Vitronectin regulates Sonic hedgehog activity during cerebellum development through CREB phosphorylation

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Accepted 13 February; published on WWW 5 April 2001

SUMMARY
During development of the cerebellum, Sonic hedgehog (SHH) is expressed in migrating and settled Purkinje neurons and is directly responsible for proliferation of granule cell precursors in the external germinal layer. We have previously demonstrated that SHH interacts with vitronectin in the differentiation of spinal motor neurons. Here, we analysed whether similar interactions between SHH and extracellular matrix glycoproteins regulate subsequent steps of granule cell development. Laminins and their integrin receptor subunit α6 accumulate in the outer most external germinal layer where proliferation of granule cell precursors is maximal. Consistent with this expression pattern, laminin significantly increases SHH-induced proliferation in primary cultures of cerebellar granule cells. Vitronectin and its integrin receptor subunits αv are expressed in the inner part of the external germinal layer where granule cell precursors exit the cell cycle and commence differentiation. In cultures, vitronectin is able to overcome SHH-induced proliferation, thus allowing granule cell differentiation. Our studies indicate that the pathway in granule cell precursors responsible for the conversion of a proliferative SHH-mediated response to a differentiation signal depends on CREB. Vitronectin stimulates phosphorylation of cyclic-AMP responsive element-binding protein (CREB), and over-expression of CREB is sufficient to induce granule cell differentiation in the presence of SHH. Taken together, these data suggest that granule neuron differentiation is regulated by the vitronectin-induced phosphorylation of CREB, a critical event that terminates SHH-mediated proliferation and permits the differentiation program to proceed in these cells.

Key words: Chick, Rat, Embryo, Cerebellum, Proliferation, Differentiation, Purkinje neuron, Granule neuron, Extracellular matrix glycoproteins, Laminin, Vitronectin, Cyclic AMP responsive element-binding protein (CREB), phosphorylated-CREB (P-CREB), CREB-binding-protein (CBP)

INTRODUCTION
The cerebellum comprises the deep cerebellar nuclei, the white matter, and the three-layered cerebellar cortex. It develops primarily from the metencephalon, although lineage studies have shown that a wedge of cells from the more anterior mesencephalic vesicle moves caudally to contribute to the developing cerebellar anlage (Hallonet and LeDouarin, 1993; Martínez and Alvarado-Mallart, 1989). The cerebellar cortex is formed from two distinct proliferative zones: a typical ventricular zone (VZ) and a displaced germinal zone, the external germinal layer (EGL). Neurogenesis of the principal output neurons of the cerebellar cortex, the Purkinje cell, occurs within the typical ventricular zone during the early phases of cerebellar development. The early migration of the Purkinje cell and the cessation of migration to form the first cell layer set up the framework for the cerebellar cortex. Concomitantly, precursors of granule cells appear in an area of the neuroepithelium just dorsal to the zone where Purkinje cells are generated. This zone, called the rhombic lip, constitutes the dorsal ridge of the cerebellar territory. Cells within this domain separate from the adjacent neuroepithelium, cross the lip, and migrate up onto the surface of the anlage. The thin layer of proliferating cells, which spreads across the roof of the anlage, is called the external germinal layer (EGL; reviewed by Hatten and Heintz, 1995; Hatten, 1999). The EGL transiently exists on the surface of the cerebellar anlage and contains many mitotically active cells (Altman and Bayer, 1997; Ramón y Cajal, 1889; Ramón y Cajal, 1911). After clonal expansion in the superficial EGL, granule cells migrate through the field of differentiating Purkinje cells and set forth three layers; an outer molecular layer (ML) of granule cell axons and Purkinje cell dendrites, a layer of Purkinje cells (PcL), and an inner layer of granule cells (IGL).

Recent studies have provided insights into the molecular nature of the signals directing the subsequent steps of cerebellum development. The initial positional restriction establishing the cerebellar territory depends on patterning signals that control regional identity along the anteroposterior axis of the neural tube, such as the expression of the engrailed (EN) homeodomain protein and the PAX2/5/8 proteins (for reviews see Lumsden and Krumlauf, 1996; Joyner, 1996).
Further signals secreted from the isthmus, such as Wnt1 (McMahon and Bradley, 1990; Thomas and Capecchi, 1990) and FGF8 (Crossley et al., 1996) are also required for the establishment of the cerebellar territory. Subsequently, dorsal midline-derived BMP signals seem to act on a regionalized cerebellum domain to induce the generation of granule neuron progenitors that migrate from the rhombic lip to populate the EGL (Alder et al., 1999). Furthermore, the secreted protein Sonic hedgehog (SHH) within the EGL and from the adjacent Purkinje neurons acts as a potent mitogenic signal to expand the granule cell progenitor population (Dahmane and Ruiz i Altaba, 1999; Wallace 1999; Wechsler-Reya and Scott, 1999).

Subsequent steps of granule cell differentiation require exit from the cell cycle, induction of differentiation, and migration through the Purkinje cell layer, events that occur within a SHH-rich environment. Therefore, termination of granule cell proliferation is probably not due to reduced exposure to SHH; rather it likely results from conversion of a proliferative response to one that directs differentiation of these cells.

SHH signals through its receptor Patched (Ptc), an eleven-pass transmembrane receptor. Under basal conditions, Ptc associates with and sequesters the activity of Smoothened (Smo), a G-protein associated receptor-like protein (for reviews see Ingham, 1998 and Ruiz i Altaba, 1999). In response to the binding of SHH, Ptc releases Smo which then activates a Gα subunit to inhibit cAMP production within the cell (Hammerschmidt and McMahon, 1998). The GLI family of zinc finger transcription factors (cubitus interruptus, Ci, in Drosophila) act at the last known step in the SHH signal-transduction pathway. Within the SHH receiving cell, GLI/Ci proteins are regulated in the cytoplasm through multiple distinct molecular mechanisms. The cyclic AMP-dependent protein kinase (PKA) acts as a common negative regulator such that GLI/Ci repressor forms in vertebrates and flies are generated by PKA-mediated phosphorylation and processing by the proteasome. Conversely, inhibition of PKA activity releases GLI/Ci activated forms that move to the nucleus where, acting together with co-factors such as the CREB-binding-protein (CBP), they activate target genes (Ingham, 1998; Ruiz i Altaba, 1999).

