF network in cultured mouse astrocytes, which has been also confirmed biochemically (Fedoroff et al., 1983; Quinlan and Franke, 1983; Sharp et al., 1982; Wang et al., 1984). Immunohistochemical studies combined with electron microscopy revealed GFAP and vimentin monomers in the same filaments of primary cultured astrocytes (Abd-el-Basset et al., 1992). Therefore, to address more directly the role of vimentin in GFAP network formation, we performed astrocyte cultures from E18 Vim − embryos. Vimentin was then reintroduced in these cells by transfection of a plasmid expressing human vimentin. Strikingly, in those cells where vimentin was reexpressed, double labeling immunofluorescence indicated that GFAP network formation was restored and apparently colocalized with the vimentin network. Thus, it seems clear that in some subpopulations of cells, the absence of vimentin precludes the formation of a GFAP/vimentin network. Interestingly, when the GFAP gene was inactivated by targeted mutagenesis, it could be shown in two independent studies that vimentin IR was not perturbed either in the brain (Pekny et al., 1995) or in the cerebellum, particularly in the Bergmann glia (Gomi et al.,
Figure 6. Vimentin (A and C) and GFAP (B and D) network detection by immunofluorescence in primary cultured astrocytes from control mice (A and B) and vimentin-deficient (C and D) mice. Note the abnormal GFAP network in the vimentin-deficient cells; some of them are negative (arrowhead), whereas other cells, even though positive, show disrupted GFAP filaments (arrow) (D). Bar, 23 μm (A and B); 20 μm (C and D).

1995). Furthermore, vimentin upregulation took place in reactive astrocytes generated either by brain stab wound or after scrapie infection, although in the latter case, subtle differences were noticed in stainability with antivimentin antibodies between wild-type and GFAP-reactive astrocytes (Gomi et al., 1995). Thus, it appears that whereas vimentin seems to be necessary for GFAP network formation, the converse does not hold true, at least in astrocyte populations.

The observation of the absence of GFAP IR in some subpopulations of astrocytes raised the important question of the underlying mechanisms: was it caused by the repression of GFAP gene activity or by some posttranscriptional or posttranslational mechanisms? Two lines of evidence suggest that, in fact, absence of GFAP IR is caused by a posttranslational mechanism. First, analysis of GFAP mRNA content in Vim− and Vim+ cultured astrocytes, as measured by RT-PCR, indicated that it was definitely present in the former. In several independent experiments, however, it was found that the level of GFAP mRNA was lower in Vim− cultured astrocytes compared to that of the Vim+ astrocytes. If this result was confirmed by quantitative RT-PCR analysis, it could give support to the hypothesis put forward by Traub and his colleagues that vimentin could act to regulate GFAP transcription (Traub and Shoeman, 1994). Second, Western blot analysis of GFAP content of cerebellar extracts did not uncover a significant difference in total GFAP content between Vim− and Vim+ mice. Whereas GFAP could be revealed in the cytoskeletal fraction of Vim− mice (as could be anticipated from the presence in the cerebellum of astrocytes with a network made of GFAP only), however, the amount of soluble GFAP appeared about twice that found in cerebellar extracts from Vim+ mice. It has been shown that in the rat brain, soluble GFAP is maintained as a constant small pool that does not increase during development as does the cytoskeletal GFAP (Malloch et al., 1987). These authors hypothesized that soluble GFAP is a steady-state pool of material involved in GFAP synthesis and assembly. Moreover, both vimentin (Blikstad and Lazarides, 1983; Soellner et al., 1985) and GFAP (Malloch et al., 1987) exist as a soluble pool before their incorporation.
Figure 7. Analysis by RT-PCR of GFAP mRNA content in cultured astrocytes from control (Vim+) and vimentin knockout (Vim-) mice. Total RNA was prepared from astrocyte cultures, reverse transcribed, and the cDNA product was amplified by PCR using the appropriate oligonucleotide (see Materials and Methods). (A) RT-PCR. A band is found at the expected size (281 bp) in both Vim+ and Vim- astrocytes (left line, molecular weight marker). (B) The gel presented in A was blotted onto a Hybond membrane, and the RT-PCR products were hybridized with a GFAP gene-specific oligonucleotide probe.

