Sonic hedgehog synergizes with the extracellular matrix protein Vitronectin to induce spinal motor neuron differentiation

Sebastián Pons and Elisa Martí*
Instituto Cajal de Neurobiología, CSIC, Avenida Doctor Arce 37, E-28002 Madrid, Spain
*Author for correspondence (e-mail: marti@cajal.csic.es)

Accepted 4 November; published on WWW 20 December 1999

SUMMARY

Patterning of the vertebrate neural tube depends on intercellular signals emanating from sources such as the notochord and the floor plate. The secreted protein Sonic hedgehog and the extracellular matrix protein Vitronectin are both expressed in these signalling centres and have both been implicated in the generation of ventral neurons. The proteolytic processing of Sonic hedgehog is fundamental for its signalling properties. This processing generates two secreted peptides with all the inducing activity of Shh residing in the highly conserved 19 kDa amino-terminal peptide (N-Shh). Here we show that Vitronectin is also proteolytically processed in the embryonic chick notochord, floor plate and ventral neural tube and that this processing is spatiotemporally correlated with the generation of motor neurons. The processing of Vitronectin produces two fragments of 54 kDa and 45 kDa, as previously described for Vitronectin isolated from chick yolk. The 45 kDa fragment lacks the heparin-binding domain and the integrin-binding domain, RGD, present in the non-processed Vitronectin glycoprotein. Here we show that N-Shh binds to the three forms of Vitronectin (70, 54 and 45 kDa) isolated from embryonic tissue, although is preferentially associated with the 45 kDa form.

Furthermore, in cultures of dissociated neuroepithelial cells, the combined addition of N-Shh and Vitronectin significantly increases the extent of motor neuron differentiation, as compared to the low or absent inducing capabilities of either N-Shh or Vitronectin alone. Thus, we conclude that the differentiation of motor neurons is enhanced by the synergistic action of N-Shh and Vitronectin, and that Vitronectin may be necessary for the proper presentation of the morphogen N-Shh to one of its target cells, the differentiating motor neurons.

Key words: Chick, Sonic hedgehog, Extracellular matrix, Vitronectin, Spinal motor neuron, Neural differentiation

INTRODUCTION

The dorsoventral patterning of the central nervous system (CNS) is controlled by intercellular signals. In the vertebrate neural tube, ventral polarity is regulated by signals emanating from the underlying notochord and from the ventral midline cells of the neuroectoderm, the floor plate. The embryonic expression pattern of Sonic hedgehog (Shh) is closely linked to the development and differentiation of the entire ventral neuraxis (Martí et al., 1995a). Exposure to the amino-terminal peptide (N-Shh) is required for the induction of spinal motor neurons (Martí et al., 1995b; Roelink et al., 1995), midbrain dopaminergic neurons (Hynes et al., 1995) and basal forebrain cholinergic neurons (Ericson et al., 1995). Moreover, mouse embryos deficient in functional Shh, show a perturbed ventral patterning of the CNS (Chiang et al., 1996). The Shh protein is proteolytically cleaved to produce two secreted proteins (Bumcrot et al., 1995; Porter et al., 1995) and all the signalling activity of Shh seems to reside in the 19 kDa N-Shh (reviewed in Hammerschmidt et al., 1997). N-Shh is covalently coupled to cholesterol during autoproteolysis of the immature Shh protein (Porter et al., 1996), a modification that constrains the diffusion of the active N-Shh. It is therefore not clear how N-Shh exerts its effect over longer distances, for example in the differentiation of spinal motor neurons.

Interactions between extracellular matrix proteins (ECM) and growth factors occur in various developmental paradigms. Vitronectin (VN) is an ECM glycoprotein that fulfils multiple functions in serum, and in both adult and embryonic tissues (reviewed in Preissner, 1991). We have previously shown that VN expression in the ventral neural tube is induced by N-Shh at the time of motor neuron differentiation, and that VN can promote motor neuron differentiation in neural explants. Treatment of embryos with anti-VN antibodies almost completely inhibits motor neuron formation, without altering other aspects of the dorsoventral patterning. These results strongly indicated that VN has a specific role in motor neuron differentiation (Martínez-Morales et al., 1997). However, the mechanism of VN action as well as the functional relationship between VN and N-Shh in the induction of motor neuron differentiation remains to be determined.

To address these questions, we have set up a model system where dissociated neuroepithelial precursor cells from the chick spinal cord can be assayed for their direct response to
either N-Shh, VN or a combination of the two. In our culture conditions, addition of N-Shh is sufficient to induce differentiation of the motor neuron phenotype, whereas VN alone has no effect. However, the combined addition of N-Shh and VN significantly increases the extent of motor neuron differentiation. We show that this effect may be the result of the biochemical association between N-Shh and a processed form of VN that lacks the heparin-binding domain (HBD). On the basis of these data, we propose a model in which VN is necessary for the proper presentation of N-Shh to the prospective spinal motor neurons.

MATERIALS AND METHODS

Chick embryos
White-Leghorn eggs were incubated at 38.5°C in an atmosphere of 70% humidity. Embryos were staged as described previously (Hamburger and Hamilton, 1951).