Extracellular matrix (ECM) glycoproteins constitute critical components of cerebellar development as signals from these factors direct the migration and differentiation of certain regional cell populations (reviewed by Hatten, 1999). The integrin heterodimers are the principal cell-surface receptor for the ECM proteins (reviewed by Howe et al., 1998). We have previously described a direct protein-protein interaction between SHH and the ECM protein vitronectin (VN) (Pons and Martí, 2000). Based on these earlier observations, our current study tests the hypothesis that ECM glycoproteins down-regulate the response of granule cell precursors to SHH activity, thereby promoting their differentiation to granule neurons. Here we demonstrate that ECM glycoproteins differentially regulate the SHH-mediated proliferation of granule cell precursors. Furthermore, we demonstrate that in the presence of SHH, laminin (LN) and VN differentially mediate the phosphorylation of cyclic AMP responsive element-binding protein (CREB); cells cultured on LN and treated with SHH contain much lower levels of phosphorylated CREB than those grown on VN and exposed to SHH. These observations suggest that granule neuron differentiation is mediated by VN-induced CREB phosphorylation, which provides the signal to terminate SHH-induced proliferation, thereby allowing differentiation to proceed in this cell population.

**MATERIALS AND METHODS**

**Chick and rat embryos**

White Leghorn chick eggs were incubated at 38.5°C in an atmosphere of 70% humidity. Embryos were staged as described previously (Hamburger and Hamilton, 1951). Wistar albino rats from our inbred colony were kept under standard housing, feeding and lighting conditions (22°C, 12 hours light-12 hours dark). The day of birth was taken as day 0 of postnatal life (P0). All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23).

**Antibodies and chemicals**

Anti-vitronectin antiserum was generated and tested as described previously (Martínez-Morales et al., 1997). The monoclonal antibodies 5E1, directed against N-SHH, anti-TAG1 and anti-bromodeoxyuridine (BrdU) were all obtained from the Developmental Studies Hybridoma Bank (Iowa). The monoclonal antibody against a unique β-tubulin (Tuj-1) was obtained from MECOD S.A. de L. (Grand Duché de Luxembourg) and used to identify postmitotic neurons. The mouse monoclonal anti-calbindin-D (28K), the polyclonal antilaminin directed against the basement membrane of mouse sarcoma (EHS) and the monoclonal anti-fibronectin directed against human plasma fibronectin, were all purchased from Sigma. The rabbit polyclonal anti-GFAP (AB1980) was purchased from Chemicon. The rabbit polyclonal anti-phosphohistone 3 (P-H3) was purchased from Upstate Biochemicals. Phospho-specific antibodies that recognize the activated forms of MAPK (phospho-Thr180/Tyr182 of p44/42 MAPK) and CREB (phospho-Ser133 of CREB) were purchased from NEB.

The monoclonal antibody against human integrin αvβ3 (mAb1976) was purchased from Chemicon. The anti-αv monoclonal antibody was a generous gift from Dr C. Martínez. The anti-cyt06 purified polyclonal antibody was a gift from Dr I. de Curtis (de Curtis and Reichardt, 1993). The autocleaved 19 kDa amino-terminal fragment of recombinant Sonic hedgehog (SHH) used in this study was a gift from Biogen Inc. Basic FGF/FGF2 was purchased from Boehringer, FGF4 and FGF8 were obtained from R & D Systems.

**In situ hybridisation and immunohistochemistry**

In situ hybridisation was performed on 50 μm vibratome sections according to the method of Wilkinson (Wilkinson, 1992). Chick Sonic hedgehog (SHH) probe has been described previously (Riddle et al., 1993). Chick vitronectin (VN) probe was described by Martínez-Morales et al. (Martínez-Morales et al., 1997). Hybridisation was revealed by alkaline phosphatase-coupled anti-digoxigenin Fab fragments (Boehringer Mannheim). Immunohistochemistry was performed on free-floating vibratome sections (50 μm) based on standard procedures. After single or double staining, sections were mounted and photographed in a Leica DMR compound microscope.

