Regulation of Immunoreactive GAP-43 Expression in Rat Cortical Macroglia is Cell Type Specific

Anna da Cunha and Ljubiša Vitković

Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892

Abstract. Growth-associated protein 43 (GAP-43) is an abundant, intensely investigated membrane phosphoprotein of the nervous system (Benowitz, L. I., and A. Routtenberg. 1987. *Trends Neurosci.* 10:527–532; Skene, J. H. P. 1989. *Annu. Rev. Neurosci.* 12:127–156), with a hitherto unknown function. We have previously demonstrated that astrocytes, brain macroglial cells, contain GAP-43 (Steisslinger, H. W., V. J. Aloyo, and L. Vitković. 1987. *Brain Res.* 415:375–379; Vitković, L., H. W. Steisslinger, V. J. Aloyo, and M. Mersel. 1988. *Proc. Natl. Acad. Sci. USA.* 85:8296–8300; Vitković, L., and M. Mersel. 1989. *Metab. Brain Dis.* 4:47–53). Results from double immunofluorescent labeling experiments presented here show that oligodendrocytes also contain GAP-43 immunoreactivity (GAP-43ir). Thus, all three macroglial cell types of the central nervous system (type 1 and type 2 astrocytes and oligodendrocytes) contain GAP-43. Whereas immunoreactive GAP-43 is expressed by progenitors of all macroglial cell types, the developmental regulation of its expression is cell type specific. Immunoreactive GAP-43 is downregulated in type 1 astrocytes, and constitutively expressed in both type 2 astrocytes and oligodendrocytes. These results may be relevant to potential function(s) of GAP-43.

GROWTH-associated protein-43 (GAP-43)[1] is an abundant, membrane-bound phosphoprotein present in the central nervous system of many species. The expression of this protein is dramatically elevated during development and regeneration (Jacobsen et al., 1986; Basi et al., 1987; Benowitz and Routtenberg, 1987; Skene, 1989). GAP-43 appears to play a role in synaptic plasticity underlying memory and learning (Benowitz and Routtenberg, 1987; Snipes et al., 1987), although its precise function in this process remains poorly understood. GAP-43 does not appear to be exclusively expressed in neurons, as previously suggested (Benowitz and Routtenberg, 1987; Benowitz et al., 1988; Goslin et al., 1988). Recent evidence based on electrophoretic, chemical and antigenic properties, indicate that GAP-43 is also present in rat cortical astrocytes (Steisslinger et al., 1987; Vitković et al., 1988; Vitković and Mersel, 1989). Astrocytes, together with oligodendrocytes, constitute the two major macroglial cell types in the vertebrate CNS (Miller et al., 1989). Two morphologically and antigenically distinct types of astrocytes, type 1 and 2, have been characterized in dissociated cell suspensions of rat embryonic and postnatal brain (Abney et al., 1981) and optic nerve (Raff, 1989; Williams et al., 1985). Immunostaining and silver impregnation of the optic nerve suggest that these two kinds of macroglial cells exist in vivo (Miller et al., 1989).

Whether type-1 and 2 astrocytes in the cortex (Miller and Raff, 1984) correspond to protoplasmic and fibrous astrocytes, respectively, (Williams et al., 1985) remains unclear (Miller et al., 1989). The three types of macroglia, type 1 astrocytes, type 2 astrocytes and oligodendrocytes, can be distinguished in cultures of embryonic rat brain, where, remarkably, the cells develop on the same schedule as in vivo (Abney et al., 1981; Williams et al., 1985). Some distinguishing properties of the three major types of macroglia have been summarized in recent reviews (Miller et al., 1989; Raff, 1989). We are investigating the regulation of GAP-43 gene expression to elucidate the role of this phosphoprotein in CNS function. Here, we report results on the developmental expression of GAP-43ir in primary cultures of rat cortical macroglia, which demonstrate that all three known macroglial cell types and their progenitors express GAP-43ir. Our results further show that the regulation of macroglial GAP-43 is cell type-specific in that type 1 astrocytes downregulate it and type 2 astrocytes and oligodendrocytes appear to express it constitutively.

Materials and Methods

Astrocyte Cultures

Brain cortices removed from 1-d-old Sprague-Dawley rats served as a source of the macroglial cells. Astrocyte cultures were prepared as previ-

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1. *Abbreviations used in this paper:* GAP-43, growth-associated protein-43; GFAP, glial fibrillary acidic protein.

ously described (Sensenbrenner et al., 1980; Vitković et al., 1988; Vitković and Mersel, 1989). Briefly, 1-d-old Sprague-Dawley rat newborns were decapitated and their brain hemispheres were removed. The hemispheres, freed of meninges, were soaked in glutamine-free DME with 50 U/ml penicillin, 50 μg/ml streptomycin, and 20% FCS, gently dissociated mechanically by pipetting, and filtered through 80-μm nylon mesh (Nytex; Tetko, NJ). The resulting dispersed cells were seeded at a density of 1-2 × 10⁴ cells per cm², on 13-mm-wide glass coverslips that had been precoated with poly-L-lysine at 3 μg per cm² (Sigma Chemical Co., St. Louis, MO), for 15 min. The medium consisted of glutamine-free DME with 50 U/ml penicillin, 50 μg/ml streptomycin and 20% FCS (Hazelton, St. Lenexa, KS). After first 3 d of seeding, the percent FCS in the above medium was lowered to 10%. The medium was changed every 2 d.