Vitronectin
Chick Serum Vitronectin (S-VN) was purified as described by Kitagaki-Ogawa et al. (1990) and was used in neural explant cultures as previously reported (Martínez-Morales et al., 1997). Yolk Vitronectin (Y-VN) was purified following the procedure described in Nagano et al. (1992), except that the last chromatographic column of anti-VN-Sepharose was not utilised. In brief, the low-density lipoprotein (LDL) fraction from the yolk plasma was applied to a hydroxylapatite column, and Y-VN was eluted with 200 mM sodium phosphate (pH 7.4). The eluate was then diluted with an equal volume of distilled water to decrease its ionic strength and applied directly to a DEAE-cellulose column (2 ml bed volume). Purified Y-VN was eluted with 0.25 M NaCl, 5 mM β-mercaptoethanol and 10 mM sodium phosphate (pH 7.4). Fractions were collected and analysed by SDS-PAGE. Fractions containing only the two bands, 54 and 45 kDa, as detected by staining with Coomassie Brilliant Blue R250, were pooled and dialysed against PBS buffer.

Sonic hedgehog
The E. coli-produced 19 kDa amino-terminal fragment of recombinant Shh (based on the Human sequence) used in this study was a gift from Biogen Inc., Cambridge, Massachusetts.

Neurotrophin-3
Recombinant NT3 used in this study was a gift from Prof. Y.-A. Barde and produced as previously described (Gotz et al., 1992).

Antibodies
Chicken VN was purified from serum and used to immunise rabbits. Anti-VN antiserum was antigen purified and tested as previously described (Martínez-Morales et al., 1997). The monoclonal antibody 5E1 directed against N-Shh was obtained from the Developmental Studies Hybridoma Bank (Iowa) and used for Immuno precipitation. Anti-mouse N-Shh polyclonal antibody (Ab 80) (Martí et al., 1995a) was used 1:500 for western blotting. This antibody was a generous gift from Prof. A. P. McMahon. The monoclonal antibody 40.2D6 directed against islet 1/2 was obtained from the Developmental Studies Hybridoma Bank (Iowa). Immunocytochemistry using this antibody (1:1000) from ascites fluid identifies postmitotic spinal motor neurons (Thor et al., 1991). A monoclonal antibody against a unique β-tubulin (Tuj-1) was obtained from MEDPASS S.a.r.l. (Grand Duché de Luxembourg). Immunocytochemistry using this antibody (1:5000) identifies postmitotic neurons. A monoclonal antibody against bromodeoxyuridine (BrdU) was obtained from the Developmental Studies Hybridoma Bank, to identify proliferating cells by immunocytochemistry (at 1:5000).

Whole-mount in situ hybridisation
Whole-mount in situ hybridisation was performed according to Wilkinson (1992). The VN cDNA was transcribed with T3 and with T7 RNA polymerases to generate digoxigenin-labelled antisense and sense RNA probes, respectively, as described by the manufacturer (Boehringer Mannheim). The location of the hybridised probes was revealed by alkaline phosphatase-coupled anti-digoxigenin Fab fragments (Boehringer Mannheim). Whole-mount hybridised embryos were sectioned on a vibratome (50 µm), sections were mounted, coverslipped and photographed.

RNA isolation and northern blotting
Chick tissues were collected from embryonic stages 13 and 25 (notochords and neural tubes), and from embryonic day 12 liver as control tissue. Under RNAse-free conditions, tissues were placed immediately on dry ice and stored at −70°C. Poly(A)+ was isolated by using the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia) according to the manufacturer’s instructions. RNA was separated by agarose gel electrophoresis; RNA loading and integrity were verified by ethidium bromide staining before transfer to a Nytran membrane. The filter was hybridised for 16 hours at 42°C in 50% formamide containing 1% SDS, 1% dextran sulfate, 0.5% NaCl and salmon sperm DNA, in a rotating oven. Filters were washed twice at 42°C with 2× SSC containing 1% SDS, and twice at room temperature with 0.2× SSC containing 1% SDS. The probe contained the full-coding chick VN (EMBL accession number Y11030).

Immunoprecipitation and western blotting
Stage 10-12 embryos (notochords and neural tubes) were collected in PBS and lysed in: 20 mM Tris (pH 7.5), 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 1% Nonidet P-40, 10% glycerol, 10 mM Na2HPO4, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF). Insoluble material was removed by centrifugation and supernatants were incubated with the monoclonal anti-N-Shh (5E1), with affinity-purified anti-VN, or with a non-relevant monoclonal antibodies as control (anti-Flag-tag M2, IBI, Kodak; anti-Thy-1, Cohen et al., 1986), at 4°C for 16 hours. The immunocomplexes were collected with an Agarose-conjugated anti-mouse antibody (Sigma) for 4 hours at 4°C. Immunoprecipitates were washed three times in lysis buffer before separation by SDS-PAGE. For western blotting (immunoblotting), proteins were transferred to nitrocellulose membranes; blocked and probed with anti-VN as described previously (Martínez-Morales et al., 1995). Membranes were consecutively probed with anti-N-Shh as described previously (Martí et al., 1995a).

