

Vitronectin is expressed in the ventral region of the neural tube and promotes the differentiation of motor neurons

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SUMMARY

The extracellular matrix protein vitronectin and its mRNA are present in the embryonic chick notochord, floor plate and in the ventral neural tube at the time position of motor neuron generation. When added to cultures of neural tube explants of developmental stage 9, vitronectin promotes the generation of motor neurons in the absence of either notochord or exogenously added Sonic hedgehog. Conversely, the neutralisation of endogenous vitronectin with antibodies inhibits over 90% motor neuron differentiation in co-cultured neural tube/notochord explants, neural tube explants cultured in the presence of Sonic hedgehog, and in committed (stage 13) neural tube explants. Furthermore, treatment of embryos with anti-vitronectin antibodies results in a substantial and specific reduction in the number

of motor neurons generated *in vivo*. These results demonstrate that vitronectin stimulates the differentiation of motor neurons *in vitro* and *in vivo*. Since the treatment of stage 9 neural tube explants with Sonic hedgehog resulted in induction of vitronectin mRNA expression before the expression of floor plate markers, we conclude that vitronectin may act either as a downstream effector in the signalling cascade induced by Sonic hedgehog, or as a synergistic factor that increases Shh-induced motor neuron differentiation.

Key word: spinal cord, motor neurons, vitronectin, Sonic hedgehog, chick

INTRODUCTION

The molecular mechanisms by which mitotic progenitor cells in the developing nervous system cease division and differentiate into specific phenotypes are still poorly understood. Cell lineage studies have shown that in some regions of the central nervous system (CNS), progenitor cells maintain their pluripotentiality to differentiate into neurons and glia (reviewed by Temple and Qian, 1996). For example, in the embryonic neural tube, motor neuron progenitors remain multipotent until shortly before the motor neurons are born and can give rise to other cell types including glial and ependymal cells (Leber and Sanes, 1991). Sonic hedgehog (Shh) is a notochord- and floor plate-derived morphogen involved in dorsoventral axis formation which specifies the differentiation of some ventral neurons in the neural tube. These include cholinergic neurons in the basal forebrain (Ericson et al., 1995), midbrain dopaminergic neurons (Hynes et al., 1995) and spinal cord motor neurons (Roelink et al., 1994, 1995; Martí et al., 1995a). However, it is still unclear whether Shh exerts this effect by direct interaction with motor neuron precursors (Ericson et al., 1996) or alternatively if it regulates their interactions with downstream secondary inducers (Johnson and Tabin, 1995).

Different components of the extracellular matrix (ECM)

have previously been shown to play central roles in the differentiation of many cell types including endothelial cells, myoblasts, macrophages, bone cells, monocytes and embryonic kidney cells (Adams and Watt, 1993; Juliano and Haskill, 1993). Although ECM molecules have been shown by numerous studies to affect neuronal survival and neurite outgrowth (reviewed by Reichardt and Tomaselli, 1991), there is little evidence for a role in neuron differentiation (Frade et al., 1996). The ECM component vitronectin is a multifunctional glycoprotein present in serum and in both adult and embryonic tissues (Hayman et al., 1983; Preissner, 1991). Vitronectin exerts multiple effects on cells by binding to integrin receptors bearing the α_v subunit which are expressed by many cell types (Hynes, 1992). While it has been shown that vitronectin promotes neurite outgrowth from cultured retinal neurons (Neugebauer et al., 1991) and the appearance of neurons in cultured retinal neuroepithelial cells (Martínez-Morales et al., 1995), no role in the differentiation of specific neuronal phenotypes has been established.

We have cloned chick vitronectin and studied its expression pattern in the embryonic spinal cord. In addition to being present at the time of motor neuron differentiation, we also show that vitronectin promotes the differentiation of neuroepithelial cells into motor neurons both *in vivo* and *in vitro*.

The functional relationships of vitronectin with Shh leading to motor neuron differentiation are discussed.

