Growth Arrest Specific Gene 1 Is a Positive Growth Regulator for the Cerebellum

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Postnatal cerebellum development involves the generation of granule cells and Bergmann glia (BGs). The granule cell precursors are located in the external germinal layer (EGL) and the BG precursors are located in the Purkinje layer (PL). BGs extend their glial fibers into the EGL and facilitate granule cells’ inward migration to their final location. Growth arrest specific gene 1 (Gas1) has been implicated in inhibiting cell-cycle progression in cell culture studies (G. Del Sal et al., 1992, Cell 70, 595–607). However, its growth regulatory function in the CNS has not been described. To investigate its role in cerebellar growth, we analyzed the Gas1 mutant mice. At birth, wild-type and mutant mice have cerebella of similar size; however, mature mutant cerebella are less than half the size of wild-type cerebella. Molecular and cellular examinations indicate that Gas1 mutant cerebella have a reduced number of granule cells and BG fibers. We provide direct evidence that Gas1 is required for normal levels of proliferation in the EGL and the PL, but not for their differentiation. Furthermore, we show that Gas1 is specifically and coordinately expressed in both the EGL and the BGs postnatally. These results support Gas1 as a common genetic component in coordinating EGL cell and BG cell proliferation, a link which has not been previously appreciated. © 2001 Academic Press

Key Words: Gas1; growth; cerebellum; Bergmann glia; external granular layer.

INTRODUCTION

The cerebellum is located on the brain stem at the midbrain–hindbrain junction and functions to control motor coordination. Precise numerical matching and synapse formation among various neuronal cell types within the cerebellum are required for such function (Altaman and Bayer, 1997). Mature cerebella contain approximately 10^8 granule cells which constitute 80–85% of the neuronal population within the mouse central nervous system (CNS). Mouse mutants lacking granule cells display severe ataxia (Kofuji et al., 1996; Hamre and Goldowitz, 1997; Mullen et al., 1997), demonstrating the functional importance of these cells. Granule cells regulate cerebellum function by controlling the activity of the Purkinje cells, the only outputs of the cerebellum (Altaman and Bayer, 1997).

Precursors to the granule cells are specified during embryogenesis (Alder et al., 1996). They first arise from the rhombic lip at the anterior hindbrain and migrate anteriorly to form a secondary proliferative zone covering the outer layer of the cerebellum anlage around E13.5 in the mouse. This layer is called the external germinal layer or the EGL (Miale and Sidman, 1961; Fujiita et al., 1966). Within the first 3 weeks of birth, cells in the superficial layer of the EGL (oEGL) proliferate extensively to generate a large number of granule cell precursors. Concurrently, some of these cells exit cell cycle, migrate transversely through the deeper layers of the EGL, and form an inner granule layer (iEGL). These cells migrate further inward, past the Purkinje layer, and terminally differentiate as granule cells in their final location, the internal granular layer (IGL) (Hallonet et al., 1990; Ryder and Cepko, 1994). At the end of this process, virtually all precursor cells in the EGL are depleted. The mature cerebellum’s outer layer consists mainly of Purkinje dendrites, granule cell axons, glial fibers, and stellate cells and is called the molecular layer.

The cellular interactions that direct the proliferation, differentiation, and migration of the granule cells are well described (reviewed by Hatten and Heintz, 1995). At least two independent mechanisms can regulate the proliferative activity of the granule cell precursors. Using in vitro high-density cultures, Gao et al. (1991) showed elegantly that a
homotypic contact-dependent paracrine signal between the EGL cells can stimulate their proliferation. It has also been shown by mutant analysis (Sonmez and Herrup, 1984; Vogel et al., 1989; Mullen et al., 1997) and ablation studies (Smythe et al., 1995) that the Purkinje cells are important for the growth and survival of the EGL cells. On the other hand, granule cell differentiation and survival have been shown to be influenced by the Bergmann glial (BG) cells in vitro (Hatten et al., 1988). BGs interpose between the Purkinje cells and send out radial fiber scaffold spanning the molecular layer. Postmitotic granule cells associate with the BG fibers during their migration, consistent with the proposal that these fibers serve as their migration substrate (Rakic, 1971; Hatten, 1993; Zheng et al., 1996). It is not clear whether there is a molecular mechanism to match the numbers between the granule cells and BG fibers to ensure proper migration.