Tryptic analysis
S-VN (70 kDa), Y-VN (54-45 kDa) and VN from stage 12-13 embryos (notochords and neural tubes) were immunoprecipitated with anti-VN antibody as described above. For non-denaturing trypsin degradation, immunoprecipitates were further washed in lysis buffer without protease inhibitors and finally in trypsin reaction buffer: 50 mM Tris-HCl pH 7.4, 1 mM CaCl2. The Sepharose pellet was dried and subjected to trypsin cleavage for 30 minutes in a 100 µl reaction volume. For denaturing reaction, the dried Sepharose pellet was denatured for 20 minutes at 100°C in 8 M Urea, 4 mM DTT, 50 mM Tris-HCl pH 8.0. Samples were renatured in 10 volumes of 50 mM Tris-HCl pH 7.4, 1 mM CaCl2 and trypsin treated as above. Trypsin-digested VN samples were analysed in western blots with the anti-VN antibody.

Explant cultures
Intermediate neural tube explants (150×200 µm) were dissected from stage 10 embryos and cultured for 48 hours in Dulbecco’s Modified Eagles Medium, F-12 (DMEM/F12, at 1:1) containing 25 units/ml penicillin, 25 µg/ml streptomycin, glutamine and N2 nutrient supplement (Gibco BRL), in a collagen gel matrix as described in Martí et al. (1995b). Explants were labelled with anti-islet 1/2 mAb,
then incubated with Cy3-conjugated anti-mouse antibody (Amersham). Labelled samples were observed by confocal microscopy and islet 1/2-positive cells were counted.

**Dissociated neuroepithelial cells cultures**

Homogeneous dissociated neural tube cultures were performed following the protocol in Kinoshita et al. (1993) with several modifications. The trunk region from stage 10 embryos was isolated in Hanks’ balanced salt solution (HBSS) (Gibco BRL) and treated with 10 mg/ml Trypsin (Worthington, Freehold, NJ) for 20-30 minutes at 4°C. Neural tubes, extending approximately 8 somites from the level of the last-formed somite, were freed from surrounding tissues in HBSS, the most ventral part (floor plate) was removed and the remaining neural tissue was then transferred to a 2.5 mg/ml solution of trypsin in Ca2+-, Mg2+-free HBSS (CMF-HBSS) (Gibco BRL), for 5 minutes at 37°C. Adding culture medium containing 10% serum terminated the digestion. Cells were sedimented by centrifugation, washed once with serum-free culture medium and dissociated into single cells in a small volume (100-200 μl) of culture medium by triturating through a standard yellow micropipette tip.

Neural tube cells thus prepared were counted with a haemocytometer, diluted into culture medium and plated on a fibronectin substratum (10 μg/ml) (Fraade et al., 1996; Kalyani et al., 1997, 1998), usually at a density of 10,000-20,000 cells in 50 μl. The culture medium used was same as above. Cultures were incubated at 37°C in a humidified atmosphere of 95% air, 5% CO2. Additives (N-Shh, purified S-VN, purified Y-VN and NT3) were freshly diluted into culture medium for each experiment. Typically 1 hour prior to fixing the cultures, 50 ng/ml of BrdU (Boehringer Mannheim) was added to the culture medium. Alternatively, cultures were grown in the presence of the same concentration of BrdU, or in the presence of 0.5 μCi/ml of [3H]methyl thymidine ([3H]Thy) during the entire culture period. For immunofluorescence detection of Tuj-1 cultures were fixed in 4% paraformaldehyde, whereas for immunostaining with anti-BrdU, cultures were fixed in methanol: DMSO (1:1). To determine the total cell number, cultures were incubated with bisBenzimide (Sigma).

**RESULTS**

**VN mRNA is expressed in CNS patterning centres**

We have previously shown that VN is expressed in the notochord and floorplate of stage 14 chick embryos (Martínez-Morales et al., 1997). Because VN is involved in the differentiation of spinal motor neurons, we wanted to determine the precise expression pattern of VN at earlier stages of embryonic development, when dorsoventral patterning of the neural tube is taking place. In whole mount in situ hybridisation, a restricted and specific pattern of VN mRNA was observed in presomatic and early somitic chick embryos. VN mRNA is expressed in extraembryonic membranes, as shown in Fig. 1A. In embryonic tissues, VN is highly expressed in the notochord as soon as it exits Hensen’s node, and in the floor plate as soon as it is formed (Fig. 1A-D). Expression in the notochord and in the floor plate is maintained at least until stage 25 (not shown). From stage 13-15, VN mRNA is also expressed in the ventral half of the neural tube at the time and position where motor neurons are being generated (Fig. 1E,F). Thus the early expression pattern of VN partially overlaps the expression pattern of the morphogen Sonic hedgehog (N-Shh) (Martí et al., 1995a) an important requirement for an interaction between N-Shh and VN to occur during the induction of motor neuron differentiation (Martínez-Morales et al., 1997).

**VN is proteolytically processed in CNS patterning centres**

In a previous study, we have shown that VN isolated from neural retina at various embryonic ages migrates as a single band of 70 kDa. This single band corresponds to the 70 kDa form of the chick serum VN (Martínez-Morales et al., 1995). However, the presence of two proteolytic forms of VN (54 and 45 kDa, respectively) has been reported in the yolk of chick eggs (Nagano et al., 1992). To determine the nature of the VN protein present in the early chick embryo, we prepared total extracts from various embryonic tissues. Western blot analysis revealed that, in addition to the 70 kDa form of VN, two smaller forms of VN existed in early embryonic tissues, which under reducing conditions migrate exactly as the 54-45 kDa forms of the egg yolk (Fig. 2B). Furthermore, the expression of these shorter forms of VN is regulated in a tissue-specific way during development. As such, the 54-45 kDa forms are present in the notochord at stages 10-13 (Fig. 2A), in the neural plate, from stage 10 to stage 20 (Fig. 2B), and in the neural tube, although less abundant, from stage 13 to stage 20 (Fig. 2C). In each of these tissues, the two short forms of VN are no longer detected at stage 25 (Fig. 2A-C). Interestingly, the
transient expression of the short forms of VN in the notochord and the floor plate precisely corresponds in time with the ventral midline tissue-inducing activities previously described (see table 1 in Martí et al., 1995a). These results suggest that the active form of VN involved in motor neuron specification would be either one or both of these short forms.