Enriched Oligodendrocyte Cultures

Oligodendrocytes were prepared by modifying the published method of Dubois-Dalcq et al. (1986). 1-d-old Sprague-Dawley rat cortices were dissected, meninges were removed, and tissue was triturated 10 times through a 10-gauge needle, and passed through an 80-μm Nytex nylon mesh. Cells derived from four cortices and suspended in 10 ml of medium (DME containing 20% FCS, 50 U/ml penicillin, and 50 ug/ml streptomycin) were seeded in 75-cm² plastic flasks (Costar Corp., Cambridge, MA) at 1.2 to 2.4 × 10⁵ cells per cm². After 3 d incubation at 37°C, the cultures were shaken on a rotary shaker at 100 rpm for 1 h at room temperature. The medium containing oligodendrocytic progenitors was removed and centrifuged at 77 g for 5 min. The cells were resuspended in the medium described above but containing 10% FCS and replated at the initial density in one 75-cm² plastic flask. Cells were grown for another 3 d and then shaken overnight at 37°C at 100 rpm. Nonattached cells were harvested as described above and suspended in glutamine-free DME with 25 μg/ml gentamycin and 4.5 g/liter glucose enriched in 2 mM glutamine, 50 μg/ml transferrin, 5 μg/ml insulin, 30 nM selenium, 30 nM triiodothyronine (Eccleston and Silberberg, 1984). To this basic medium we added 0.5% FCS as recommended by Dubois-Dalcq et al. (1986) to facilitate the early adhesion and growth of progenitors. The resulting dispersed cells were seeded at a density of 10⁵ cells per cm², on 13-mm wide glass coverslips which had been precoated with 3 μg/cm² poly-L-lysine as described above.

Neuron Cultures

Primary cultures of rat cerebellar granule neurons were prepared as previously described (Novelli et al., 1988) and provided by R. C. Henneberry (National Institute of Neurological Disorders and Stroke [NINDS], National Institutes of Health).

Purification of GAP-43

GAP-43 was isolated from 8-d-old rat brain by alkaline extraction, ammonium sulphate precipitation and isoelectric focusing (IEF, Sephadex G-200 flat bed), as previously described (Oestreicher et al., 1983). IEF-derived fractions were passed through mixed bed AG 501 X8 resin to remove ampholines and provided to us by V. J. Aloyo (Medical College of Pennsylvania, Philadelphia). We resolved the proteins in GAP-43-enriched fraction by preparative SDS-PAGE using 10-20% gradient gels (Integrated Separation Systems, Hyde Park, MA). The band corresponding to GAP-43, identified by Western blotting, was excised and electrophoretically eluted using the Elutrap (Schleicher & Schuell, Keene, NH) according to the manufacturer's instructions. Protein recoveries were estimated using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA).

Fixation and Immunolabeling

Individual macroglial cell types were identified by double immunolabeling of cells in culture with antibodies described below. Cells were incubated at room temperature in 40-50 μl of each antibody solution for 30 min, followed by an incubation in the appropriate rhodamine- or fluorescein-conjugated second antibody solution. Antibodies were diluted in DME (Whittaker Bioproducts, Walkersville, MD), containing 0.02 M Hepes and 10% FCS. After every incubation, cells were washed several times with DME containing 0.02 M Hepes. Surface antigens rat neural antigen-2 (Ran-2), A2B5, and galactocerebroside (GC) were visualized on living cells (Wolswijk and Noble, 1989). Intracellular antigens glial fibrillary acidic protein (GFAP) and neurofilament protein, NF-68, were detected after fixation in methanol at −20°C for 10 min (Wolswijk and Noble, 1989). The membrane

associated antigen GAP-43 was detected after both, fixation in methanol at −20°C for 10 min followed by permeabilization with 0.01% (wt/vol) digitonin for 30 min and direct exposure to primary antibody (Vitković et al., 1988). Cells were immunolabeled with a combination of the following: mouse IgM A2B5 monoclonal antibody obtained from M. Dubois-Dalcq (NINDS, NIH) (ascites, 1:100 or fresh, undiluted hybridoma supernatant; Eisenbarth et al., 1979), mouse IgM Ran-2 (fresh, undiluted hybridoma supernatant; Bartlett et al., 1981), mouse IgG anti-GC monoclonal antibody (hybridoma supernatant, 1:10, Ranscht et al. 1982), polyclonal rabbit anti-GFAP antiserum (obtained from R. Lipsky, NINDS, NIH) (1:500; Temple and Raff, 1985), mouse IgG monoclonal antineurofilament 68 kD (undiluted, Boehringer-Mannheim Biochemicals, Indianapolis, IN). Two polyclonal antibodies against rat GAP-43 were used: a rabbit serum obtained from G. J. Snipes and J. A. Freeman (Vanderbilt University School of Medicine; Skene et al., 1986) was partially purified by adsorbing out the IgGs specific to cortical plasma membrane proteins other than GAP-43 (Vitković et al., 1988). An affinity-purified anti-GAP-43 antibody raised in sheep was from L. I. Benowitz (Mailman Research Center, McLean Hospital, Belmont, MA; Benowitz et al., 1988). The binding of A2B5 or Ran-2 antibodies was detected with fluorescein- or rhodamine-conjugated secondary antibodies. The fluorescein detection method used donkey anti-mouse IgG, biotinylated species-specific F(ab')2 fragment (1:200; Amersham Corp., Arlington Heights, IL), followed by streptavidin-FITC (1:200, Amersham). The rhodamine detection method used goat anti-mouse IgG lissamine rhodamine conjugate (1:1,000; Boehringer-Mannheim Biochemicals). The binding of rabbit anti-GFAP was detected with donkey anti-rabbit IgG, biotinylated species-specific F(ab')2 fragment (1:200; Amersham Corp.), followed by streptavidin-FITC (1:200; Amersham Corp.) or goat anti-rabbit IgG lissamine rhodamine conjugate (1:1,000; Boehringer-Mannheim Biochemicals). The binding of mouse anti-NF-68 was detected with goat anti-mouse IgG lissamine rhodamine conjugate (1:1,000; Boehringer-Mannheim Biochemicals). The binding of rabbit anti-GAP-43 was detected with donkey anti-rabbit IgG, biotinylated species-specific F(ab')₂ fragment (1:200; Amersham Corp.) followed by streptavidin-FITC (1:200; Amersham Corp.) or ABC-phycoerythrin (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. In some experiments, goat anti-rabbit IgG lissamine rhodamine conjugate (1:1,000; Boehringer-Mannheim Biochemicals) was used. The binding of sheep anti-GAP-43 was detected with biotinylated anti-sheep IgG (1:400; Vector Laboratories) followed by either streptavidin-FITC (1:200; Amersham Corp.) or ABC-phycoerythrin (Vector Laboratories).