**Plate coating**

Tissue culture dishes or glass coverslips were incubated for 4 hours in 20 mM borate buffer, pH 8.4 containing 10 μg/ml of pol-y-L-Lys (PLL; 90-120 kDa) (GIBCO, BRL). Subsequently, they were washed three times in water, air dried and stored at 4°C until needed. Extracellular matrix glycoproteins laminin (LN), fibronectin (FN) and vitronectin (VN) were incubated (10 μg/ml) on PLL pre-coated dishes or glass coverslips. LN and FN were purchased from GIBCO, BRL. Rat serum VN was purified as described by Kitagaki-Ogawa et al. (Kitagaki-Ogawa et al., 1990). Just before plating the cells, solutions were aspirated and plates washed once in PBS.
Culture of granule cells
Cerebellar cultures were performed using a modification of the procedure described by Meyer-Franke et al. (Meyer-Franke et al., 1995). Chemicals and incubation times were optimised to process simultaneously four P6 rat cerebella. Cerebella were aseptically removed, washed once in Earl’s balanced salt solution (EBSS) (GIBCO, BRL), cut into small pieces (1 mm) and transferred to a 50 ml screw cap tube. Tissue fragments were allowed to settle, the excess EBSS aspirated and 4 ml of EBSS containing 100 U/ml of DNase (Worthington, Lake Wood N.J.), 1 mM CaCl₂ and 1 mM MgCl₂ were added and gently mixed with tissue fragments. Finally, 100 U of papain (Worthington) that was pre-activated for 30 minutes at 37°C in 1 ml of activation buffer (EBSS containing 5 mM L-Cys, 2 mM EDTA and 0.067 mM β-mercaptoethanol) was added, the air in the tube was displaced with 95% O₂-5% CO₂ and incubated for 90 minutes on a shaking platform at 37°C. At the end of this period, the tube was vortexed at low speed for 1 minute and undigested fragments were allowed to settle, the supernatant was transferred to a fresh 15 ml screw cup tube and centrifuged at 800 rpm for 5 minutes. The supernatant was aspirated and the pellet resuspended in 3 ml of EBSS (GIBCO, BRL), cut into small pieces (1 mm) and transferred to a 50 ml screw cap tube. Tissue fragments were allowed to settle, the supernatant was aspirated and 4 ml of EBSS containing 100 U/ml of DNase (Worthington), layered onto an albumin cushion consisting of 5 ml of EBSS aspirated and 4 ml of EBSS containing 100 U/ml of DNase (Worthington, Lake Wood N.J.), 1 mM CaCl₂ and 1 mM MgCl₂ were added and gently mixed with tissue fragments. Finally, 100 U of papain (Worthington) that was pre-activated for 30 minutes at 37°C in 1 ml of activation buffer (EBSS containing 5 mM L-Cys, 2 mM EDTA and 0.067 mM β-mercaptoethanol) was added, the air in the tube was displaced with 95% O₂-5% CO₂ and incubated for 90 minutes on a shaking platform at 37°C. At the end of this period, the tube was vortexed at low speed for 1 minute and undigested fragments were allowed to settle, the supernatant was transferred to a fresh 15 ml screw cup tube and centrifuged at 800 rpm for 5 minutes. The supernatant was aspirated and the pellet resuspended in 3 ml of EBSS (GIBCO, BRL), and the cell number and viability was assessed using an hemocytometer. The typical yield from this protocol ranges between 12 and 15 million cells per cerebellum and cell viability is higher than 90%. Cells were plated at 100,000 cells/cm² in neurobasal medium supplemented with B27 (GIBCO, BRL) containing 20 mM KCl and they were then maintained in a humidified incubator at 37°C in 5% CO₂. This culture medium has been optimized to support neuronal survival and minimize glial proliferation; more than 95% of the cells display neuronal markers after 48 hours in culture. Four hours prior to fixation, cultures were treated with 50 ng/ml bromodeoxyuridine (BrdU) and then fixed in 4% paraformaldehyde.

DNA constructs
The coding region of wild-type CREB and the dominant negative mutant CREB (CREB S⁻¹³³,A) (Gonzalez and Montminy, 1989) were subcloned from RSV-CREB into PCDNA 3.1 (Invitrogen) using the polymerase chain reaction with the proof reading DNA polymerase Pfu (GIBCO, BRL). The PCDNA 1-α-c2 construct used as a control was purchased from Invitrogen. The vector expressing EGFP used to assess transfection efficiency was from Clontech.

Transfection of granule cell precursors
After 24 hours in culture, granular cell preparations were transfected using FuGENE6 reagent (Roche). Briefly, P6 granular cells platted onto PLL/LN coated coverslips were grown in neurobasal-B27 containing 3 μg/ml of N-SHH for 24 hours. For the transfection, 50 μl of neurobasal medium containing 2 μl of FuGENE plus 0.5 μg of DNA vectors coding for CREB, CREB S⁻¹³³,A or β-galactosidase and 0.1 μg of GFP (used as a transfection marker) were added to each well of a 12-well culture dish. The cultures were allowed to grow for 48 hours in the same medium and then fixed in 4% paraformaldehyde. In a preliminary study to test these transfection conditions, we determined that a ratio of 5:1 ensures a co-transfection index higher than 90%. Moreover, under these conditions, the concentration of FuGENE is extremely low and does not effect cell viability.

Western blotting
At embryonic days (E) 10, 12, 14 and 16, chick cerebella were collected in PBS and lysed in a buffer of 20 mM Tris (pH 7.5), 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1% Nonidet P-40, 10% glycerol, 10 μg/ml of aprotinin, 10 μg/ml of leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF). Insoluble material was removed by centrifugation and the protein content of the supernatants standardised at 2 mg/ml. Cell cultures were directly lysed in 1× SDS loading buffer (10% glycerol, 2% SDS, 100 mM DTT and 60 mM Tris-HCl pH 6.8) and the DNA disrupted by sonication. Samples were separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose membranes; blocked with 8% non fat dry milk in TTBS (150 mM NaCl; 0.05% Tween 20 and 20 mM Tris-HCl pH 7.4) and probed with the different antibodies. The blots were developed using anti-rabbit coupled peroxidase plus the ECL system (Amersham). Quantifications were performed using a Molecular Dynamics Densitometer.

Statistical analysis
Quantitative data were express as mean ± s.e.m. Significant differences among groups were tested by two-way ANOVA followed by the Tukey’s test.

RESULTS
Sonic hedgehog and vitronectin are expressed in the developing cerebellum
Sonic hedgehog (SHH) is expressed in migrating and settled Purkinje neurons (Pc) (Fig. 1B,C) (see also Dahmane and Ruiz i Altaba, 1999; Traiffort et al., 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999) and is directly responsible for proliferation of granule cell precursors in the external germinal layer (EGL) (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Since we have recently reported biochemical and functional interactions between SHH and vitronectin (VN) in patterning of the neural tube (Pons and Martí, 2000), we analysed whether VN is also expressed in the developing cerebellum as a prerequisite for a functional interaction between these two molecules in this process. To study VN expression during cerebellar development, we first prepared total extracts from chick cerebella at various stages of embryonic development. As revealed by western blot analysis, VN was present in lyses of all cerebella (Fig. 1A), migrating as a single band of 70 kDa. This single band represents the 70 kDa serum form of vitronectin (S-VN; Martinez-Morales et al., 1995). Interestingly, the two proteolytic forms of chick yolk vitronectin (Y-VN; Nagano et al., 1992) that we have recently reported in early neural tube, notochord and floorplate do not appear during development of the cerebellum (Fig. 1A; Pons and Martí, 2000).