Although the cellular interactions are well described, the molecular components that mediate these interactions are just beginning to be identified. The EGL proliferation-stimulating signal derived from the Purkinje cells has recently been attributed to the growth factor Sonic Hedgehog (SHH) based on antibody-blocking and cell culture experiments (Wallace, 1999; Wechsler-Reya and Scott, 1999; Dahmane and Ruiz-i-Altaba, 1999). Furthermore, analysis of several mutant mice has assigned the contributions of the following genes in cerebellum development. Math1, a basic helix-loop-helix (bHLH) containing gene, is essential for the genesis of granule cell precursors during embryogenesis (Ben-Arie et al., 1997). The Zn-finger transcription factor Zic1 is implicated in controlling the proliferation of granule cells at early stages (Aruga et al., 1998). Another bHLH transcription factor, NeuroD, is expressed in the iEGL and is required for granule cell differentiation (Miyanaka et al., 1999). Brain-derived neurotrophic factor (BDNF) controls cerebellar morphogenesis (Schwartz et al., 1997) by regulating Purkinje cell dendrites and granule cell survival. Last, the cell-cycle-regulator cyclinD2 is required for the postnatal production of the granule cells and stellate cells (Huard et al., 1999).

In contrast to the multiplicity of genes identified to direct EGL proliferation and differentiation (reviewed by Hatten et al., 1997), factors that regulate BG production are less well documented. Applications of function-blocking antibodies to SHH (Dahmane and Ruiz-i-Altaba, 1999) and brain lipid-binding protein (BLBP) (Feng et al., 1994) have been shown to interfere with aspects of BG differentiation in vivo and in vitro, respectively. There is, as of yet, no known genetically nor biochemically defined component that regulates BG precursor cell proliferation.

Growth arrest specific gene 1, Gas1, was cloned as one of a group of enriched cDNAs in serum-starved NIH3T3 cells (Schneider et al., 1988). It encodes a membrane-associated glycoprotein. Since it is highly expressed during G0, it has been used as an indicator for G0 arrest of cultured cells. Importantly, it has been shown to negatively regulate growth of both transformed and nontransformed cells when overexpressed (Del Sal et al., 1992; Evdokiou and Cowled, 1998). Despite its well-characterized in vitro function, its in vivo role in growth regulation is not known. We are interested in the growth regulation of the CNS and have investigated whether this negative growth regulator plays a role in CNS development. To our surprise, Gas1 mutant animals are viable and do not grow larger or develop excessive tumors, as would be expected for the inactivation of a growth inhibitor. Below we describe that Gas1 is in fact positively required for the growth of postnatal cerebellum.

**EXPERIMENTAL PROCEDURES**

**Generation of mutant Gas1 mice.** R1 ES cells were kindly provided by Drs. Nagy and Rossant. The targeting construct and the 5’ and 3’ probes are described in Lee et al. following standard protocols (Wasserman and DePamphilis, 1993). ES cell clones were used to generate chimeras with CD1 mouse. The mutant animals are viable and maintained in a mixed 129/CD1 background. Genotypes are determined by Southern and/or PCR as described in Lee et al.

**Cerebellum staging.** The day of birth is designated P0. The embryo heads or the postnatal cerebella were dissected in L-15 media (GIBCO-BRL). They were either photographed as whole-mount images or fixed in the Carnoy’s, dehydrated in ethanol, paraffin embedded, and sectioned (6 μm) for analysis. Mutant and control (wild-type or heterozygous) cerebella of the same litter were used for comparative analysis. They were processed in parallel for reliable comparison. Typically, the central vermis regions were used for statistical comparative analysis; however, some in situ hybridization data were from more sagittally located sections so that multiple probes could be used on the same cerebellum. Quantification of the cerebellum size is described in Huard et al. (1999) using digital images of the histological sections and presented as pixel numbers by the Photoshop program. Specific cell types were counted based on their histological position or molecular markers.

**Histology.** Paraffin sections (6 μm) of the cerebellum were typically counterstained with hematoxylin for 5 min in Gill’s II (Surgipath) after in situ hybridization. For histological comparisons, 20 s of eosin (Surgipath) staining was included after hematoxylin to improve elucidation of cellular morphology. Slides from in situ hybridization, histological analysis, BrdU labeling, and TUNEL assay were mounted in Permukon solution (VWR), while slides from immunofluorescence studies were mounted in Fluoru-mount G (Sigma).

**Immunostaining.** Immunostaining was performed using Abs according to manufacturer’s recommendations. TAG-1 was purchased from Hybridoma Bank (Farley et al., 1990); calbindin from Chemicon; NeuN from Chemicon (Mullen et al., 1992), and GFAP from Sigma (Bignami et al., 1972), peroxidase, alkaline phosphatase, Cy3, or FITC-conjugated secondary Abs were also acquired from commercial sources (Sigma and NEN). DAB (Sigma) and BCIP/NBT (GIBCO-BRL) were used for color development in conjunction with the peroxidase-secondary Abs and alkaline phosphatase-secondary Abs, respectively.