This double-immunofluorescence procedure was carried out using two cell type-specific antigens to distinguish individual macroglial cell types and their progenitors. Bipolar A2B5⁺,GFAP⁻; A2B5⁺,Ran2⁻; A2B5⁺,GC⁻ cells were considered to be oligodendrocyte-type 2 astrocyte (O-2A) progenitors and bipolar Ran-2⁺,GFAP⁻; Ran-2⁺,GC⁻; Ran-2⁺,A2B5⁻ cells were considered to be type 1 astrocyte progenitors (Raff et al., 1979; Miller et al., 1989). Cell with many narrow processes, A2B5⁺,GFAP⁺, Ran-2⁻,GFAP⁺ were considered type 2 astrocytes and cells with few broad processes, Ran-2⁺,GFAP⁺; A2B5⁻,GFAP⁺, type 1 astrocytes (Raff et al., 1979; Miller et al., 1989). GC⁺,GFAP⁻; GC⁺,Ran-2⁻; GC⁺,A2B5⁻ cells were considered oligodendrocytes (Raff et al., 1978). Primary cultures were NF-68⁻ and therefore considered free of neurons (Vitković et al., 1988).

Once individual macroglial cell types were characterized, the double immunofluorescence procedures were used to identify cells containing GAP-43ir. The method used for staining cultured cells with anti-GAP-43 was that published by Meiri et al. (1986), modified by Vitković et al. (1988). Cultures were exposed to anti-GAP-43 followed by antibody against the cell type-specific antigen, followed by fluorescein- and rhodamine-conjugated secondary antibodies, respectively. Cells that were GFAP⁺,GAP-43⁺ were considered type 1 and type 2 astrocytes (Bignami et al., 1972). Whereas type 1 astrocytes and their progenitors were recognized by their cell-specific antigen Ran-2, type 2 astrocytes and oligodendrocyte-type 2 astrocyte progenitors were recognized by antigen A2B5 (Raff et al., 1979; Miller et al., 1989). Oligodendrocytes were recognized by their cell specific antigen GC (Raff et al., 1978). In double stainings involving the surface antigens, and membrane associated GAP-43 cells on coverslips were sequentially surface labeled first for either A2B5, Ran-2 or GC, followed by anti-mouse rhodamine, and then GAP-43 was detected as described above. In these experiments cells that were rhodamine⁺ (A2B5⁺, Ran-2⁺ or GC⁺) were easily distinguished from cells that were fluorescein⁺ (GAP-43⁺).