Using in situ hybridisation, we next compared the precise expression pattern of VN with that of SHH. Vibratome sections of chick E12 cerebellum revealed the expression of Shh mRNA in Purkinje neurons (Fig. 1B,C, PcL); expression of VN protein was detected in close apposition to Pc, both in the most internal EGL (iEGL) and in the IGL (Fig. 1D). To determine which cells synthesize VN, we then analysed the expression of VN mRNA. VN mRNA was present in the deep cerebellar nuclei and in the cerebellar cortex (Fig. 1E). Double-labelling with the anti-calbindin antibody to specifically stain Pc (García-Segura et al., 1984) revealed that VN mRNA was expressed in a layer of cells just beneath the PcL (Fig. 1F), most likely in Bergmann gli cells including Bergmann glia, revealed that that VN mRNA was expressed in a layer just beneath GFAP expression (Fig. 1G), ruling out the possibility that Bergmann glial were synthesizing
VN. Interestingly, the expression of both Shh mRNA and protein was maintained in Pc at E14 (Fig. 1H,I), whereas the expression of VN was increased upon differentiation of granule neurons (Fig. 1I). Double immunostaining with the calbindin antibody revealed the expression of VN in growing parallel fibres (Fig. 1J), as has been reported previously (Murase and Hayashi, 1998). Thus, these studies demonstrate that during cerebellar development VN is expressed in the EGL and the IGL just below the SHH-containing Pc. The close structural proximity of these cell populations raised the possibility of an interaction between SHH and VN during cerebellum development.

**Extracellular matrix glycoproteins regulate SHH-induced proliferation of cerebellar cells in culture**

SHH induces a potent and long-lasting proliferative response in granule cell precursors. To test whether SHH-induced proliferation could be modulated by VN, granule cell precursors were plated onto different ECM glycoproteins: poly-L-Lys (PLL), laminin (LN), fibronectin (FN) or vitronectin (VN). Cells were cultured for 72 hours in the presence or in the absence of purified N-SHH. Four hours prior to fixation, cultures were pulsed with BrdU to label proliferating cells. Cells were then immunostained with anti-BrdU antibody and examined by phase-contrast microscopy (Fig. 2). In untreated cultures (plated onto PLL, LN, FN or VN), only a small percentage of cells (0.6%) incorporate BrdU after 3 days in culture, and there were no significant differences related to ECM (Fig. 2A-E). In contrast, all cultures treated with N-SHH increased the percentage of BrdU-labelled cells in a dose-dependent manner (Fig. 2A,F-I). Interestingly, the various matrix glycoproteins regulated N-SHH proliferating activity differentially. LN as a substrate glycoprotein significantly increased BrdU-labelled cells (46% at 3 μg/ml), whereas both FN and VN produced fewer BrdU-labelled cells (19% and 17% respectively, at 3 μg/ml) than PLL (25% at 3
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mg/ml; Fig. 2A). To confirm that these granule cell precursors were indeed completing their cell cycle, we also immunostained treated cultures with an anti-BrdU antibody. Analysis of each culture was done by counting total cells stained with Haematoxylin, and BrdU immunostained cells, in a minimum of 25 randomly selected microscope fields. (A) Statistical analysis of cultures treated with different concentrations of N-SHH (0, 1, or 3 μg/ml), and plated onto LN (blue), PLL (yellow), FN (red) or VN (green) substrates. An increase of BrdU-positive cells was noted in cultures treated with 1 μg/ml of N-SHH (plated onto any substrate; LN 32±10%; PLL 19±9%; FN 18±10%; VN 14±4%) as compared to cultures grown in the absence of N-SHH (0.6±1). A further increase in proliferation was seen in cultures treated with 3 μg/ml of N-SHH. Furthermore, LN significantly increased the percentage of proliferation (47±12%) as compared to cultures grown onto PLL (18±9%), whereas VN significantly decreased N-SHH induced proliferation (14±4%) as compared to PLL (18±9%).

(B-E) Granule cells grown for 3 days on each substrate, in the absence of N-SHH, and immunostained with anti-BrdU. (F-I) Granule cells grown for 3 days on each substrate, in the presence of 3 μg/ml of N-SHH, and immunostained with anti-BrdU. Proliferating cells form clones that are particularly visible on plates coated with LN. (J-M) Granule cells grown for 5 days on each substrate, in the presence of 3 μg/ml of N-SHH, and immunostained with the mitosis marker anti-P-H3. Cultures grown onto LN-coated dishes frequently reach confluence.

μg/ml; Fig. 2A). To confirm that these granule cell precursors were indeed completing their cell cycle, we also immunostained treated cultures with the mitotic marker phospho-H3 (Fig. 2J-M). Although percentages of anti-P-H3 immunostained cells were similar to those detected with BrdU labelling for each condition (quantification not shown), the total numbers are smaller since P-H3 is expressed within a short time-frame during the cell cycle. Interestingly, cells plated onto LN and treated with 3 μg/ml N-SHH reach confluence after 5-6 days in culture (Fig. 2J).

Since it has been reported (Wechsler-Reya and Scott, 1999) that bFGF and Forskolin (FK) can suppress the proliferative response to N-SHH by 90% and 85% respectively, we next tested the effects of FK and three different FGFs; bFGF/FGF2, FGF4 and FGF8 on N-SHH induced proliferation of granule cell precursors cultured on different substrates. Independent of the substrate, FK was the strongest inhibitor of N-SHH induced proliferation, consistent with a direct increase of intracellular cAMP levels and PKA activation (Fig. 3). FGF2 enhances N-SHH proliferative effect, particularly in cultures plated on laminin. FGF4 slightly reduces proliferation in cultures plated onto either PLL or LN, whereas it has no significant effects onto VN. Finally FGF8 causes the strongest inhibition, although not reaching FK levels, and this inhibition does not occur in cultures plated on laminin (Fig. 3). In conclusion FGFs do not exert a synergistic effect with VN, nor do they have a stronger effect on the inhibition of N-SHH-induced proliferation than that of VN itself. We are aware that in our hands FGFs and FK do not induce as strong inhibition of proliferation as that reported by Wechsler-Reya and Scott (1999), however this discrepancy might be due to differences in culture conditions and/or reagent sources.