**RNA in situ hybridization.** Paraffin sections were subjected to standard radioactive in situ hybridization using [35S]-UTP-labeled probes (Fan and Tessler-Lavigne, 1994). [35S]-UTP-labeled Gas1 3’UTR antisense probes were used for hybridization. No specific
pattern was observed when sense probe was used. Photographs of the detected transcripts (silver granules) were taken as darkfield images with a red filter. Corresponding phase pictures were taken to reveal the histology with a blue filter. Each photograph presented here is an overlay of a darkfield image and a phase image revealing the histology and expression signal. Whenever possible, alternating sections were used to compare gene expression patterns. Mutant and control cerebella of the siblings were processed in parallel for comparison. The Zic1 (Aruga et al., 1994), NeuroD (Miyata et al., 1999), BLBP (Feng et al., 1994), and Smo (Chidambaram et al., 1998) probes were generated by PCR according to their original descriptions.

**BrdU and TUNEL assays.** Mice were injected with 10 mg/ml BrdU (Sigma) at the dose of 0.01 ml/g body weight 1 h before sacrifice. BrdU-positive cells were revealed using a BrdU staining kit (Zymed). TUNEL assays were performed using the ApopTag Peroxidase kit (Intercon). Positive cells were counted and averaged over three midsections of at least three animals each of mutants and controls. All statistics were performed using Statview software and the comparisons were based on the two-tailed Student t test. Statements using statistical differences were based on P < 0.005.

**RESULTS**

**Gas1 Mutant Mice Have Cerebellum Defects**

To elucidate the growth regulatory role of Gas1 during development, we created a null mutation of Gas1 generated by the ES cell/homologous recombination-based gene disruption method (Wassarman and DePamphilis, 1993). A PGK-neo cassette was used to replace the entire coding region of Gas1. Gas1 mutants were born at mendelian ratio, indicating no embryonic lethality (Lee et al.). In the 129sv pure background, most of the mutants die perinatally; in the mixed CD1/129sv background the mutants are viable to adulthood. The phenotypes associated with the mutants but not the wild-type and heterozygous siblings (referred to as controls (con) from here on) are reduced postnatal cerebellar growth and reduced eye pigmentation (Lee et al.). Here, we focus on the cerebellar defects.

**Gas1 Mutant Is Defective in Cerebellum Growth Postnatally**

Mutants’ body growth curve is similar to that of the controls. To minimize the growth regulatory role of variables, only mutants and controls of similar body weight were used for comparison. At P2, mutant cerebella have size and morphology similar to those of the controls (Figs. 1A and 1B). Histological analysis indicates that the foliation of the mutant cerebella is slightly shallower (Figs. 1C and 1D), although the area it covers (comparing the transverse sections of the central vermis) appears equal to the control. P2 is the first time point at which we can begin to distinguish the mutant and control cerebellum by histology. The variability due to fluid content makes wet weight an unreliable measure of mass at this stage.

The mutant cerebella show gradual size retardation after P2. At maturity (P30), the mutant cerebella are visibly smaller than the control (Figs. 1E and 1F). The mutant cerebella have an average mass (0.037 ± 0.003 g; n = 3) less than half of the controls (0.076 ± 0.006 g; n = 3). Comparisons of the length and width between the mutant and control cerebella are summarized in Fig. 1I. Midsagittal sections through the vermis region were examined. As shown in Fig. 1H, the defect is primarily reflected by reduced IGL. Since the granule cells are too numerous to count, we resorted to using an arbitrary unit to compare the IGL area in sections (see Huard et al., 1999). Images of the central vermis region were taken digitally, and the areas of the IGL and the white matter (WM) were quantified as pixels in the Photoshop program. The analysis revealed that the mutant IGL occupies only 47% of the area as the control (19,162 ± 760 vs 40,869 ± 2316 pixels/mutant vs control; n = 4). In contrast, there is no significant difference of the WM between the mutant and the control (8381 ± 1062 vs 8964 ± 813 pixels/mutant vs control; n = 4; see below).

When the central vermis region was examined (compare Figs. 1G and 1H), we found the mutant’s folia to be smaller and fissures to be shallower than those of the control. Based on the pixel measurements of each folium area, mutant folia IV and IX are disproportionately smaller and less than 50% of the controls (Fig. 1J). The uvular sulcus in folium IX is missing and the precentral and precuminate fissures are reduced. By simple observation, Gas1 mutant mice show balance failures as well as wobbling and halting behaviors during walking. They do not exhibit an overt ataxic gait, indicating that their cerebellar function is compromised but not grossly impaired.