As a result of these analyses, we wanted to determine whether the two short forms of VN present in early chick embryos were identical to those previously reported in Yolk-Vitronectin (Nagano et al., 1992). Interestingly, the analysis of the two fragments of Y-VN led the authors to propose a model whereby an initial cleavage generates a 54 kDa fragment and a second cleavage generates the 45 kDa fragment. Accordingly, the 54 kDa form would lack the HBD and the 45 kDa form would lack both the HBD and the integrin-binding domain, RGD (Fig. 3A), implying that signalling through this 45 kDa form would not require an integrin type receptor.

In order to compare the identities of the VN proteins from serum, yolk and early embryonic tissues, we performed a tryptic analysis. Samples were immunoprecipitated with anti-VN

![Fig. 2](image)

**Fig. 2.** Different forms of the VN protein are present in embryonic chick tissues. Tissue extracts from various developmental stages were fractionated by SDS-PAGE electrophoresis and transferred onto nitrocellulose membranes. Membranes were probed with affinity purified VN antibody, which identified a 70 kDa band corresponding to the full VN, as well as two shorter forms of VN running at 54 and 45 kDa, respectively. (A) Notochord tissue extracts show the presence of processed forms of VN at stage 10 and 13, but not at stage 25. (B) Floor plate tissue extracts show the presence of processed forms of VN at stage 10 and 13, but not at stage 25. 70, 54 and 45 kDa VN forms are also present in chick yolk total proteins. (C) Neural tube tissue extracts show the presence of processed forms of VN at stage 13 and 19, but not at stage 25.

![Fig. 3](image)

**Fig. 3.** Tryptic analysis of VN forms. (A) Proposed model for VN cleavage, as in Nagano et al. (1992). S-VN suffers a first cleavage event (black arrow) at the amino-terminal part of the heparin-binding domain (HBD-box), generating a 54 kDa form lacking HBD. Second cleavage event (second black arrow) at the carboxyl-terminal portion of the integrin-binding domain (RGD-box) generates a 45 kDa form, lacking both HBD and RGD. (B-D) Purified S-VN (S), purified Y-VN (Y) and VN present in the notochord and neural tube of stage 12-13 chick embryos (E) were immunoprecipitated with anti-VN and subjected to trypsin degradation. (B) Titration of the proteolytic activity of trypsin on non-denatured VN proteins. Lanes 1,2 show native 70 kDa VN from Serum (S) and 54-45 kDa VN from Yolk (Y). Lanes 3-8 show identical amounts of S-VN and Y-VN treated with increasing concentrations of trypsin. Note the final conversion of all VN forms into the 45 kDa form. (C) Titration of the proteolytic activity of trypsin on urea-denatured VN proteins. Lanes 1, 2 show denatured 70 kDa VN from Serum (S) and 54-45 kDa VN from Yolk (Y). Lanes 3-8 show identical amounts of denatured S-VN and Y-VN treated with increasing concentrations of trypsin. (D) Comparison of S-VN, Y-VN and E-VN degradation patterns under non-denaturing and urea-denaturing conditions. Lanes 1-3 show untreated Y-VN, S-VN and E-VN. Lanes 4-6 show non-denatured VN proteins treated with 5 μg/ml of trypsin. Lanes 7-9 show urea-denatured VN proteins treated with 0.01 μg/ml of trypsin. Note that the peptidic map of E-VN represents the combined maps of S-VN and Y-VN, as expected if the 54-45 kDa forms of E-VN were identical to those of Y-VN.
antibody and subjected to trypsin degradation in non-denaturing and urea-denaturing conditions. Under non-denaturing conditions, treatment of S-VN with low concentrations of trypsin gives rise to three degradation fragments ranging from 65 to 50 kDa. Under the same conditions, the 54 kDa Y-VN is mainly converted into the 45 kDa fragment. Increasing the concentration of trypsin resulted in the conversion of both S-VN and Y-VN into the 45 kDa fragment (Fig. 3B). Under denaturing conditions, the 70 kDa, 54 kDa and 45 kDa forms of VN are equally degraded, and no conversion into the 45 kDa form is observed (Fig. 3C). The degradation pattern of VN isolated from embryonic tissue represents a summation of the fragments generated from S-VN and Y-VN (Fig. 3D). This provides evidence that the 54-45 kDa forms of VN present in embryonic tissues are the same to those in Y-VN.