After immunolabeling, coverslips were washed and mounted on a drop of aqueous mounting medium (Chemicon International, Inc., Temecula, CA). Cells were examined in a Zeiss axioplan microscope equipped with phase contrast, epifluorescence, and rhodamine and fluorescein optics and equipped for photography.
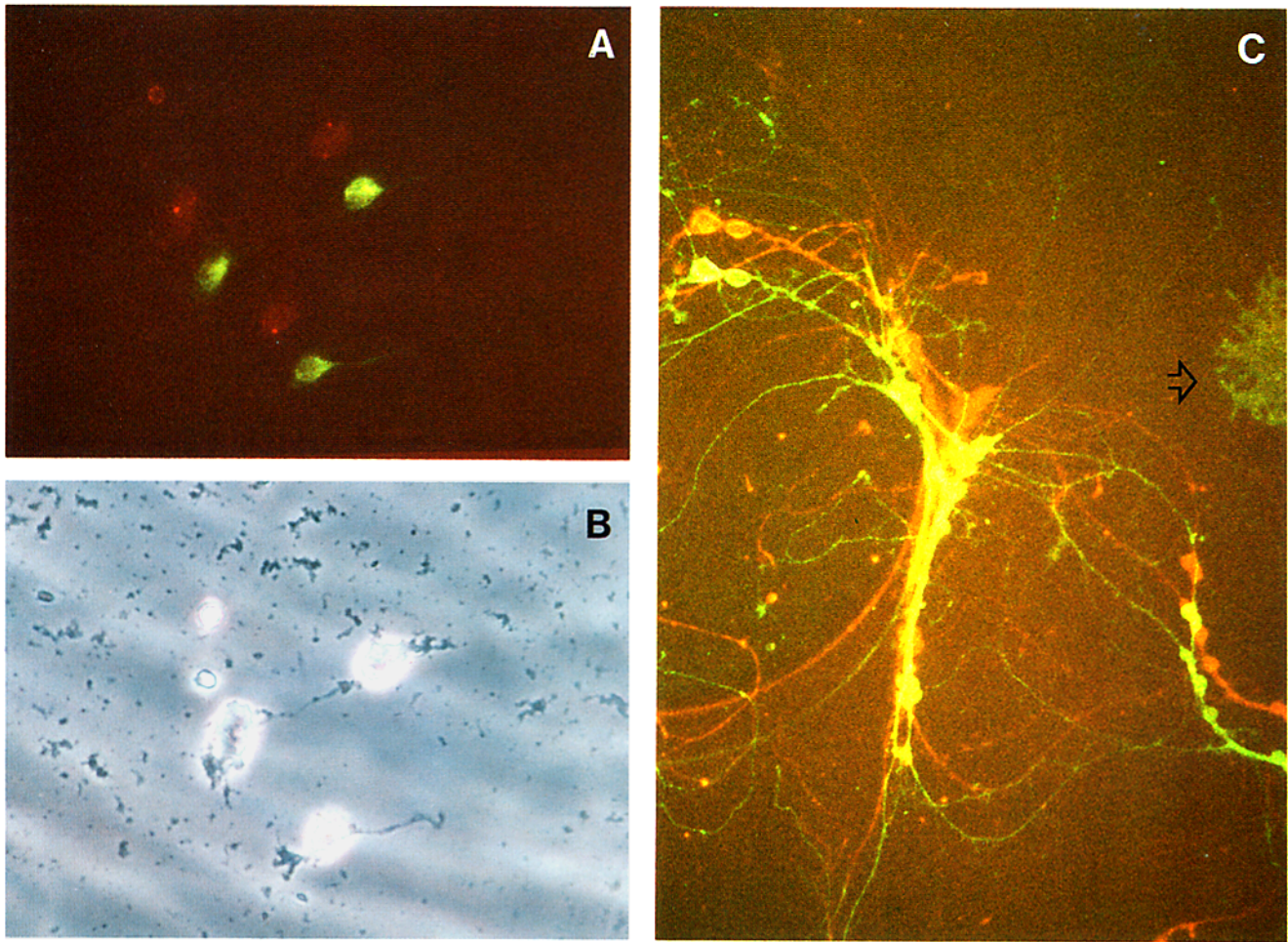
Figure 1. Cells in primary astrocytic culture do not stain with antibody to 68-kD neurofilament (NF68) but do stain with antibody to GFAP. Cells were cultured for 2 days and immunostained with anti-NF68 and anti-GFAP antibodies followed by rhodamine- (red) and FITC-conjugated (green) secondary antibodies, respectively. Double exposure photomicrograph is shown (*A*). Phase-contrast micrograph reveals several round NF68$^-$, GFAP$^-$ cells, which are probably astrocytic progenitors (*B*). In contrast, anti-NF68 antibody stained intensely cerebellar neurons in primary culture (*C*). Cells were cultured 8 d and immunostained with anti-NF68 and anti-GAP-43 antibodies followed by rhodamine and FITC-conjugated secondary antibodies, respectively. Double-exposure photomicrograph demonstrates the ease of labeling with anti-NF68 as well as no mutual interference between rhodamine and FITC signals. Note an NF68$^-$, GAP-43$^+$ cell with glial morphology (*arrow, C*). ×186.

Photomicrography and Quantitation

The time course of GAP-43ir expression in cultured rat cortical astrocytes was assessed immunocytochemically as follows: three coverslips were randomly selected and cells stained with anti-GAP-43 and anti-GFAP followed by fluorescein- and rhodamine-conjugated secondary antibodies, respectively, as described in "Fixation and Immunolabeling." Three fields were photographed from each coverslip using T-Max 400 (Eastman Kodak Co., Rochester, NY) film exposed for 20 s at 1,600 ASA using filters for either rhodamine or fluorescein at a magnification of 250. Contact prints such as the ones in Fig. 3 were made and cells counted with the aid of a magnifying glass. On the average, 517 cells were counted per time point or from 18 to 237 per field. The number of GFAP$^+$ cells expressing GAP-43ir were estimated as percentage of total cells expressing GFAP. The number of oligodendrocytes expressing GAP-43 was assessed after double immunostaining with anti-GC and anti-GAP-43ir as described in "Fixation and Immunolabeling." Cells were photographed as described above and percent cells staining for both GC and GAP-43ir calculated as percent total GC$^+$ cells. To determine the expression of GAP-43ir separately in types 1 and 2 astrocytes, cells were double immunostained with anti-GFAP and either anti-A2B5 or anti-Ran-2. The percent A2B5$^+$,GFAP$^+$ with many narrow processes (type 2 astrocytes; Raff et al., 1979; Miller and Raff, 1984; Miller et al., 1989) was calculated with reference to the number of Ran-2$^+$,GFAP$^+$