**Gas1 Mutant Cerebella Contain Major Cell Types of the Cerebellum**

We next determined whether there were also histological abnormalities or alterations of other cell types in the mutant. Hematoxylin/eosin staining of the P30 cerebellum sections revealed that the mutant granule cells are of the same size and distribution density as the control (Figs. 2A and 2B). Thus, the reduction of IGL is mainly due to the reduction in cell number. There is a slight reduction of stellate cell density in the mutant molecular layer (ML) (Figs. 2C and 2D), which may be a secondary defect caused by the reduction of the ML (see Discussion). Using anti-GFAP (Bignami et al., 1972) to monitor the astrocytes and BGs, we found that the mutant astrocytes appear normal based on GFAP expression level and pattern in the WM (Figs. 2E and 2F). In contrast, we found a greatly reduced number of GFAP+ radial fibers in the mutant ML (Fig. 2F). The mutant fibers are difficult to trace as “radial” fibers and often appear fasciculated and immature in morphology. This is not due to the obliqueness of the sample as sections at different angles and from multiple mutants display the same pattern. The control, on the other hand, displays well-organized radial fibers and high levels of GFAP expression (Fig. 2E). We next examined the Golgi cells in the IGL.
Gas1 mutant cerebellum is retarded in growth postnatally. At P2, the mutant (−/−, B) cerebellum is of size and pattern similar to those of the wild-type control sibling (con., A) at a gross level. Histological section of the wild-type control cerebellum (C) is compared to the mutant (D) to show that the sizes of the cerebella are almost identical. At P30 wild-type cerebellum (E, G) is compared to the mutant (F, H) by whole-mount (E, F) and by histology (G, H). For whole-mount, cerebella were dissected out in PBS and photographed. For histology, cerebella were Carnoy fixed, paraffin embedded, sectioned (6 μm) sagittally, and stained with hematoxylin. The central vermis region is used for comparison. In G and H, the folia III–X are as labeled. The disproportionally reduced fissures are indicated by arrowheads: uvu,
using HNK-1 (Figs. 2G and 2H). HNK-1 also labels other cell types weakly (Werneke et al., 1985; Eisenman and Hawkes, 1993). Only the cells in the IGL with high levels of staining and branched morphology were counted as the Golgi cells (Huard et al., 1999). The mutant Golgi cell morphology is normal and present at a slightly higher density than in the control. The basket cells were monitored by antiphosphorylated NF200 (Huard et al., 1999; Figs. 2I and 2J). As in the control, mutant basket cell bodies are located underneath the Purkinje cells with neurites wrapping around the Purkinje cell body (Montiero, 1989). The mutant basket cells are more densely distributed and their wrapping area is smaller. Consistently, anti-calbindin (marker for the Purkinje cells) revealed that the mutant Purkinje cell bodies are smaller and more densely distributed (Figs. 2K and 2L). The dendritic trees of the mutant are less organized and elaborated, particularly the tertiary branches near the pia surface. Quantification of each of these cell types from central vermis sections is presented in Figs. 3A and 3B. Taking the reduced PL length and IGL area into consideration, the increased density of Purkinje cell, basket cell, and Golgi cell reflect that the total numbers of each of these cell types are not affected. Since the Purkinje axons and astrocytes are the major constituents of the WM, the observation that they are of normal numbers in the mutant/wild-type is labeled on the top. The error bars are standard deviations. All measured differences are subjected to student’s t test and P values ≤ 0.01.

**Defects of the Purkinje and BG Fibers in Gas1 Mutants**

The Gas1 mutant has morphological defects in the Purkinje cells and BG cells. These cell types are known to influence the proliferation, survival, differentiation, and migration of the granule cells. We next examined whether their dysmorphology is an early event or a late secondary event due to reduced granule cells. We monitored the Purkinje cells and the BG fibers at early, mid, and late stages. Mutant Purkinje cells are normal in number compared to the control at P2, P5, and P11. To compare the dendritic trees in detail, we use anti-calbindin to mark the Purkinje dendrites. At P5, mutant and control Purkinje cells just begin to develop their immature dendrites and they look similar (not shown). At P11, the mutant Purkinje cells are already slightly more compacted, but their primary and secondary dendrite morphology is relatively normal compared to the control (Figs. 4E and 4F). A time-course study between P2 and P30 indicates that the Purkinje dendrite dysmorphology is a gradual change and a secondary late event. In contrast, the abnormality of the radial fibers in the mutant is observed throughout development. At P5 when the BGs just begin to establish radial network for granule cell migration, there is a greatly reduced GFAP expression in the mutant ML compared to the control ML (Figs. 4A and 4B). The GFAP radial fibers are not visible in the mutant prior to P5. At P11 there are much fewer radial fibers which also tend to be more fasciculated in the mutant (Figs. 4C and 4D). The other glial cells, the astrocytes, appear to be normal in their structure and levels of GFAP expression. Consistently, BLBP expression is reduced in the mutant (Figs. 4G and 4H). BLBP expression in the IGL is at the normal level, indicating that its reduction is specific to the BGs. Thus, Gas1 is involved in regulating the proliferation and/or the maturation of the BGs at the beginning of their generation.