To further confirm that the two VN immunoreactive 54-45 kDa bands were indeed generated by the processing of a single VN transcript, we performed a northern blot analysis with poly(A)+ RNA from various embryonic tissues. These tissues included the notochord and neural tube from stage 13 embryos (tissues containing the 54-45 kDa forms of VN), notochord and neural tube from stage 25 embryos (tissues where only the single 70 kDa form of VN was previously detected), and embryonic day 12 liver as control tissue. The blot was hybridised with a full-length VN cDNA probe that revealed a single mRNA species of approximately 1.6 kb. The mRNA from liver and notochord migrated slightly below the mRNA from the neural tube, which might reflect tissue-specific different polyadenylation (Fig. 4). These results excluded the possible existence of different spliced forms of VN mRNA as reported for other species (Preissner, 1991; Seiffer et al., 1993), providing further evidence that the 54-45 kDa forms of VN present in early chick tissues are indeed products of a proteolytic processing.

**The 54-45 kDa forms of VN have motor-neuron-inducing activities**

We have previously shown that in vivo blockade of VN activity with anti-VN leads to a specific loss of islet 1/2-positive neurons in the spinal cord. Furthermore, we showed that the 70 kDa form of VN was sufficient to induce the generation of islet 1/2-positive neurons in neural explant cultures. (A) Stage 10 neural tube explant grown in the presence of VN purified from chick serum (10 µg/ml). S-VN increased the number of islet 1/2-positive cells to a mean of 65±15 cells per explant (n=6 out of 10). (B) Neural tube explant grown in the presence of VN purified from chick egg yolk (10 µg/ml). Y-VN increased the number of islet 1/2 immunoreactive cells to a mean of 78±18 cells per explant (n=10 out of 17). (C) Explant grown in the presence of purified human N-Shh (1 µg/ml) gave a strong induction of islet 1/2-expressing cells, mean of more than 250 cells per explant (n=8 out of 10). (D) Control explant grown in culture medium.

**Fig. 5. Effect of purified forms of VN on the differentiation of motor neurons in cultured neural tube explants.**

\[ \text{S-VN, Y-VN, VN, N-Shh, Control} \]

To determine whether there is a physical interaction between the two proteins, we performed co-immunoprecipitation experiments. In vitro experiments were performed by incubating 1 µg of purified S-VN, purified Y-VN, or purified fibronectin (FN) as control, with increasing concentrations of N-Shh (0, 1 and 5 µg). Complexes were precipitated with the monoclonal anti-N-Shh (5E1) and subsequently detected in western blots with the anti-VN antibody, with the anti-FN antibody, and with the polyclonal rabbit anti-N-Shh (Ab 80). Both S-VN and Y-VN are co-immunoprecipitated with N-Shh in a dose-dependent fashion, whereas FN is not (Fig. 6A).

**N-Shh physically interacts with VN**

To determine whether there is a physical interaction between the two proteins, we performed co-immunoprecipitation experiments. In vitro experiments were performed by incubating 1 µg of purified S-VN, purified Y-VN, or purified fibronectin (FN) as control, with increasing concentrations of N-Shh (0, 1 and 5 µg). Complexes were precipitated with the monoclonal anti-N-Shh (5E1) and subsequently detected in western blots with the anti-VN antibody, with the anti-FN antibody, and with the polyclonal rabbit anti-N-Shh (Ab 80). Both S-VN and Y-VN are co-immunoprecipitated with N-Shh in a dose-dependent fashion, whereas FN is not (Fig. 6A).

**Fig. 4. Northern blot analysis of VN.** Poly(A)+ isolated from stage 13 and 25 notochords (Not), from stage 13 and 25 neural tubes (NT) and from liver were electrophoresed transferred onto nylon membranes and hybridised with a full-coding VN cDNA probe. Upper panel shows a single band of approximately 1.6 kb corresponding to a single VN transcript. Note that mRNA from the notochord run slightly lower that RNA from neural tube. Lower panel shows ribosomal RNA as loading control.

\[ \text{Not} \quad \text{NT} \quad \text{Liver} \]

\[ \text{VN} \quad \text{1.8 kb} \]

\[ \text{St13 St25} \quad \text{St13 St25} \]
Co-immunoprecipitation experiments in embryos extracts were performed by carefully dissecting notochords and neural tubes from stage 10-12 embryos. Lysates were immunoprecipitated with the monoclonal anti-N-Shh (5E1), with anti-VN, or by using irrelevant monoclonal antibodies (M2 and anti-Thy 1) as controls. The three forms of VN (70, 54 and 45 kDa) are co-immunoprecipitated with N-Shh, as detected in western blots with the anti-VN antibody, however the 45 kDa form of VN seems to be enriched with respect to the other forms (Fig. 6B, lane 1). Additionally, the 19 kDa N-terminal Shh is also co-immunoprecipitated with VN, as detected in western blots with the polyclonal anti-N-Shh (Fig. 6B, lane 3). Thus, both in vitro and in vivo co-immunoprecipitation experiments demonstrate a specific physical interaction between the signalling molecule N-Shh and the ECM protein VN. Furthermore, our results suggest a preferential association of N-Shh with the 45 kDa VN. This proteolytic fragment lacks the heparin-binding domain and the integrin-binding domain (Nagano et al., 1992), as such will be a highly diffusible protein unable to signal through the canonical integrin receptor.