cells with few broad processes (type 1 astrocytes; Raff et al. 1979; Miller and Raff, 1984; Miller et al., 1989). This ratio of type 1 to type 2 astrocytes was compared with values obtained from cells stained with anti-GFAP and anti-GAP-43. Photographs of cells stained with both anti-GFAP and anti-GAP-43 were assessed for percent cells that were GFAP$^+$ and having many narrow processes (type 2 astrocytes; Raff et al., 1979; Miller and Raff, 1984; Miller et al., 1989) as opposed to percent cells that were GFAP$^+$ with few broad processes (type 1 astrocytes; Raff et al., 1979; Miller and Raff, 1984; Miller et al., 1989). The values obtained for percent type 1 and type 2 astrocytes were similar to those obtained using cell type-specific markers, Ran-2$^+$/GFAP$^+$ and A2B5$^+$/GFAP$^+$ described above.

The number of astrocytes per unit surface area was determined by detaching cells with 0.05% trypsin (suspended in 0.02% EDTA in calcium, magnesium-free HBSS) at 37°C for 2 min. Viable cells were counted after dilution (1:1, vol/vol) in trypan blue (0.4%, wt/vol).

Whenever appropriate the results were analyzed by *t* test.

Results and Discussion

Astrocytic cultures used in this study are a well-defined, homogeneous population consisting of both types of astrocytes
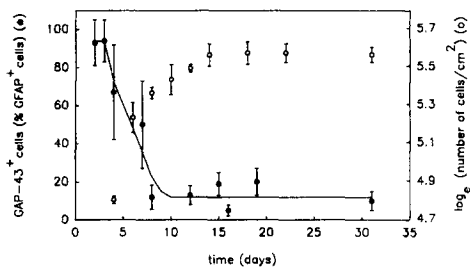
Figure 2. Time course of GAP-43 expression in cultured rat cortical astrocytes assessed immunocytochemically. Number of GAP-43$^+$ cells as a fraction of the total GFAP$^+$ cells was plotted against the time in culture ($\bullet$). Data represent an average and SD of three independent experiments. In one experiment the rabbit and in the other two, the sheep anti-GAP-43 antibodies were used. Growth of astrocytes in culture is noted by open circles (O). In each case, the number of GFAP$^+$ cells was 95–100% of cells recorded in light phase photomicrographs.

and no neurons (Sensenbrenner et al., 1980; Vitković et al., 1988). The staining of these cells from culture day 2 onwards with anti-NF68, a neuron-specific marker, has been persistently negative (Fig. 1 A and Vitković et al., 1988). This strongly suggests, although it does not prove, that there are no neurons in these cultures. In contrast to astrocytic cultures, cerebellar granule neurons in primary culture immunostained reproducibly and intensely with anti-NF68 (Fig. 1 C). These cells contained large amounts of GAP-43 as judged by intense immunostaining with anti-GAP-43 (Fig. 1 C) as well as assessed by two dimensional PAGE as we showed previously (Steisslinger et al., 1987). The pattern of immunostaining with anti-NF68 is similar but distinct from that observed with anti-GAP-43 (Fig. 1 C). This suggests no "bleed through" between rhodamine and FITC filters, which is consistent with the manufacturer's specifications.

Using double immunofluorescence labeling with antibodies to GFAP as an intracellular marker for astrocytes (Raff et al., 1979) and one of the two monospecific antibodies to GAP-43 (Benowitz et al., 1988; Skene et al., 1986; Vitković et al., 1988), we counted GAP-43 immunoreactive astrocytes present at various times during development. Results obtained with the purified rabbit anti-GAP-43 did not differ from those obtained with the sheep anti-GAP-43. Both monospecific antibodies to GAP-43 have been demonstrated, by multiple criteria, to react specifically with GAP-43 (Benowitz et al., 1988; Skene et al., 1986; Vitković et al., 1988). In control experiments we substituted sheep anti-GAP-43 with normal sheep serum (diluted 1:500) and found no staining of cells. Further, when the sheep anti-GAP-43 antibody (1 µl) was neutralized with rat GAP-43 protein (5 µg) for 30 min at 37°C and then used (1:500 dilution) to stain astrocytes, again, no immunostaining was observed (data not shown). These results together with those already published demonstrate that the anti-GAP-43 antibodies used in these studies were specific for GAP-43.

At 2 d of culture, GAP-43 was present in 80–100% of astrocytes isolated from 1-d-old cortex. During the ensuing 3 wk, the number of GAP-43 immunoreactive astrocytes decreased markedly, consistent with our earlier report (Vitković and Mersel, 1989). The number of GFAP$^+$ cells contain-

ing GAP-43ir decreased at a linear rate of ~8.4%/d (Fig. 2), whereas the amount of GAP-43 protein, as assessed by Western blotting decreased in a parallel culture at the linear rate of ~9.1%/d (data not shown). Hence, the amount of GAP-43 protein was proportional to the number of cells immunostained for GAP-43 and, thus, to the intensity of immunofluorescence. Thus, the number and intensity of immunofluorescence of cells stained with anti-GAP-43 reflected the amount of GAP-43 present in these cells.