**Gas1 Mutant Cerebella Have Decreased Proliferation in the oEGL and PL**

The reduced granule cell and glial fiber numbers likely result from decreased proliferation in the EGL and the BG precursors. Using BrdU-labeling, we observed a reduction in proliferation of the P2 mutant central vermis region: 501 ± 28 (−/−) vs 759 ± 27 (con) BrdU cells/section (n = 3). Thus, a 34% [(759–501)/759] reduction of BrdU cells per section in the mutant EGL is observed (n = 3; Fig. 5). Due to the large number of EGL cells after P2, mitotic cells were monitored by antiphosphorylated histone3 antibody (not shown). Positive cells in the EGL were counted and averaged within 1-mm segments (positive cells/1 mm): at P5 and P11, the numbers of mitotic cells in the mutant EGL were reduced by 14.5 and 17.6%, respectively. At the same time, there was also reduced BrdU incorporation in the PL; 52.1% less BrdU cell was observed in the mutant [97 ± 16 (−/−) vs 186 ± 13 (control)/section; n = 3; P < 0.001]. Similarly reduced rate (55%) was observed in the mutant PL at P5 by counting mitotic cells. Hardly any mitotic cells were observed in the PL at P11. Since Purkinje cells are postmitotic, we assign these proliferating PL cells to be the BG precursors. Consistently, these BrdU cells have small cell bodies (Figs. 5A and 5B) and we observed fewer GFAP radial fibers and reduced BLBP expression in the mutant ML (Figs. 4B, 4D, and 4H).
FIG. 2. Cell-type-specific marker analysis of the mutant cerebellum. P30 cerebellum of control (con., A, C, E, G, I, K) and mutants (-/-, B, D, F, H, J, L). The cell type of interest and the Abs used to detect them are on the left. (A, B) Histological sections of the IGL area. (C, D) Histological sections focused on the stellate cells (transparent arrowhead) in the molecular layer (ML, the bracketed area). These images were taken as 400×, while the rest were taken at 200× magnification. (A–D) Paraffin sections are of 6-μm thickness and stained by hematoxylin and eosin. (E, F) Anti-GFAP (followed by Cy3-conjugated secondary Ab) staining of the radial BG fibers (Bf, the orientation...
Gas1 Mutant Cerebella Have Increased Cell Death in the EGL

Although reduced proliferation accounts for smaller cerebellum, we cannot exclude the possibility that increased programmed cell death (PCD) may also contribute. We performed TUNEL assay at various cerebellar stages (P2, P5, P11, P14, and P17). While there is a slight increase of PCD throughout the stages examined, we observed a significant increase of cell death in the mutant EGL (Figs. 5C and 5D; 61 ± 14 (control) vs 147 ± 8 (−/−)/section; n = 3; P < 0.001) at P5. These TUNEL− cells were found in the eEGL and the iEGL. No significant difference in IGL cell death was found between the mutant and the control. These observations indicate that Gas1 mutation leads to compromised cell survival in the EGL. We rarely observed cell death in the PL of either mutant or control. Thus, unlike EGL, reduction of BGs is mainly due to reduced proliferation.

The Granule Cells' Differentiation Is Not Affected

The increased cell death in the mutant EGL may result from compromised differentiation of the granule cells. To monitor granule cell differentiation, we examined the early time point of EGL migration and differentiation at P5 (Hatten and Heintz, 1995). As shown in Figs. 6A and 6B, the control and mutant cerebella contain TAG1+ iEGL cells of equal thickness, suggesting that the early differentiation of the granule cells is not compromised. When NeuN was used to monitor the differentiated granule cells, we observed NeuN+ cells in the IGL of the mutant. Since the mutant NeuN+ cells emerge in the IGL at P5 and there is no excessive cell accumulation in the iEGL or ML, it is unlikely that there is a delay in granule cell differentiation. We next examined the expression of known genetic components in this process, Zic1 (Aruga et al., 1998) and NeuroD (Miyata et al., 1999). No alteration of Zic1 and NeuroD expression in the iEGL or the IGL (except fewer cells) was observed in Gas1 mutants compared to controls (Figs. 6E–6H), consistent with the conclusion that granule cell differentiation is not altered.