We next asked whether this differential rate of proliferation might reflect changes in neural differentiation. To test whether granule cell precursor differentiation could be modulated by ECM, cells were again plated onto the different matrix glycoproteins, cultured for 72 hours in the presence or in the absence of N-SHH, and immunostained for the neural differentiation marker β-tubulin. In the absence of N-SHH, LN, FN and VN significantly increased the rate of neural differentiation (87%, 84% and 81%, respectively) as compared with PLL (66%; Fig. 4A-C). However, in the presence of N-SHH (3 μg/ml), FN and VN increased the rate of neural differentiation (40% and 43%), whereas LN slightly reduced it (21%; Fig. 4A,D,E) compared with PLL (25%). Similar results were also obtained using TAG1 as an early granule neuron
differentiation marker (Dahmane and Ruiz i Altaba, 1999; Wechsler-Reya and Scott, 1999) (Fig. 4F-H). Together these in vitro results demonstrate that ECM glycoproteins are able to differentially regulate SHH-induced proliferation of granule cell precursors, and prompted us to further investigate their sites of expression within the developing cerebellar cortex.

**Extracellular matrix glycoproteins and their integrin receptors are differentially expressed in the developing cerebellar cortex**

Generation of the large pool of granule cell precursors takes place in the outer most part of the external germinal layer (oEGL), where proliferation is maximal. Developing granule cell precursors then exit the cell cycle while they move to the internal EGL (iEGL), extend axons and migrate inward past the Purkinje cell layer (PcL) to their final destination, the internal granular layer (IGL). Thus, maximal proliferation occurs in the oEGL, as we show here by immunostaining with

![Image](image.jpg)

**Fig. 3.** Modulation of N-SHH induced proliferation by FGFs and Forskolin. Cerebellar granule precursors were cultured on three different substrates; poly-L-lysine as control (yellow bars), vitronectin (blue bars) or laminin (purple bars) in medium containing no stimulus (control), N-SHH alone (3 μg/ml), or N-SHH (3 μg/ml) plus FGF2 (10 nM), FGF4 (10 nM), FGF8 (10 nM) or Forskolin (FK 4 μg/ml). After 40 hours in culture, cells were pulsed with [3H]thymidine for 8 hours, harvested and analysed for incorporated radioactivity.

**Fig. 4.** Effects of N-SHH and extracellular matrix glycoproteins on the differentiation of granule cell precursors in culture. Cerebellar granule cells were cultured for 3 days, in the absence or the presence of purified N-SHH. Cell were plated on poly-L-lysine- (PLL), laminin- (LN) fibronectin- (FN) or vitronectin- (VN) coated glass coverslips. At the end of the culture period, cells were immunostained with the neural differentiation markers β-tubulin or TAG1. Analysis of each culture was done by comparing the total number of cells stained with DAPI versus those staining positive for β-tubulin in a minimum of 25 randomly selected fields.

(A) Statistical analysis of cultures grown in the absence or in the presence of N-SHH (3 μg/ml) and plated onto LN, PLL, FN or VN substrates. In the absence of N-SHH, all three ECM glycoproteins significantly increase the percentages of β-tubulin-positive cell (LN 87±6%; FN 81±6%; VN 84±11%) as compared to PLL (66±8%). In the presence of N-SHH, both FN and VN significantly increase the percentage of differentiated cells (40±10% and 43±11% respectively) as compared to either PLL (25±6%) or LN (21±5%). (B-E) Granule cells cultured for 3 days and immunostained for β-tubulin.

(B) Control culture grown on PLL in the absence of N-SHH.

(C) Culture grown on VN in the absence of N-SHH.

(D) Culture grown on LN in the presence of N-SHH, where the total number of cell was significantly increased (blue nuclei), most of which do not express the neural differentiation marker β-tubulin.

(E) Culture grown on VN in the presence of N-SHH where the total number of cell was significantly increased (blue nuclei), with majority of cells expressing the neural differentiation marker β-tubulin.

(F-G) Granule cells cultured for 3 days and immunostained for TAG1.

(F) A culture grown on VN in the absence of N-SHH.

(G) A culture grown on LN in the presence of N-SHH, where the total number of cells is significantly increased (blue nuclei) but most do not express the early neural differentiation marker TAG1.

(H) A culture grown on VN in the presence of N-SHH; the majority of these cells do express the early neural differentiation marker TAG1.
the mitosis marker phospho-H3 (P-H3) in a postnatal day-6 rat cerebellum (Fig. 5A,B). The pattern of immunostaining with P-H3 is similar in a developing chick cerebellum at E14 (data not shown). Distribution of P-H3-labelled nuclei is similar to the accumulation of LN in the outer-most EGL (see arrowheads in Fig. 5D); LN are also expressed in the Purkinje cell bodies and in the meninges (Fig. 5C,D). Concomitant with exit from the cell cycle, P-H3-positive nuclei are almost absent in the iEGL (Fig. 5B). Interestingly, VN (Fig. 5I,J, see also Fig. 1I,J) and low levels of FN (Fig. 5G,H) are both expressed in the iEGL and surrounding the Pc bodies.