Dissociated neuroepithelial cells retain an undifferentiated state in culture

Our results suggest a model in which the morphogen N-Shh, by binding to the processed form of VN, might form a complex with greater facility for the presentation of N-Shh to the motor neuron progenitors. To examine this hypothesis, we wanted to compare the putative inductive activity of the N-Shh/VN complex with that of the two separate factors in motor neuron differentiation. Intermediate neural tube explants of stage 10 chick embryos are commonly used in motor neuron induction assays. However, it has been reported that, by stage 10, the neural tube has already been exposed to low levels of Shh which downregulate dorsal gene expression (Pax3 and Pax7), forming a generic population of ventral progenitors (Ericson et al., 1996). Furthermore, we previously reported that VN mRNA is itself induced by N-Shh (Martinez-Morales et al., 1997) and we have shown here that VN protein is already present in the neural tube of stage 13 chick embryos (Fig. 2C). Thus, we sought to set up a system where dissociated neuroepithelial cells could be assayed for their direct response to each factor individually.

Homogeneous dissociated cultures of neuroepithelial cells were prepared from stage 10 chick neural tubes and maintained in serum-free medium. Under these conditions, most neuroepithelial cells retain an undifferentiated phenotype (Fig. 7A), and only after 48 hours in culture a few cells show a neural phenotype with small refringent cell bodies and long neurites (Fig. 7B). The percentage of cells showing a neural phenotype increased after 72 hours (not shown). To confirm that these cells acquired a neural phenotype in vitro, we compared the extent of [3H]Thy labelling with the expression of neural markers. 4 hours after plating, approximately 76% of the cells had incorporated [3H]Thy and thus had undergone a complete cell cycle, but none of them expressed the neuron-specific marker Tuj-1. After 24 hours in culture, all the cells have undergone at least one cell division in vitro and 2% of the cells expressed Tuj-1 (Fig. 7C), of which none are positive for the motor neuron differentiation marker islet 1/2. The number of Tuj-1-positive cells increased after 48 hours in culture (approximately 5% in control cultures, Fig. 8A,D), but again no neurons are islet 1/2 positive (Figs 7E, 8B,E). Taken together these data suggest that only a minor percentage of the plated neuroepithelial cells differentiate into neurons, and that those that have acquired a neural phenotype have done so in vitro (Fig. 7G-I). Thus, it seems that this is an appropriate system to study the activities of N-Shh and VN proteins.
Sonic and Vitronectin in neural differentiation of motor neurons.

**Shh and VN act synergistically in the induction of motor neurons**

Addition of high concentrations of N-Shh (5 µg/ml) to dissociated neuroepithelial cells stimulated, after 48 hours, a significant increase in the percentage of differentiated neurons (8%) compared to either control cultures (4.2%) or to lower concentrations of N-Shh (50 ng/ml and 0.5 µg/ml; 4.8% and 5%, respectively) (Fig. 8A). Furthermore, addition of N-Shh significantly induced the acquisition of the motor neuron phenotype, as assessed by immunostaining for islet 1/2 (2.5% in cultures treated with 50 ng/ml; 3.5% in cultures treated with 0.5 µg/ml; and 7% in cultures treated with 5 µg/ml) (Fig. 8B). The stimulation of Tuj-1 and islet 1/2 immunoreactive cells by N-Shh appeared to be restricted to the differentiation of new neurons and not to an increase in the total cell number, since N-Shh had no effect on the rate of proliferation. Indeed, approximately 20% of cells incorporate BrdU during a 1 hour pulse prior to fixation of the culture, in both control and treated cultures (Fig. 8C).

In contrast to these results, neither S-VN (70 kDa), nor Y-VN (54-45kDa) displayed any activity at any of the concentrations tested (2.5, 5 and 10 µg/ml). Neither the rate of proliferation nor that of neural differentiation changed in control or treated cultures (Fig. 8 and data not shown).

However, when cultures were treated with N-Shh/VN complexes, neural differentiation raised up to approximately 16% (Figs 7D, 8D), and the acquisition of the motor neuron phenotype to approximately 14% (Figs 7F, 8E). This was particularly significant given that the concentrations of N-Shh (0.5 µg/ml) and VN (5 µg/ml) used in these combinations were only partially (3.5% for N-Shh) or not effective (VN) in inducing motor neuron differentiation when applied individually. The stimulation of islet 1/2 immunoreactive cells via a synergism between N-Shh and VN appeared to be restricted to the differentiation of new neurons and not to an increase in total cell number, since no effect on the rate of proliferation was observed. Approximately 21% of neuroblasts incorporate BrdU after a 1 hour pulse prior to fixation, both in control and treated cultures (Fig. 8F).

It has been reported that NT3 enhances....
the number of motor neurons in dissociated neural tube cultures (Averbuch-Heller et al., 1994) and collaborates with N-Shh to induce motor neuron differentiation (Dutton et al., 1999; Roelink et al., 1995). Therefore, we tested whether NT3 would enhance motor neuron differentiation induced by N-Shh in our culture conditions. The addition of 50 ng/ml of NT3 to the medium in either control or experimental cultures did not significantly change the data reported above (not shown). The difference of our results with those of others might reside in our use of very early, undifferentiated neural plate tissue. This suggests that NT3 may be implicated in the later but not in the initial steps of motor neuron differentiation. Furthermore, in vivo, NT3 activity over spinal motor neurons can be substituted probably by other neurotrophins, as suggested by the analysis of the NT3-deficient mice (Fariñas et al., 1994).