Double immunofluorescence labeling of cells with anti-GAP-43 and anti-GFAP showed that at 3 d in culture most astrocytes stained with both anti-GAP-43 and anti-GFAP. Most of these cells looked like type 1 astrocytes, they were flat and nonprocess bearing (Fig. 3, A–C). After 8 d, ~80% of the GFAP$^+$ cells stained weakly and 20% intensely with anti-GAP-43. The weakly stained GAP-43$^+$ cells had type 1 morphology (Fig. 3, D–F), whereas those that stained intensely had type 2 morphology (process bearing, Fig. 3, D–F). By day 14 of culture the distinction was even more clearly discernible (Fig. 3, G–I). These observations suggest that the regulation of GAP-43ir expression in astrocytes is cell type specific.

Using as criteria antiastrocyte type-specific antibody binding (Ran-2 and A2B5; Miller and Raff, 1984) and morphology (Raff et al., 1979), we scored type 1 and 2 GFAP$^+$ astrocytes for expression of GAP-43 immunoreactivity. 10–20% of GAP-43 immunoreactive astrocytes cultured for 8 d were predominantly, if not exclusively, type 2 astrocytes (Fig. 4). While the intensity of staining of type 1 astrocytes with anti-GAP-43 progressively decreased over 8 d, type 2 astrocytes constantly expressed this protein to ages corresponding to the adult cortex (Figs. 3 and 4). Because ~80–90% of the cells in these cultures were type 1 astrocytes, the developmental pattern of immunoreactive GAP-43 astrocytes described above, and the decrease in GAP-43 protein (Vitković and Mersel, 1989) correspond predominantly to type 1. These results suggest that GAP-43 is downregulated in type 1 astrocytes and constitutively expressed in type 2 astrocytes. The adult rat cerebellum is reported to contain predominantly type 1 astrocytes (Raff et al., 1983) and low levels of GAP-43 mRNA (Skene et al., 1986), which is consistent with our observations.

Oligodendrocytes and type 2, but not type 1, astrocytes develop from a common A2B5$^+$ progenitor cell known as the bipotential O-2A progenitor (Temple and Raff, 1985; Miller et al., 1989; Raff, 1989). Because type 2 astrocytes appeared to constitutively express GAP-43ir, it was of interest to determine whether oligodendrocytes also express GAP-43ir. When oligodendrocytes were exposed to antibodies to their cell type marker, GC, and GAP-43, followed by appropriate secondary antibodies, the coincidence between the two antigens was observed (Fig. 5). Both the rabbit and sheep anti-GAP-43 antibodies have been found to react specifically with rat GAP-43 as demonstrated by multiple criteria (Benowitz et al., 1988; Skene et al., 1986; Vitković et al., 1988). Oligodendrocytes stained at 9, 11, 22, 23, and 28 d in culture were GAP-43ir$^+$. These results were obtained with two monospecific polyclonal antisera obtained from two independent sources, as described above. The intensity of labeling with anti-GAP-43 was independent of when the cells were stained. This suggests that oligodendrocytes contain GAP-43ir and that its expression is independent of time in
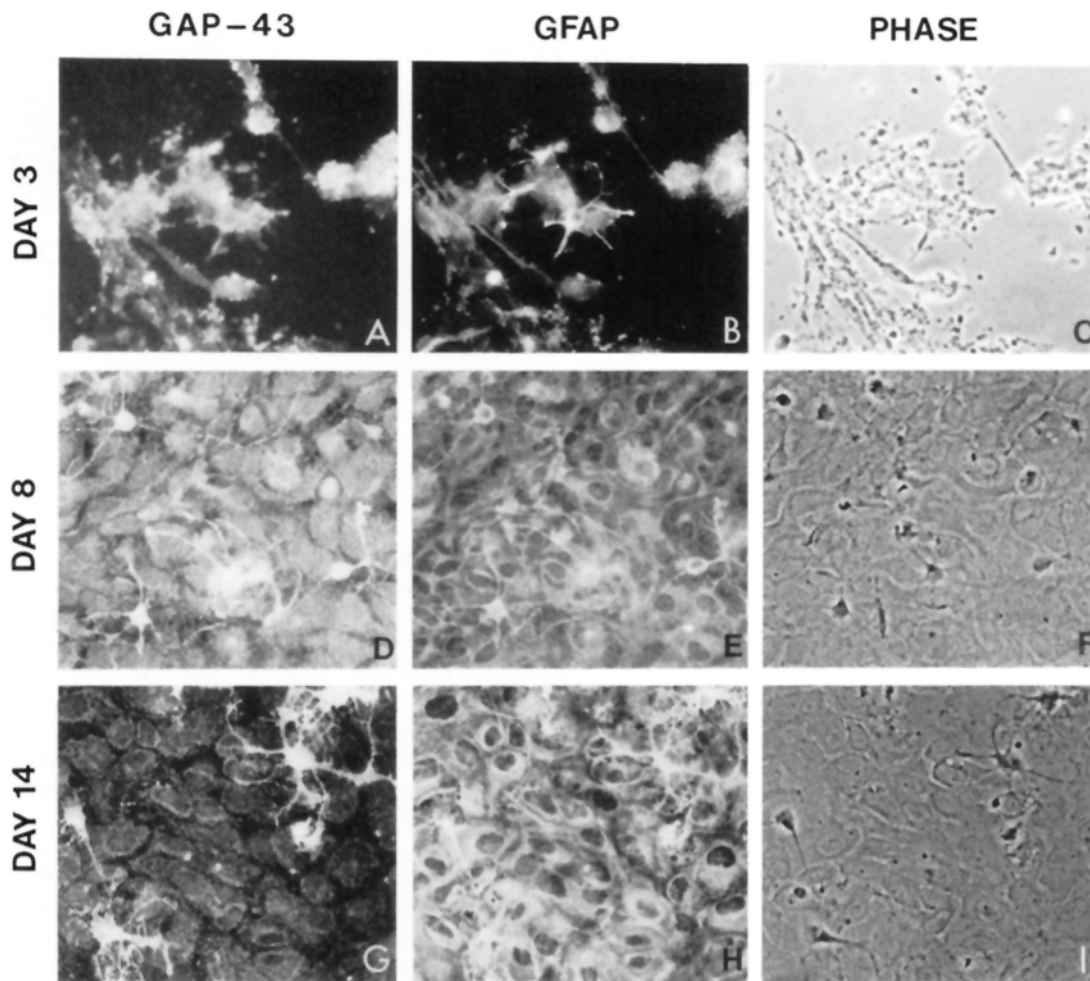
Figure 3. Differential expression of GAP-43ir in two types of astrocytes during development. A fluorescent photomicrograph of cells 3 d in culture stained with anti-GAP-43 (*A*). The majority of cells were GFAP$^+$ (*B*) with the morphology of type 1 astrocytes (*C*). A fluorescent photomicrograph of astrocytes 8 d in culture stained with anti-GAP-43 (*D*). Approximately 80–90% of these cells stained weakly for GAP-43, and had the type 1 morphology (*E*). 10–20% of the cells intensely stained for GAP-43 (*F*) and GFAP (*E*) and had the type 2 morphology (*F*). A fluorescent photomicrograph of astrocytes stained with anti-GAP-43 after 14 d in culture (*G*). Approximately 10–20% of the cells intensely stained for GFAP (*H*) and had the type 2 morphology (*I*). The distinct morphology of the two types is clearly visible. ×250.
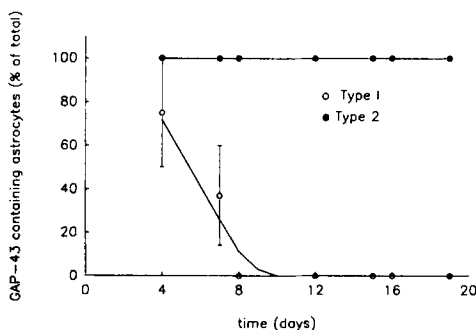