It is into the insoluble cytoskeletal scaffold. We suggest that the excess of soluble GFAP in Vim- cerebellar extracts corresponds to polypeptides that specifically need vimentin to build filaments and might be able to coassemble in situ with vimentin into IFs. In any event, taken together, the results of RT-PCR and Western blot analysis strongly suggest that the inability of some populations of cells to build up a GFAP network originates from a posttranslational mechanism.

One intriguing aspect of our results is the identification in the living mice of two populations of cells, one that normally coexpresses vimentin and GFAP and needs vimentin to build filaments and might be able to coassemble in situ with vimentin into IFs. In any event, taken together, the possibility that the Vim+ mice showed a higher GFAP level than Vim- mice indicates that one of the hallmarks of astrogliosis is the upregulation of GFAP. It has also been demonstrated that while the astrocytes distal to the wound upregulate GFAP only, those in the immediate vicinity of the wound recover the capacity to express vimentin that is lost during normal CNS development (Rataboul et al., 1988; Takamiya et al., 1988; reviewed in Eng and Ghirnikar, 1994; Hatten et al., 1991). When the Vim- mice were submitted to stab wound injury, we could show that no GFAP IR could be demonstrated in the region where both GFAP and vimentin are normally upregulated. In contrast, astrocytes distal to the wound retained the characteristic ability of overexpressing GFAP. These results reinforce the contention that there exists a subpopulation of astrocytes that need vimentin to assemble a GFAP network. But they also show that GFAP and vimentin may not be essential for astrocytes to become reactive, although vimentin gene induction took place in Vim- astrocytes near the wound, as demonstrated by the expres-

Figure 8. Restored GFAP network after vimentin expression in Vim- cultured astrocytes. (A) Cellular distribution of human vimentin after transient transfection of vimentin-deficient astrocytes visualized by mouse antibodies followed by rhodamine-conjugated secondary IgG. (B) GFAP detection in the same cells, using a rabbit antiserum to GFAP and an FITC-conjugated secondary antibody. Coexpression of human vimentin with endogenous GFAP drives efficient assembly of GFAP in mutant astrocytes. Bar, 20 μm.
Figure 9. GFAP expression after a stab wound injury of wild-type and vimentin-deficient CNS. Coronal sections (40-μm) of brains from 3-mo-old wild-type (A and C) and Vim− (B and D) mice. Arrows indicate the sites of stab wound injury. (A) GFAP detection in control mice. Reactive astrocytes both in the vicinity of and distal to the lesion are characterized by a strong GFAP IR. (B) GFAP detection in vimentin-deficient mice. In contrast to the wild-type mice, GFAP IR was absent in astrocytes located near the lesion. (C) Vimentin detection in wild-type mice. As expected, reactive astrocytes in the vicinity of the lesion exhibit a strong vimentin IR. Vimentin IR can be superimposed to the blank space observed in B. (D) β-Galactosidase detection in vimentin-deficient mice. Blue nuclei of astrocytes in the vicinity of the lesion indicate that vimentin gene is upregulated and, consequently, that these astrocytes are in a reactive state. Bar, 300 μm.

It is interesting to note that two groups who recently generated mice lacking GFAP (Gomi et al., 1995; Pekny et al., 1995) came to the same conclusion, namely that in the absence of the IF protein, reactive gliosis seems to be induced in a similar way to the one observed in wild-type mice. Thus, neither GFAP nor vimentin seems to be an absolute prerequisite for reactive gliosis to take place after brain injury. In a very recent study, it was shown that not only vimentin and GFAP are overexpressed in reactive astrocytes, but that nestin is also overexpressed in these cells (Frisén et al., 1995). It will be very interesting to check whether nestin assembly also depends on the presence of vimentin in some way.

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