The integrin heterodimers are the principal surface receptor for the ECM glycoproteins. In addition to directly generating signals, integrins can modulate signalling responses to secreted growth factors (reviewed by Howe et al., 1998). To provide support to our in vitro data, we next examined the localisation of the appropriate integrin subunits within the developing cerebellar cortex. Thus, using a polyclonal antibody against \( \alpha_6 \), the integrin subunit that specifically binds laminins (reviewed by Hynes, 1992), we detected the expression of \( \alpha_6 \) in the outermost EGL (Fig. 5E,F). In addition, using a monoclonal antibody against \( \alpha_v \), the integrin subunit that specifically binds VN, we detected expression of this receptor subunit in the iEGL as well as in the area surrounding the Pc bodies and in the IGL (Fig. 5K,L). Furthermore, staining with a monoclonal antibody against the integrin complex \( \alpha_v \beta_3 \), the main vitronectin signalling receptor, gave the same pattern of expression (not shown). These data support the idea that differentiating granule cells possess the appropriate integrins to respond to VN-mediated signals.

**Vitronectin and laminin differentially regulate phosphorylation of cyclic-AMP responsive element-binding protein (CREB)**

Recently, much research has been directed at elucidating the intracellular signalling cascades that govern the events of cell proliferation, survival and differentiation during development. SHH has been reported to stimulate proliferation through a decrease in cyclic AMP-dependent protein kinase (PKA) activity (reviewed by Ruiz i Altaba, 1999). Activation of MAPK and CREB also occur during this process of neuronal differentiation, although the precise role(s) of these pathways in the differentiation program is not well understood (Cowley et al., 1994). Since both MAPK and CREB are PKA targets, we investigated whether either of these signalling components may contribute to the differential modulation of SHH signalling by LN and VN. We first evaluated the phosphorylation status of CREB and MAPK in granule cells grown on a matrix of either LN or VN for 48 hours in the presence or absence of N-SHH. Total cell lysates from granular cell cultures were separated by SDS-PAGE and the resulting nitrocellulose membranes were blotted sequentially with antibodies against phosphorylated-CREB (P-CREB) and phosphorylated-MAPK (P-

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**Fig. 5.** Localisation of extracellular matrix glycoproteins and their integrin receptors in the developing rat and chick cerebellum. Vibratome sections of postnatal day (P) 6 rat cerebellum and embryonic day (E) 14 chick cerebellum were immunostained with several antibodies. (A-B) P6 rat cerebellum stained for the mitosis marker phosphorylated histone3 (P-H3). The sparse P-H3-stained nuclei within the Purkinje cell layer (PcL) and the internal germinal layer (IGL) are not well understood. (C-D) P6 rat cerebellum stained with a polyclonal anti-laminin antibody. (D) LNs are expressed in the Pc bodies and in the IGL (red arrowheads). (E-F) E14 chick cerebellum stained with a polyclonal anti-integrin subunit \( \alpha_6 \), showing expression in the Pc bodies and in the oEGL. (G-H) E14 chick cerebellum stained with a polyclonal anti-FN antibody. FN is highly expressed in the meninges and low levels of FN are present in the iEGL. (I-J) E14 chick cerebellum stained with a polyclonal anti-VN antibody. VN is totally absent from the oEGL and is expressed in the iEGL, surrounding the Pc bodies (white shadows) and in the IGL. (K-L) P6 rat cerebellum stained with a monoclonal anti-\( \alpha_v \) antibody. The integrin dimer \( \alpha_v \) is totally absent from the oEGL but is expressed in the meninges and in the iEGL, surrounding the Pc bodies. Red arrowheads point to migrating granule cell precursors within the layer of higher \( \alpha_v \) expression.
cells in the culture express detectable levels of P-CREB. Under these culture conditions P-CREB expression was restricted to fully differentiated cells bearing long processes, at least twice as long as the cell soma, and showing elevated levels of β-tubulin (see arrows in Fig. 6C). In contrast, proliferating precursors typically not expressing β-tubulin (identified by DAPI staining, not shown) or showing low levels of β-tubulin and short processes, did not contain detectable levels of P-CREB (arrowheads in Fig. 6C). Interestingly, in cultures grown on VN and treated with N-SHH, up to 23.5±2.3% of cells showed expression of P-CREB, and these were not restricted to fully differentiated cells (arrows), as P-CREB was also present in cells that still show very low levels of β-tubulin (arrowheads). (E) In cultures plated onto LN, treated with N-SHH and stimulated for 30 minutes with DBA, up to 43±4.5% cells showed expression of P-CREB. Elevated levels of P-CREB were present in differentiated (arrows) and in non-differentiated (arrowheads) cells. (F) E14 chick cerebellum vibratome section immunostained with anti-P-CREB antibodies. Granular cells (arrows) begin to express P-CREB during their migration throughout the Pc layer (red arrowheads). Although an accumulation of P-CREB-positive cells was detected just beneath the Purkinje cell bodies, the number of positive cells was also relatively high in the internal granular layer (IGL) (see inset).

MAPK; Fig. 6A). Interestingly, treatment with N-SHH significantly decreased the levels of P-CREB only in cultures plated onto LN, whereas N-SHH did not alter the levels of P-CREB in cells grown on VN (Fig. 6A). However, when CREB phosphorylation was directly induced by treatment of cultures (for 30 minutes) with the PKA activator, dibutyryl cAMP (DBA), no differences in CREB phosphorylation were detected under either culture condition. It is also worth noting that, in contrast to CREB phosphorylation, MAPK activity was not affected by either N-SHH treatment or culture on the different ECM substrates. These data demonstrate that LN but not VN reduces the levels of CREB phosphorylation within granule cells. Moreover, our results suggest that the differential effects of LN and VN on CREB phosphorylation may represent the signal by which these ECM proteins differentially modulate the proliferation induced by N-SHH.

Based on these results, we next studied the in situ expression of phospho-CREB in granular cell cultures plated on either LN or VN and cultured in the presence or absence of N-SHH (Fig. 6C-E). Cultures were grown for 48 hours, fixed and double-stained with anti-phospho-CREB and the neuronal marker β-tubulin. In cultures exposed to LN and N-SHH, only 13±2%
express P-CREB during their migration through the Pc layer (PcL, arrow). Although, there was an accumulation of P-CREB-positive cells just beneath the Purkinje cell bodies (red arrowheads), the number of positive cells was also relatively high in the IGL (see inset in Fig. 6F). In conclusion, these results strongly implicate a role for P-CREB in the regulation of granular cell differentiation.