Figure 4. Developmental expression of GAP-43ir differs in the two astrocytic cell types. Number of GAP-43$^+$ cells as a fraction of all GFAP$^+$ cells of the type 1 (o) and type 2 (●) morphology was plotted against the time in culture. In these cultures containing both type 1 and type 2 astrocytes, cells of the type 2 morphology stained A2B5$^+$, GAP-43$^+$, whereas type 1 stained A2B5$^-$, Ran-2$^+$. Data represent an average number of cells and standard deviation from three independent experiments.

culture. Thus, the regulation of GAP-43ir expression in oligodendrocytes appears to be constitutive.

We also wanted to know how early GAP-43ir is expressed in the differentiation of each macroglial cell type. To address this question we double-stained cells in either astrocytic or oligodendrocytic cultures early in development with combinations of cell type–specific antibodies. We observed that bipolar Ran-2$^+$ GFAP$^-$ and A2B5$^+$ GFAP$^-$ or A2B5$^+$ GC$^-$ cells characteristic of type 1 and 0–2A progenitors, respectively (Federoff, 1985; Temple and Raff, 1985), stained very intensely with anti-GAP-43 antibody, indicating that they contained high levels of GAP-43ir (Fig. 6). This result suggests that GAP-43ir is expressed during the differentiation of macroglial progenitors before GFAP in astrocytes and GC in oligodendrocytes are expressed.

The developmental regulation of GAP-43ir expression, the hallmark of this protein (Jacobsen et al., 1986; Basi et al., 1987) may be, at least in part, determined by macroglial differentiation. Increase in GAP-43 during the first postnatal
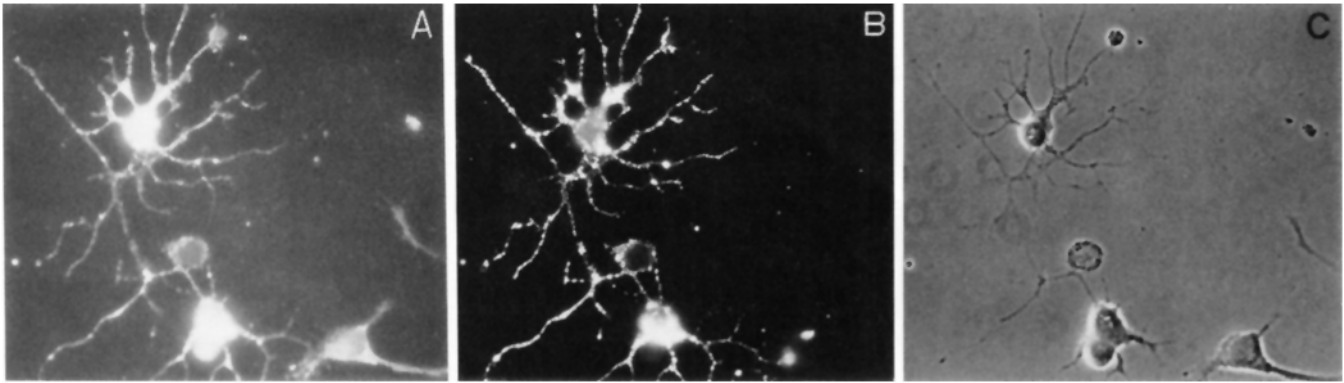
Figure 5. Oligodendrocytes express GAP-43ir. Fluorescent photomicrograph of rat cortical cells 23 d in culture stained with anti-GAP-43 antibody (*A*) and anti-galactocerebroside antibody (*B*), display the oligodendrocyte morphology (*C*).