Gas1 Is Expressed in the eEGL and the PL

Gas1 is a general growth inhibitor in cell lines. It is surprising that the phenotype uncovered in the mutant cerebellum is not overproliferation. This raises the possibility that Gas1 plays an indirect role in the proliferation of EGL and BG of the fiber is indicated by white lines) and the astrocyte fibers (As). The exposure time for the mutant GFAP staining was twice as long as that for the controls in order to observe fiber staining. Arrowheads indicate the fasciculated fibers. (G, H) HNK-1 staining (followed by a Cy3-conjugated secondary Ab) of the Golgi cells (Gc) (white arrowhead). Of note, HNK-1 also labels immature glia, granule cell precursors, and Purkinje cells (Werneke et al., 1985; Eisenman and Hawkes, 1993). At P30, the strongest HNK-1 positive cells are the Golgi cells (Huard et al., 1999), although weak staining can be seen in the PL (Pc) and the speckled pattern throughout the IGL, presumably other glia cells. (I, J) Antiphosphorylated NF-200 (followed by a HRP-conjugated secondary Ab and color development using DAB) were used to examined the basket cells (Bc). (K, L) Anti-calbindin (followed by an AP-conjugated Ab and color development using BCIP/NBT) recognizes the Purkinje cells (Pc) and their dendrites. Their dendritic trees are shorter and disorganized, and the terminal branches near the pia surface are less obvious (arrows and brackets). Scale bars = 50 μm for A–F, I, J; 100 μm, for K, L; 200 μm, for G, H.
The defects of Purkinje cells and BG fibers arise at different times. Control (con.) and mutant (−/−) cerebellum are examined at P5 (A, B) and P11 (C–H) for BG fiber (A–D), Purkinje cell morphology (E, F), and BLBP expression (G, H). Sections were stained with anti-GFAP (followed by goat anti-mouse Cy3, in red) to monitor the BG fibers (Bf) (A–D). The exposure time for the GFAP staining was twice as long for the mutant as for the control in order to observe staining. To examine Purkinje dendrites and cell bodies, E and F were stained by calbindin followed by HRP color development (brackets indicate the extension of the primary and secondary dendrites). (G, H) 35S-UTP-labeled antisense BLBP probe was used for in situ hybridization to compare its expression levels in the BG cells. Note that the IGL expression of the BG cells is of the same level in the mutant and the control.
cells. Yet, we did not observe a clear early deficit of any other cell types (Figs. 2 and 3). To clarify its role, we examined where Gas1 is expressed. Surprisingly, Gas1 expression in the cerebellum is first found to be restricted to the EGL and PL at P2 and persisted in P5, P11, and P22 (Figs. 7A–7D). As the EGL becomes multilayered, Gas1 expression is detected only in the EGL and PL.

**FIG. 5.** Gas1 mutant cerebellum has proliferation and cell survival defects. (A, B) Short-term in vivo BrdU labeling of P2 cerebellum of the control and mutant animals, respectively. (C, D) TUNEL assay shows that there is an increased cell death (arrowheads in the EGL) throughout the vertical layers of the EGL in the P5 mutant cerebellum. Cell death was also observed in the IGL (arrows). (E) BrdU and TUNEL-positive cells were counted (per section) and compared. Three alternating sections of the central vermis region (8 μm) of each cerebellum were used for counting and the numbers were averaged. Three mutants and controls were used for each counting. Error bars represent the standard deviation. The differences are significant by student’s t test (P ≤ 0.001).
FIG. 6. Granule cell differentiation is not interrupted. Control (con.) and mutant (−/−) P5 cerebellum were examined for their granule cell differentiation. To monitor whether there is a disruption of oEGL to iEGL transition, anti-TAG-1 (followed by goat anti-mouse Cy3) was used as an iEGL marker (A, B). NeuN (followed by goat anti-mouse HRP and DAB) was used to monitor the differentiating to differentiated granule cells (C, D). Anti-sense Zic1 (E, F) and NeuroD (G, H) probes were also used to monitor granule cell development.
where the proliferating precursors reside, but not in the differentiating EGL or the mature IGL. The EGL expression is persistent throughout postnatal development (also examined at P4, P7, and P14, not shown). At P22, there are few Gas1 cells at the pia surface, possibly the residual EGL cells.

Gas1 expression in the PL is presumed to localize to the BG cells as the silver granules are over the small cells, not over the large Purkinje cells (Figs. 7B–7D). This is particularly clear at P22 when the BG cells are aligned next to the Purkinje cells (Fig. 7D). The timing of expression also follows the timing of BG cell production. Due to the weak expression of Gas1 in these cells, double in situ with two-color development was not informative. We thus performed a comparative study using alternating sections hybridized to BLBP and Gas1 probes to confirm their coexpression. As shown in Figs. 8A–8E, the layer of Gas1 cells coincides with BLBP cells. In high-power images (Figs. 8F–8I), for the BG cells that are identified as the same on the adjacent sections [using the Purkinje (black dots) and stellate (red dots) cells as references], they coexpress BLBP and Gas1 (yellow arrows). Due to the section thickness (6 μm) and the cell size (10–15 μm), about half of the BG cells can be identified as the same in adjacent sections. This result strongly supports the fact that Gas1 is expressed in the BG cells. These results together with the phenotype described above indicate that Gas1 acts directly within the EGL and BG cell lineages to regulate their proliferation.