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 was purified as described by Kitagaki-Ogawa et al. (1990). In short, after a brief centrifugation, chicken serum (Seralab) was passed through a heparin-agarose column (Biorad), previously equilibrated with 10 mM sodium phosphate buffer, pH 7.7, 130 mM NaCl and 2 mM p-methyl sulfonyl fluoride. The eluate was collected and urea added to give a final concentration of 8 M. After 2 hours incubation at 4°C, the eluate was passed through the heparin-agarose column equilibrated with the same buffer containing 8 M urea. The eluate was discarded and vitronectin was eluted using a linear gradient 150–500 mM NaCl. Fractions were collected and analysed by SDS/PAGE. Fractions containing only a doublet of M_r 70 and 68, as detected by staining with Coomassie Brilliant Blue R250, were pooled and dialysed against PBS buffer. The double band was expected because pure vitronectin preparations of mammals and chicken has been shown to migrate as a doublet, the lower band being a proteolytic fragment of the upper (Preissner, 1991).

The final vitronectin preparations did not contain detectable contaminants as assessed by examination of Coomassie Blue-stained gels. The identity of vitronectin was established by Edman degradation of selected cyanogen bromide fragments which corresponded to the deduced amino acid sequence after vitronectin cDNA cloning (see below).

Sonic hedgehog

The autocleaved 19K amino-terminal fragment of recombinant Shh (Martí et al., 1995a; Roelink et al., 1995) used in this study was a gift from A. P. McMahon, Harvard University.

Antibodies

Purified chicken vitronectin was used to immunize rabbits. Anti-vitronectin antiserum was purified by affinity chromatography on a vitronectin-bound agarose column (Martínez-Morales et al., 1995). The blocking capacity of the antibody was assessed by inhibiting the attachment of cells dissociated from embryonic day (E) 6 chick retinas to vitronectin tissue culture substrate following a published assay protocol (Hall et al., 1987). The monoclonal antibody (mAb) 40.2D6, directed against Islet-1/2 was obtained from the Developmental Studies Hybridoma Bank (Iowa). Immunohistochemistry using this antibody at a 1:1000 dilution in ascites fluid identifies postmigratory motor neurons in the ventral horn (Thor et al., 1991). The hybridoma culture supernatant of mAb BEN was used to label notochord, floor plate and motor neuron at a dilution of 1:5 (Pourquie et al., 1990), and was also obtained from the Hybridoma Bank. Anti-chicken Pax 6 polyclonal antibody (Macdonald et al., 1994) was used for immunohistochemistry at 1:600 dilution of the serum and was a gift from S. W. Wilson King College, London. Anti-chicken Shh polyclonal antibody has been described previously (Martí et al., 1995a). This antiserum was used at a dilution of 1:200 for immunohistochemistry and 5 µg/ml purified IgGs to block the activity of Shh in explant cultures as previously described (Martí et al., 1995a). The blocking capacity of the antibody was tested by inhibiting the appearance of motor neurons in cultured stage 9 neural tube/notochord co-explants. This antibody was a generous gift from A. P. McMahon.

Immunohistochemistry

12 µm cryostat transverse sections of the brachial region of stage 16 and stage 27 embryos were used for immunohistochemistry following standard procedures. Primary antibodies were detected by either goat anti-mouse IgG or goat anti-rabbit IgG, conjugated to either Cy2, Cy3 or Cy5 (Amersham). Sections were mounted with PBS/glycerol and analysed with a TCS laser scanning confocal imaging system equipped with a krypton-argon ion laser (Leica).

Cloning of chick vitronectin

A fragment of chick vitronectin cDNA was amplified by RT-PCR using degenerate primers derived from the bases 101–119 and 890–871 of the rabbit vitronectin sequence (Sato et al., 1990): sense 5'-ARGGIMGITGYGAYGARGG-3', antisense 5'-GGCTGRTGIGC-GAARTCGTA-3'. The amplified 783 bp fragment was cloned in pBlueScript and sequenced. The deduced amino acid sequence displayed 66% identity with that of rabbit vitronectin. In addition, the sequence included 19 amino acids identical to the sequence obtained by Edman degradation of chick vitronectin (Nakashima et al., 1992). We used this fragment as a probe to screen a cDNA library from E6–7 chick retinas. Full length cDNAs were found and sequenced (EMBL accession number Y11030).

In situ hybridisation

The vitronectin 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 riboprobe for chick Pax 3 was prepared by transcription from a cDNA clone (Goulding et al., 1993). Hybridisations on neural tube 14 µm cryostat sections were carried out following a published protocol (Schäeren-Wiemers and Gerfin-Moser, 1993). The location of the hybridised probes was revealed by alkaline phosphatase-coupled anti-digoxigenin Fab fragments (Boehringer Mannheim).