DISCUSSION

Signalling in the generation of spinal motor neurons
In vertebrate embryos, N-Shh promotes the differentiation of both floor plate cells and motor neurons (reviewed by Placzek, 1995; Hammerschmidt et al., 1997). However, the induction of motor neuron differentiation appears to be independent of that of the floor plate (Tanabe et al., 1995; Ericson et al., 1996). Although the N-Shh peptide is a secreted protein, it seems to remain anchored to the membrane of the synthesising notochord and floor plate cells by means of a cholesterol moiety, attached to the active peptide (Porter et al., 1996). As such, N-Shh protein has never been detected outside the domain of Shh-expressing cells (Martí et al., 1995a). It is clear however that motor neuron progenitors depend on exposure to local N-Shh signalling until late into their final cell cycle (Ericson et al., 1996). Furthermore, in this study, we provide evidence that N-Shh is sufficient to directly induce motor neuron differentiation in isolated neuroepithelial cells. Thus, resolving the biological mechanism by which N-Shh could exert its effect over long distances, seems crucial for understanding the biology of motor neuron differentiation.

Based on the results here presented, we propose a model in which the ECM glycoprotein VN will actively participate in the presentation of the N-

Shh signal to motor neuron progenitors. VN is a multifunctional ECM protein that contains specific-binding sites for integrins, collagen, and heparin, complement components and plasminogen. VN is present in focal adhesions of cultured cells, is a major adhesive glycoprotein in plasma and also plays an important role in the regulation of the complement, coagulation and fibrinolytic systems (reviewed in Preissner, 1991).

![Activity of N-Shh and VN on the differentiation of dispersed neuroepithelial cells.](image-url)

(A-C) Cultures treated with different concentrations of N-Shh (0; 0.05; 0.5; 5 μg/ml) for 48 hours and assessed for neural differentiation (immunostaining with anti-Tuj-1), for motor neuron differentiation (immunostaining with anti-islet 1/2) and for cell proliferation (immunostaining with anti-BrdU, after 1 hour BrdU incorporation prior fixation). (A) A slight but significant increase on the percentage of Tuj-1-immunostained cells in cultures treated with 5 μg/ml of N-Shh (8±2.9%, X0 =X 5 , P<0.02, Students t-test), as compared to control cultures (4.2±2.2%) and to cultures treated with lower concentrations of N-Shh (4.8±1.4%, X0 =X Shh , P>0.2). (B) A significant increase on the percentage of islet 1/2 immunostained cells in cultures treated with 5 μg/ml of N-Shh (7±2.9%, X0 =X Shh , P<0.0002), as compared to control cultures where there are no islet 1/2-positive cells, and as compared to cultures treated with the lowest concentrations of N-Shh (2.5±1.1%, X0 =X 0.05 =X 5 , P<0.002). (C) No significant difference in the proliferation rate, at any of the concentrations tested. (D-F) Cultures treated with 0.5 μg/ml of N-Shh, cultures treated with 5 μg/ml of VN (VN) and treated with a combination of some of the concentrations of the two factors (Shh/VN) for 48 hours. (D) No significant increase on the percentage of Tuj-1-immunostained cells in cultures treated with each factor separately (4.6±2.3% in control cultures; 5.3±2.0% in Shh treated cultures; 5.2±2.1% in VN-treated cultures; X0 =X Shh , P>0.5; X0 =X VN , P>0.5, Students t-test). A very significant increase in the number of differentiated neurons is observed in cultures treated with a combination of the two factors (16±4.3%, X0 =X Shh/VN , P=0.0002). (E) Shows a very significant increase in the percentage of islet 1/2-immunostained cells in cultures treated with the combination of the two factors (14±4.8%) as compared to cultures treated with 0.5 μg/ml of N-Shh (2.8±1.3%, X0 =X Shh/VN , P<0.0002). Islet 1/2-positive cells do not develop in control cultures or in cultures treated with 5 μg/ml of VN. (F) No difference in the proliferation rate, in any of the treatments analysed. Standard errors are indicated, as the numbers (n) of cultures analysed. Analysis of each culture was done by counting total cells stained with bisBenzimide versus immunostained cells, in a minimum of 25 randomly selected microscopic fields.
However, very little is known about the role of VN during embryonic development. The distribution of VN mRNA has been described during murine development (Seiffert et al., 1995) and, in this study, we report the early sites of expression of VN in developing chick embryos. During this period, VN transcripts were observed in domains partially overlapping those of the signalling molecule N-Shh. In a previous study, we provided the first insight into the involvement of VN in the pathway leading to motor neuron differentiation. We showed that in vivo blockage of VN almost completely inhibits motor neuron formation, without affecting floor plate development or dorsoventral patterning of the neural tube (Martínez-Morales et al., 1997). In this study, we now show that VN associates with the signalling molecule N-Shh, and that this interaction facilitates the differentiation of motor neuron progenitors. We believe that these results might indicate a role for VN in the transportation or presentation of N-Shh to its target cells, the motor neurons. It is possible thought that, in vivo, this VN activity might be substituted by other ECM molecules also expressed in the notochord and floor plate of various vertebrates (Gotz et al., 1995; Higashijima et al., 1997; Tucker et al., 1997; Urry et al., 1998), since mice lacking VN displayed an apparently normal phenotype (Zheng et al., 1995). Our results suggest that interaction with the ECM is important for the efficient activity of N-Shh in vertebrates. Indeed in the fly, an interaction between Hh and ECM components has been previously described. The product of the gene tout-velu, a molecule implicated in the processing of the ECM, is required in the receiving distant cell for the signal to be correctly interpreted (Bellaiche et al., 1998; The et al., 1999).