CREB phosphorylation is sufficient to induce granule cell differentiation

Our initial studies suggested a close relationship between the effects of LN and VN on N-SHH-induced proliferation signals and the level of CREB phosphorylation. Therefore, we next tested whether elevated levels of CREB were sufficient to induce granule neuron differentiation in vitro. To address this question, we assessed the effects of overexpressing CREB or its dominant negative form CREB S133-A on N-SHH induced proliferation (Fig. 7). Granular cell cultures plated onto LN and treated with N-SHH were transfected with DNA vectors containing lacZ (A,B), CREB (C,D) or CREB S133-A (E,F) cDNA expressing green fluorescent protein (GFP) was included as transfection marker. After fixation, cultures were stained with anti β-tubulin. (G) The percentage of differentiated cells (cells showing processes longer than two cell bodies) among the GFP-expressing ones was evaluated in each transfection group. Overexpression of CREB increased the percentage of differentiated cells more than twofold when compared to the control group transfected with lacZ (from 27±1.5% to 60±4%). Expression of the dominant negative form of CREB reduced the percentage of differentiated cells to less than half compared to control cells (from 27±1.5% to 12.1±0.4%).

DISCUSSION

Sonic hedgehog (SHH) is a potent signalling molecule responsible for a number of early patterning processes; it is involved in the control of left-right asymmetry, dorsoventral patterning of the central nervous system and the somites, and in patterning of the limb, as well as some aspects of organogenesis (reviewed by Hammerschmidt et al., 1997). Most of SHH’s early developmental roles involve the acquisition and regulation of cell fates. However under various experimental conditions, ectopic and long-lasting expression of Shh itself or components of the SHH signalling pathway resulted in over-proliferation of the target neural tissue (Echelard et al., 1993; Epstein et al., 1996; Hynes et al., 1997; Hynes et al., 2000; Rowitch et al., 1999), suggesting that SHH may play a role in regulating mitotic activity of the CNS. In fact, late in CNS development SHH functions to induce the proliferation of granule cell precursor (GCP) in the developing cerebellar cortex (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Subsequent steps in granule cell differentiation require exit from the cell cycle, induction of differentiation and migration through the Purkinje cell layer, all processes which occur within a SHH-rich environment. Therefore, termination of granule cell proliferation is probably not due to reduced exposure to SHH; rather it is likely to result from conversion of a proliferative response into a signal for differentiation. Here, we demonstrate...
that the ECM glycoprotein vitronectin is able to down-regulate SHH-induced proliferation and thus, allow the differentiation of granule neurons.

**SHH interacts with components of the extracellular matrix**

Interactions between components of the extracellular matrix and signalling molecules are of growing interest for developmental biologist since they have been shown to perform critical roles in morphogenesis and cell differentiation. Heparan sulphate proteoglycans have been proposed to act as co-receptors at the cell surface by promoting signalling complexes for a variety of secreted proteins including fibroblast growth factors (FGFs) and members of the Wnt, transforming growth factor β (TGFβ) and hedgehog (Hh) families (reviewed by Perrimon and Bernfield, 2000; Selleck, 2000). In particular, the heparan sulphate copolymerase encoded by *tout-velu* (*ttv*) affects the transport of Hh across the *Drosophila* wing imaginal disc (The et al., 1999). Interestingly, *ttv* function is selective for Hh signalling, with FGF and Wg-dependent patterning being unaltered by loss of *ttv* gene function (Perrimon and Bernfield, 2000). Furthermore, we have previously shown a direct, protein-protein interaction between SHH and the ECM glycoprotein vitronectin, during the process of spinal motor neuron differentiation (Pons and Martí, 2000). Based on those results, we set forth in the current study to analyse putative interactions between SHH and VN during cerebellum development.

Western blot analysis revealed the presence of a 70 kDa form of VN in the developing cerebellum. This single band corresponds to the full-length 70 kDa form of the serum vitronectin (S-VN) also present in the developing chick retina (Martínez-Morales et al., 1995). In early neural tube, notochord and floorplate, VN is proteolitically processed to produce two fragments of 54 kDa and 45 kDa, as was previously described for the VN isolated from chick yolk (Nagano et al., 1992; Pons and Martí, 2000). The 45 kDa fragment lacks the heparin-binding domain and the integrin-binding domain, RGD, present in the non-processed VN glycoprotein. During patterning of the neural tube, SHH and VN are expressed in partially overlapping domains (Martí et al., 1995b; Pons and Martí, 2000) and N-SHH binds to the three forms of VN protein (70 kDa, 54 kDa, and 45 kDa), although it is preferentially associated with the 45 kDa form. This association enhances the extent of N-SHH-induced motor neuron differentiation and lead us to propose a model in which the 45 kDa VN is necessary for the proper presentation of N-SHH to the differentiating motor neurons (Martí et al., 1995a; Pons and Martí, 2000).

Observations from the current study indicate that the biological activity exerted by SHH in the cerebellum is clearly different from the function of this ligand in the developing neural tube. Furthermore, our results demonstrate that the biochemical nature of the VN protein present in the developing cerebellum (the intact 70 kDa form) is also different from the forms of VN (the proteolytic fragments of 54 and 45 kDa) that interact with SHH in the developing neural tube. Thus, based on these structural differences, we anticipated a totally different type of interaction, if any, during development of the cerebellum. Granule cell precursors exhibit a very potent, long lasting and dose-dependent proliferation in the presence of N-SHH (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Our in vitro data reveal that N-SHH induced proliferation is differentially modulated by ECM glycoproteins. In particular, plating of cultures on LN significantly increased the rate of proliferation induced by N-SHH, as compared to control cultures grown on poly-L-Lys and treated with the same concentration of N-SHH. In contrast, granule cell precursors grown on VN displayed significantly decreased rates of SHH-mediated proliferation and increased neural differentiation with respect to control cultures. To explore the in vivo relevance of these results, we then analysed the expression of ECM glycoproteins and their integrin receptors in the developing cerebellar cortex.