Antibody treatment of chick embryos

Eggs were incubated for 38 hours (stage 10) and were then opened at the blunt pole. The shell membrane was removed and 50 µl of PBS solution containing 5 µg of anti-vitronectin antibody was layered onto the chorioallantoic membrane. The incubation of the eggs was continued and the antibody application was repeated after 60 hours incubation. The embryos were sacrificed at E3. The antibody was injected into another group of embryos after 36, 60 and 84 hours incubation which were killed at E5. Controls were performed by injecting 50 µl of PBS containing 5 µg of purified rabbit immunoglobulin. These immunoglobulins did not produce any observable change in the development of neural tube (data not shown).

Explant cultures

Intermediate neural tube explants of 300×200 µm were dissected from stage 9 and stage 12 embryos and cultured for either 6 hours, 12 hours, or 3 days in N2 medium (Bottenstein and Sato, 1979) in a collagen gel matrix as described by Martí et al. (1995a). In motor neuron differentiation experiments, explants were labelled with the anti-Islet-1/2 mAb, then incubated with a Cy3-conjugated anti-mouse antibody (Amersham). Labelled samples were subsequently observed by confocal microscopy, and the Islet-1/2-positive cells were counted. For vitronectin mRNA expression experiments, total RNA was prepared from single explants using the method described by Chomczynski and Sacchi (1987). First strand cDNA was synthesized with MoMuLV reverse transcriptase (BRL) following standard protocols (Sambrook et al., 1989).

Vitronectin, HNF-3β and Islet-1 mRNA expression in Shh-induced neural tube explants

First strand cDNA was used as template for PCR with two primers derived from nucleotides 233–252 and complementary to nucleotides

547-567 of the vitronectin cDNA sequence. 0.1 mCi [³²P]dCTP (3000 Ci/mmol; Amersham) was added to each amplification mix which were then subjected to 30 PCR cycles for amplification of vitronectin cDNA. PCR products were analyzed by electrophoresis in 6% polyacrylamide gels, which were then dried and exposed to X-ray films. Parallel experiments were performed to amplify HNF-3β and Islet-1 cDNAs. For the former, primers derived from nucleotides 1053-1073 and complementary to nucleotides 1192-1216 were used (Ruiz i Altaba et al., 1995). For Islet-1 cDNA amplification, primers were derived from nucleotides 381-400 and complementary to nucleotides 751-770 (Tsuchida et al., 1994). As a control, GAP-DH was also amplified from each cDNA sample. Primers were derived from nucleotides 680-699 and complementary to nucleotides 990-1009 (Dugaiczky et al. 1983). PCR amplification was shown to be dependent on reverse transcription of RNA templates and on the presence of both forward and reverse primers in the PCR reaction. The identity of the PCR products was confirmed by restriction enzyme digestion.

RESULTS

Cloning and structure of chick vitronectin

To clone chick vitronectin, two degenerate primers were first designed based on those regions of maximum homology among mammalian vitronectin sequences. The fragment amplified with these primers from reverse-transcribed chick liver mRNA was used in turn to screen an embryonic retinal cDNA library (see Methods). The putative peptide sequence and its alignment with human and rabbit vitronectin are shown in Fig. 1. Chick vitronectin is slightly shorter than its mammalian homologues (453 versus 475-478 amino acids). Overall amino acid identity between chick and mammalian vitronectins range from 53% for the mouse to 55.6% for the rabbit. However, homology increases significantly around the known functional domains, such as the RGD domain involved in the binding to integrin receptors, and the basic region that binds to heparin, although this domain is much shorter in chick vitronectin. A specific feature of chick vitronectin peptide is the stretch of eight threonine residues at position 130-137. This string is located within a region of minimum homology among vitronectin sequences. Other proteins that also contain such an oligothreonine motif are the salivary protein SGS-3 of *Drosophila* (Garfinkel et al., 1983), chick collagen XIV (Trueb and Trueb, 1992) and different types of mucins (Hauser and Hoffmann, 1992).