**VN proteolysis is spatially and temporally regulated**

Our results show that VN is proteolytically processed in the notochord and the floor plate, and that this processing coincides temporally with the patterning activities localised to these two signalling centres (see table 1 in Martí et al., 1995a). This suggests that processing of VN might be required to generate the form active in motor neuron differentiation. Proteolytic events regulate ECM molecules as well as the release of bioactive fragments and growth factors (for review see Werb, 1998). It is noteworthy that the processing of VN releases a 54 kDa form lacking the HBD. This 54 kDa VN is subjected to a second processing event that releases a 45 kDa form of VN that lacks both HBD and the integrin-binding domain, RGD (Nagano et al., 1992). Thus, in addition to being a highly diffusible molecule, the 45 kDa VN would not be able to interact with an integrin type receptor.

Localising the enzymes that perform processing events to sites where degradation takes place is necessary for such an exquisite regulation of proteolytic events in the ECM. Members of the matrix metalloproteinase family (MMP), in particular MMP-9, MMP-10 and MMP-14, have the capacity to cleave VN (see table 1 in Werb, 1998). Furthermore, the bone morphogenetic protein 1/Tolloid family of metalloproteinases has also the potential capability of degrading ECM, and localises to CNS signalling centres (Martí, 1999) at the time and place when VN is being cleaved, thus suggesting that these might be among the proteases responsible for releasing the active form of VN.

Another molecule that may be potentially involved in the processing of VN is Shh itself, if the interaction between the two molecules and the biochemical characteristics of Shh are taken into consideration. Indeed, the carboxy-terminal peptide of Sonic hedgehog (C-Shh) catalyses the autocleavage of the hedgehog precursor protein (Porter et al., 1995), and its structure is similar to self-splicing proteins (Hall et al., 1997). In addition, the crystal structure of the amino-terminal Sonic hedgehog (N-Shh) revealed the presence of a zinc cation, coordinated in a fashion normally found in the catalytic pocket of zinc hydrolases (Hall et al., 1995). This suggests that N-Shh might also have proteolytic activity although this has not yet been demonstrated biochemically. Further studies will be required to identify the proteases involved in cleaving VN.

**N-Shh/VN complex has motor-neuron-inducing activity**

In this study, we have set up a system where isolated neuroepithelial cells can be assayed for their direct response to growth factors. In our culture conditions, the majority of the plated neuroepithelial cells maintain their undifferentiated state, even after 48 hours in culture. This powerful culture system allowed us to directly assess the response of individual progenitor cells to independent signalling molecules, without the interference of any pre-existing molecules.

In accordance with our previous study (Martínez-Morales et al., 1997), we found that VN is capable of stimulating motor neuron differentiation in neural explants. However, this activity requires either cell/cell interactions or some other molecules present in the explant, since VN alone is not able to stimulate motor neuron generation in isolated neuroepithelial cells. However, we also show that N-Shh is indeed sufficient to directly stimulate motor neuron differentiation in a dose-dependent way and independent of cell/cell interactions. These results could be explained if the explants contained low levels of trapped N-Shh protein. In support of this, it has been reported that stage 10 neural tube has already been exposed to low levels of Shh which downregulates dorsal gene expression, Pax3 and Pax7, forming a generic population of ventral progenitors (Ericson et al., 1996). Low levels of trapped N-Shh protein in the explant would explain how the sole addition of VN, possibly by binding to N-Shh, would result in the differentiation of motor neurons. The fact that the activity of VN on explants is not dose dependent, and that it never produces a strong induction of motor neurons, would further support this hypothesis.

Even though neither forms of VN (70 kDa VN; 54-45 kDa VN) show any activity on isolated neuroepithelial cells, treatment of cultures with N-Shh/VN combinations significantly increased the extent of motor neuron differentiation. In this study, we provided a biochemical basis for this combined activity, since our results demonstrate a physical interaction between N-Shh and VN. The fact that the N-Shh/VN complex is more efficient than each separate molecule, in inducing motor neuron differentiation, suggests that the formation of this complex might promote the presentation of the morphogen to its target cells. The formation of this complex could also explain why some specific antibodies are not able to detect the presence of N-Shh in the ventral neural tube, where protein should be present if it were to directly induce motor neuron differentiation (Martí et al., 1995a). Since N-Shh and the processed forms of VN (54-45 kDa) are co-expressed in the notochord and in the floor plate, we propose a model in which binding of N-Shh to VN would help transport N-Shh through the ECM.

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Authors are grateful to Dr Alfredo Rodríguez-Tébar and to Dr Paola Bovolenta for support and scientific discussions; Drs Mark Sefton, Juan Ramón Martínez-Morales, Alfredo Rodríguez-Tébar and Paola Bovolenta for critical reading of the manuscript, Prof. A. P. McMahon for the polyclonal N-Shh antibody, Prof. Y.-A. Barde for the purified NT3, and Biogen Inc., for the purified human-N-Shh protein. We are also grateful to Concha Bailón for image assistance and María Jesús Martín-Bermejo for protein purification. This research was financed by Grants from the Spanish Ministry of Education (DGICYT) PB95-0025 and PM98-0114 and from the European Union Biotech Programme Bio4C T98-0112.

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