Using a polyclonal anti-LN antibody, we demonstrate that LNs are expressed in Purkinje neurons as previously reported for laminin 2 by Powell et al. (Powell et al., 1998). LN proteins and their integrin receptor subunit α6 were also detected in the outermost part of the EGL. The presence of LNs and their receptors in the outer EGL further supports the hypothesis that these ECM proteins enhance SHH-mediated proliferation of granule cell precursors, and thus may facilitate the clonal expansion of the granule cell precursor pool. However, further investigation will be required to determine the precise form of LN present in the oEGL, the biochemical nature of the interactions between SHH and LNs in the developing cerebellum, and the signalling cascades activated by these interactions.

We have localised the expression of VN in the developing cerebellar cortex to the layer where granule cells commence their differentiation. Indeed, our in situ hybridisation studies establish that VN mRNA is expressed in the recently differentiated granule neurons located just beneath the Purkinje cell layer. However, VN protein is secreted above the Purkinje cell layer. Expression of VN protein is thereafter maintained in growing parallel fibres, the axons of granule neurons (Murase and Hayasi, 1998). Furthermore, we have localised the VN-specific integrin receptor (the αv subunit alone or the dimer αvβ3; as reviewed by Hynes, 1992) in the most internal part of the EGL, providing evidence that differentiating granule neurons possess the mechanisms to respond to VN-mediated signals. Our results show that in culture FN exerts an activity similar to that observed for VN. Moreover, in the developing cerebellar cortex, the pattern of FN expression is similar to that of VN, although FN is expressed at much lower levels. Since FN and VN utilise similar integrin receptor subunits (Hynes, 1992), compensation by FN activity may represent one of the reasons why VN null mice do not display gross defects in CNS formation (Zheng et al., 1995). It is possible that the only effect of VN is binding and sequestration of N-SHH with a consequent reduction in granule cell precursors proliferation. However, two observations argue against this hypothesis. Firstly, we have previously shown that N-SHH binds preferentially to the 45 kDa form of VN (Pons and Martí, 2000), which is not present in the developing cerebellum. Secondly, direct signalling of VN to granule precursors is supported by the expression of the integrin subunit αv. Therefore we favour the hypothesis that VN-dependent signalling, is able to terminate SHH-mediated proliferation and together with other secreted factors (Wechsler-Reya and...
Scott, 1999; SP and EM unpublished results) contribute to granule neuron differentiation.

**VN induces a differentiation signal**

ECM molecules through their integrin receptors trigger a broad array of intracellular signals. Recruitment of focal adhesion components like FAK or Pik2 appears essential to stimulate many effects involving MAPK signalling (reviewed by Howe et al., 1998). However, we have shown that in primary cultures of cerebellar granule neurons, VN stimulates CREB phosphorylation with no corresponding effect on MAPK activation indicating that in granular cells VN is able to activate CREB phosphorylation using a pathway not involving MAPK. Although at the present time the different components mediating VN stimulation of CREB phosphorylation are not known, it has been reported that integrins stimulate Ca^{2+} influx through Calreticulin family members (Coppolino et al., 1997). Ca^{2+} is a pleiotropic second messenger that activates a wide variety of kinases (reviewed by Shaywitz and Greenberg, 1999), being the phosphorylation of Ser 133 of CREB a common downstream target for many of these kinases, these data converts Ca^{2+} signalling into a priority candidate to be investigated in order to elucidate the pathway by which VN stimulates CREB phosphorylation.

The factors that have been reported to counteract SHH-induced granular cell proliferation (FGFs, Forskolin and DBA) all have the capacity to strongly stimulate phosphorylation of both CREB and MAPK. Moreover, activation of MAPK has been reported to be sufficient to induce differentiation in different neuronal types (Cowley et al., 1994). Thus, considering that CREB is one of the main downstream targets of MAPK, and as we demonstrate here, CREB signalling is sufficient to induce differentiation in granular cells, it is possible though that CREB phosphorylation is the common essential signal elicited by either VN and/or the other differentiation factors previously reported in granular cells. During development of the nervous system in general and the cerebellar cortex in particular, precursor cells proliferate until they undergo their final mitosis and subsequently differentiate. Therefore, mechanisms controlling the exit of the cell cycle and the initiation of differentiation need to be tightly regulated. It has been reported that the Ci/GLI family of zinc finger transcription factors require association with the CREB Binding Protein (CBP) in order to mediate the SHH induced proliferation (reviewed by Ingham, 1998 and by Ruiz i Altaba, 1999). However, phosphorylated CREB also requires binding to CBP in order to activate the CRE sites (reviewed by Shaywitz and Greenberg, 1999). Therefore, it is reasonable to propose a model where CREB Binding Protein (CBP) acts as a temporal switch to coordinate the end of proliferation with the initiation of differentiation. Accordingly, competition for CBP availability may represent an appropriate mechanism to integrate proliferation and differentiation signals.

The authors wish to thank Dr Paola Bovolenta for constant support and critical reading of the manuscript. We also thank Dr Angela Nieto for the use of her digital camera, Dr Deborah Burks for valuable improvement of the manuscript, and the two anonymous referees for their constructive comments on the earlier version of the manuscript. We wish to thank Dr Cliff Tabin for the chick SHH cDNA, Dr Carlos Martínez A. for the monoclonal anti-0x antibody, Dr Ivan de Curtis for the anti-68 antibody, and Biogen Inc for the purified human N-SHH. J. L. T. and J. R. M.-M. are supported by postdoctoral fellowships from the Comunidad Autónoma de Madrid (CAM). This research was supported by grant PM97-0018 to Dr Ignacio Torres-Alemán and by grants PM97-0019 and BIO4-CT98-0399 to Dr Paola Bovolenta, in whose laboratories this research was carried out.

**REFERENCES**