Vitronectin mRNA expression and immunoreactivity in embryonic neural tube

Highest levels of vitronectin mRNA expression were detected in the notochord of stage 14 embryos (Fig. 2A). Lower levels were also observed in the floor plate and in the ventral part of the neural tube neuroepithelium. As development proceeds (stage 17; Fig. 2B), vit-

ronectin mRNA expression persists, although apparently at lower levels in the notochord, the floor plate and the ventral region of the neural tube. Later in development (stage 27; Fig. 2C), vitronectin mRNA was barely detected in the notochord (not shown), whereas high levels were seen in both the floor plate and ventral third of the germinal paraventricular zone of the neural tube. Consistent with in situ hybridisation data, immunohistochemical analysis revealed strong vitronectin immunoreactivity in the notochord, floor plate and the paraventricular zone of the ventral region of stage 16 neural tube (Fig. 3A). As development proceeds (stage 27), vitronectin immunoreactivity decreased in the notochord and floor plate, while spreading in the ventral neural tube (Fig. 3B).

Effects of vitronectin and vitronectin neutralisation on motor neuron generation in cultured neural tube explants

Since the expression pattern of vitronectin is consistent with a role in the differentiation of motor neurons, we first investigated the influence of vitronectin on motor neuron generation in cultured neural tube explants. As previously reported (Martí et al., 1995a), both co-culture of stage 9 neural tube and notochord (Fig.4A), and addition of Shh to stage 9 neural tube explants (Fig. 4D), leads to generation of Islet-1/2 positive motor neurons. In the absence of notochord or added Shh, very

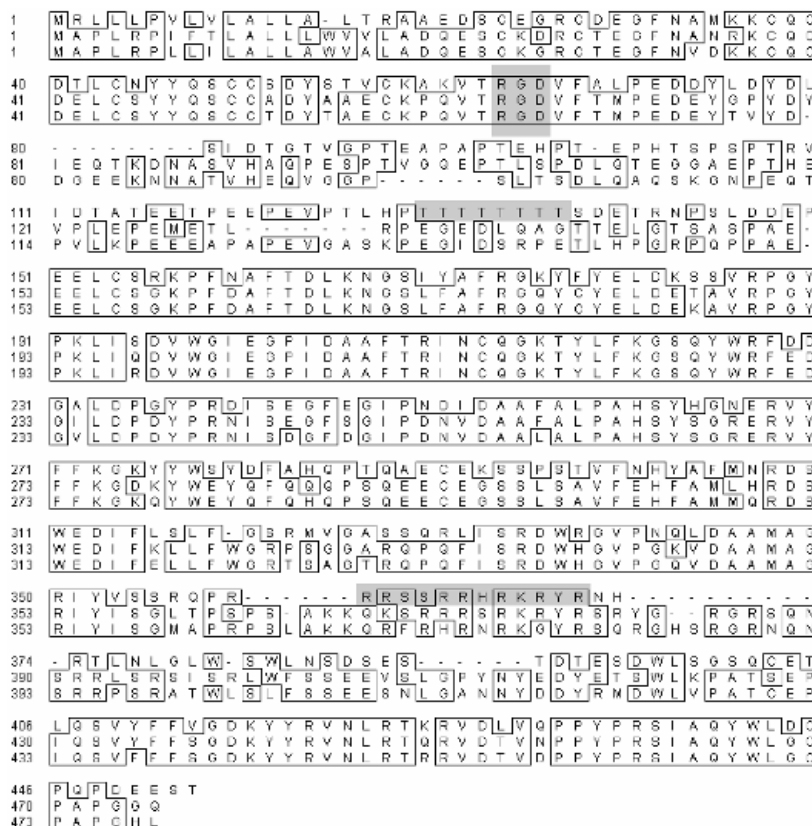


Fig. 1. Amino acid alignment of chick, rabbit and human vitronectins (first, second and third row, respectively). Shaded areas correspond to the RGD integrin binding motif, the chick-specific oligo-threonine string and the chick basic heparin-binding domains.