week (Jacobsen et al., 1986; Basi et al., 1987) coincides with the proliferation of oligodendrocyte type 2 astrocyte progenitors in addition to neurons. Its precipitous decrease over the next 2 wk of development coincides with the down-regulation observed in type 1 astrocytes. This is supported by the observations that brain regions presumed rich in type 1 astrocytes, such as cerebellum, express little GAP-43 mRNA in adult rat brain (Raff et al., 1983; Oestreicher et al., 1986; Neve et al., 1987; Benowitz et al., 1988). The high amounts of GAP-43 that persist in adult brain (Oestreicher et al., 1986; Karns et al., 1987; Snipes et al., 1987; Benowitz et al., 1988) may be partly due to constitutive expression of GAP-43 in oligodendrocytes and type 2 astrocytes. We have also found that granule cells from the cerebellum produce high levels of GAP-43 protein (Steisslinger et al., 1987) and their long neurites in culture intensely stain with anti-GAP-43 (Fig. 1 *C*). These macroglial cell types and granule neurons are process-bearing with large surface-to-volume ratios. In contrast, type 1 astrocytes in confluent culture are polygonal cells without long processes. Thus, constitutive GAP-43 expression occurs in highly arborescent cells with branched or unbranched processes, and not in cells with small surface-to-volume ratio. It appears as if the number of

GAP-43 molecules per unit area of membrane may be held constant. This suggests that GAP-43 may function during differentiation in the synthesis and, after differentiation, in the maintenance of neural cell processes or neurites. These processes do not have to necessarily form synapses (Goslin et al., 1988) but may form nodes of Ranvier or myelin sheaths (Miller et al., 1989; Raff, 1989). The observation that three immortalized, non-neural cell lines transfected with the GAP-43 gene in an expression vector grew long thin processes reminiscent of filopodia, supports this view (Zuber et al., 1989).

After the submission of this manuscript it has come to our attention that others have detected GAP-43ir in nonneuronal cells. GAP-43ir was detected using one of the two antibodies we used, in satellite/Schwann cells in cultured adult rat dorsal root ganglia (Woolf et al., 1990.) Thus, nonneuronal cells of the peripheral nervous system are also capable of expressing GAP-43ir.
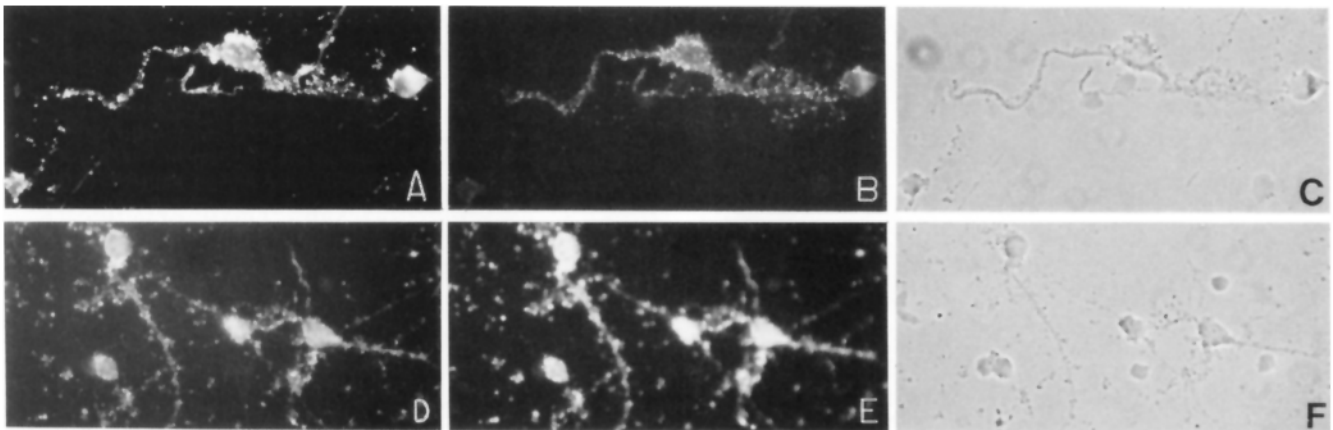
Figure 6. Expression of GAP-43 immunoreactivity in type 1 astrocyte and oligodendrocyte-type 2 astrocyte progenitors. The cells stained with anti-GAP-43 (*A*) were considered to be type 1 astrocyte progenitors because they were A2B5⁻, Ran-2⁺ (*B*), and bipolar (*C*). Oligodendrocyte-type 2 astrocyte progenitors stained with anti-GAP-43 (*D*). They were A2B5⁺ (*E*) and bipolar (*F*). Cells were prepared as described in the text and immunostained 2 d after seeding.

providing monospecific anti-GAP-43 sera; Dr. V. J. Aloyo for providing partially purified GAP-43; and Dr. R. C. Henneberry for granule neurons.

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