**DISCUSSION**

Gas1 has been suggested to be a growth suppressor gene based on its G0-specific expression and its ability to induce growth arrest in cultured cells (Schneider et al., 1988; Del Sal et al., 1995). In contrast to this identified role, we provide evidence that Gas1 is positively required for growth in the postnatal cerebellum. Gas1 mutation is the first genetic component reported to date that causes reduced proliferation in both the EGL and the BG cells. This realization provides the novel insight that there is a previ-
**FIG. 8.** Comparison of Gas1 and BLBP expression. Alternating 6-μm adjacent sections of P22 cerebellum were hybridized to Gas1 and BLBP antisense probes (labeled on the top). (A, B) 100× phase photos and (C, D) their corresponding in situ dark filed images of sections hybridized to Gas1 (in red) and BLBP (in green) probes. The exposure for the BLBP probe was 3 days and the exposure for Gas1 probe was 2 weeks. The images were taken with color filters and were overlaid to show their overlapping expression patterns (E, in yellow). 400× images were used to identify the cells that express both transcripts. (F, G) Phase images and (H, I) dark filed images of Gas1 and BLBP in situ results without color filters. BG cells that can be identified as the same are labeled by yellow arrows based on their corresponding position in the adjacent sections and the landmark neighboring Purkinje cells (black dots) and stellate cells (red dots). The yellow dots in the image are marks used initially to identify the signal-positive BG cells. Images of H and I are false-colored (Gas1 in red and BLBP in green) and overlaid in J. Due to the scattering of radioactivity, the silver granules do not precisely overlap if they are within the same cells. Note that only a subset of each cell type can be identified as the same in adjacent sections due to the relative thickness between the section (6 μm) and the size of the cells.
ously unappreciated link of growth regulation between these two cell types. Such a coordinated program may help to match the number between newly generated granule cells and BG fibers to ensure proper inward migration of the granule cells.

**Gas1’s Action in the EGL**

Prior to P2, the mutant EGL is almost identical to the control. It is unlikely that there are fewer EGL precursors being specified embryonically. We also did not observe an early onset or a delay of EGL depletion in the mutant (examined at P0, 1, 2, 4, 5, 7, 8, 11, 1, 4, 17, 22), indicating that the proliferation potential is maintained in a temporally correct fashion. The unaltered expression of Zic1 (Aruga et al., 1994), NeuroD (Miyata et al., 1999), NeuN, and TAG-1 (Furley et al., 1990) in the mutant supports the fact that Gas1 does not act at the step of differentiation. These observations indicate that Gas1 contributes specifically to the proliferation rate of the postnatal oEGL cells, consistent with its restricted expression in the eEGL. Since Zic1 is also involved in EGL proliferation and acts early (Aruga et al., 1998), Gas1 may act downstream of Zic1.

Although Gas1 mutation affects EGL cell survival, this is likely a secondary outcome. First, cell death occurs throughout the EGL while Gas1 is only expressed in the oEGL. Second, the dramatic cell death bursts right around P5, a time corresponding to the earliest stage of granule cell migration. Coincidently, mutant BG fibers are very scarce and poorly defined at this stage. Since BGs have been suggested to contribute to granule cell survival (Hatten et al., 1988), we propose that the stage-specific EGL cell death is elicited by insufficient mature BG fibers in the ML.

**Gas1’s Action in the Purkinje Layer**

Gas1 mutants have compromised proliferation and disorganized fibers of BGs. Our data are consistent with a direct action of Gas1 in the BGs: First, BG precursors arrive in the PL at P2 and their proliferation occurs mainly between P2 and P8 in the rat (Altman and Bayer, 1997). Gas1 expression in the PL at P2 corresponds to the location of BG precursors and there is a reduced proliferation in this location in the mutant. Second, its expression in this layer increases as glial cells differentiate and becomes more abundant. Third, its expression can be colocalized to the mature BG cells expressing BLBP. Since BG proliferation is affected prior to Purkinje cell abnormality and IGL formation, we conclude that the defect is a direct consequence of Gas1 mutation.

The disorganization of the radial fibers can be a primary defect of the mutant cells or a secondary defect due to compromised ML. CyclinD2 mutant has similarly defective ML yet its radial GFAP fiber morphology is normal, suggesting that the phenotype we observed is specific to Gas1 mutation. Since Gas1 is expressed continuously in the differentiated BG cells, our data suggest that it contributes to their maturation.

**Cerebellar Foliation Is Altered in the Gas1 Mutant**

Gas1 mutant cerebella provide additional support for the role of granule neurons in cerebellar foliation in the late period. There are two primary hypotheses concerning primary cerebellar foliation. One is that the Purkinje monolayer and dendritic arbor lay out the basic pattern (reviewed by Jacobson, 1991), as the loss of Purkinje cells eliminates the foliation (Feddersen et al., 1992). The other model proposes that the expansion of EGL and the IGL cell masses are essential to induce physical foliation (Mares and Lodin, 1970; Millen et al., 1995), as the Math1 mutant cerebellum has no granule cells and fails to foliate (Ben-Arie et al., 1997). Since these two cell types interact extensively, depletion of one leads to defects in the other and does not allow assessment of their contributions in later foliation process. In Gas1 mutant, the initial (before P2) foliation pattern is normal, consistent with the normal number of Purkinje cells and EGL cells. Our data suggest that the late foliation defect is due to reduced IGL mass. The foliation depth and the loss of terminal branches of elaborate folia are likely consequences of IGL reduction. Consistently, shallow foliation is also found in CyclinD2 and BDNF mutants (Huard et al., 1999; Schartz et al., 1997), both of which have levels of IGL loss similar to that of Gas1 mutant. However, the affected folia are distinct in these mutants, suggesting that the final foliation pattern is dictated not only by the IGL mass but also by specific gene action either in timing or in cell types.

**Other Cerebellar Defects of Gas1 Mutant**

Besides the granule and BG cell defects, Gas1 mutant phenotype also provides insight into regional development. Studies have shown that the granule cells are important for Purkinje dendritic spine formation (Baptista et al., 1994) and the ratio between Purkinje and granule cells (normally 1:778; Korbo et al., 1993) may be important for the elaboration of the Purkinje dendrites. Granule cells have also been documented to provide trophic cues for the Purkinje cells (Baptista et al., 1994). The stunted Purkinje dendritic arbor and the shrunken Purkinje cell body observed are consistent with ~50% reduced granule cells in the Gas1 mutants. It is surprising that such a phenotype was not reported in the CyclinD2 mutant (Huard et al., 1999), which has similar levels of IGL loss.

There is also a reduction of stellate cells in the mutant. Stellate cells derive from the same precursors as the basket and Golgi cells (Zhang and Goldman, 1996). Since the latter two cell types are normal, the precursors are likely specified normally. However, there is not yet a marker for them to address this question directly. As Gas1 is not expressed in the three cell types, we propose that the final stellate cell number is regulated through a secondary homeostatic mechanism due to the compromised mutant ML.
Gas1 Is a Positive Regulator of Cerebellar Growth

Our data support the fact that Gas1 acts positively in EGL and BG cell proliferation. In contrast, in vitro studies using the 3T3 fibroblast have assigned Gas1 as a negative growth regulator: Its expression is upregulated at G0 and it induces growth arrest cell autonomously when overexpressed (Del Sal et al., 1992). Recently, it was shown that GAS1 acts on the cell surface and the C-terminal half of the protein partially contains this inhibitory function (Ruaro et al., 2000). Since GAS1 has no intracellular domain (Del Sal et al., 1992; Ruaro et al., 2000), it must signal through another unidentified membrane component(s). Although its signaling pathway is not known, a proline-rich domain of p53 is required to mediate this negative function (Ruaro et al., 1997). How do we reconcile our findings to the documented growth arrest function of Gas1? It is important to note that the in vitro studies were performed on immortalized and tumor cell lines (Del Sal et al., 1992; Evdokiou and Cowled, 1998). Not all cell lines respond to GAS1 overexpression (at least one nonresponsive cell line expressed p53; Evdokiou and Cowled, 1998) and no primary cells have even been tested. It is thus possible that during the immortalizing/tumorigenic process, some cell lines adopt a specific cellular state that allows them to respond to GAS1 overexpression negatively in conjunction with p53. During normal development when p53 activity is minimal, Gas1 may in fact couple to the same or distinct cell-cycle regulators to promote proliferation, e.g., in the cerebellum. The seemingly opposing functions may represent differences in the cell types examined. Consistently, Gas1 is generally expressed (Lee and Fan, in preparation) but the mutant defects are restricted to the eye and the cerebellum. Alternatively, overexpression may elicit a dominant negative effect of GAS1 by titrating/inhibiting its signaling partner on the cell surface. This hypothesis is supported by the fact that high levels of GAS1 achieved by single-cell RNA injection appeared to be required to obtain this inhibitory function (Del Sal et al., 1992). Importantly, GAS1 appears to mediate both the positive and the negative functions in a cell autonomous manner. In the future, it will be necessary to define whether the two opposite activities of GAS1 reside on distinct domains of GAS1 and/or on the signaling components that it couples to.

Although GAS1’s growth arrest function does not require a ligand(s), we cannot exclude the possibility that its in vivo function does. To that end, we have tested the responsiveness of the mutant EGL cells to SHH, FGF, and IGF-II (growth factors known to stimulate EGL proliferation) both in slice cultures and in dissociated cell cultures (Wechsler-Reya and Scott, 1999) and found them to be equally responsive as the wild-type cells (data not shown). Mutant granule precursor cells also display normal growth response stimulated by cell–cell contact using aggregated cultures (Gao et al., 1991) (data not shown). Although cyclinD2 is the only cyclin reported to date to have a cerebellum growth defect, CyclinD2 expression is not altered in Gas1 mutant (not shown). Thus, GAS1 appears to mediate a pathway that has not been explored. Whether Gas1 is a receptor/coreceptor for a specific signaling pathway or an integral cell-cycle regulator remains to be determined. Nevertheless, Gas1 mutant provides a foundation for future investigation of this unique pathway in regulating cerebellum growth.

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REFERENCES


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A hypothesis for the trophic rescue of granule cells from target-related cell death. J. Neurosci. 9, 3454–3462.

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