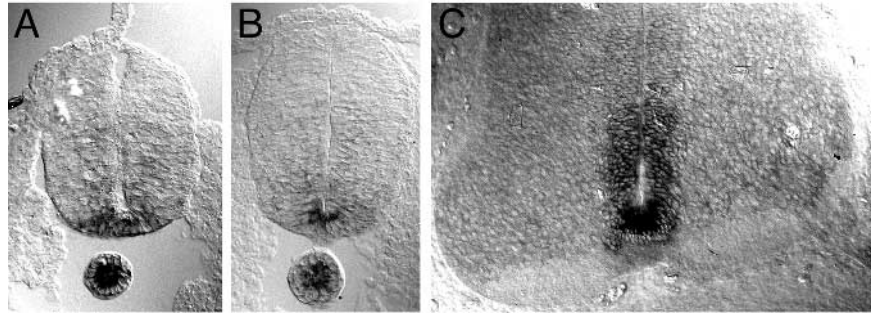


Fig. 2. Expression of vitronectin mRNA in brachial neural tube sections from stage 14 (A), stage 17 (B) and stage 27 (C) chick embryos, detected by in situ hybridisation.

few motor neuron appeared during culture (Fig. 4C). However, the motor neuron-inducing activity both of the notochord and of Shh was greatly inhibited by the anti-vitronectin blocking antibody (Fig. 4B,E). The role of vitronectin in motor neuron differentiation was then directly assessed by application of vitronectin to neural tube explants. In the absence of either notochord or added Shh, exogenous vitronectin promoted motor neuron generation in stage 9 explants (Fig. 4F), and this increase was not inhibited by anti-Shh blocking antibodies (Fig. 4G). Furthermore, in cultures of neural tube explants from stage 13 embryos, where motor neurons differentiate in the absence of either notochord or exogenous Shh (Fig 4H; Ericson et al., 1996), the presence of anti-vitronectin antibody also inhibited the appearance of motor neurons to a large extent (Fig. 4I). The addition of identical amounts of rabbit preimmune immunoglobulins to the cultures did not affect the generation of motor neurons, neither did the addition of fibronectin to neural tube explants result in the appearance of Islet-1/2-positive cells (data not shown).

Anti-vitronectin treatment of chick embryos

To demonstrate the role of vitronectin in vivo, affinity-purified anti-vitronectin antibodies were applied to the chorioallantoic membrane of chick embryos at stage 10 following the protocol described in Materials and Methods. The application of anti-vitronectin antibodies to the embryos was followed by a reduction in motor neuron number at stage 16, as seen by staining with the BEN mAb, specific for floor plate cells and motor neurons in the neural tube (Tanaka and Obata, 1984; Yamada et al., 1993) (Fig. 5A,B). These data indicate that vitronectin neutralisation impaired the generation of motor neurons, although BEN staining had no apparent effect on

either the floor plate or the notochord (Fig. 5A,B). Reduction in motor neuron number was also detected with the anti-Islet-1/2 antibody that stains late migrant and terminally migrated motor neurons in the ventral horns of the neural tube (Thor et al., 1991) (Fig. 5C,D). When examined, at stage 27, the number of Islet 1/2-positive motor neurons in the ventral horns remained low in embryos that had been treated with anti-vitronectin antibody at stage 10 (Fig 5E,F).

To determine the specificity of the effects induced by vitronectin deprivation, we studied the effects of vitronectin neutralisation on other areas of the neural tube. To this end, three dorsoventral markers were used. First, immunoreactivity of Shh, which is specifically expressed in the floor plate (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994), was not affected by the anti-vitronectin treatment (Fig. 6A,B). Second, the distribution of immunoreactivity of Pax 6, the mRNA of which is expressed in the medio-lateral region of the neural tube after closure (Goulding et al., 1993), was not affected by neutralisation of endogenous vitronectin (Fig. 6C,D). And third, there was no change in either Pax 3 mRNA, a gene selectively expressed in the dorsal half of the neural tube, which is restricted by signals from the notochord, floor plate (Goulding et al., 1993) or Shh (Liem et al., 1995) (Fig. 6E,F).

These data indicate that the consequences of vitronectin neutralisation are restricted to motor neuron generation, and possibly to other cells in the ventral neural tube, but do not appear to involve the general dorso-ventral patterning of the neural tube, which might secondarily lead to motor neuron differentiation.

Effect of Shh on the expression of vitronectin mRNA

The results described previously suggest that vitronectin could be a downstream element in the signalling cascade of Shh leading to motor neuron differentiation. To test whether vitronectin expression is under the control of Shh, stage 9 neural tubes devoid of floor plate were cultured in the absence or in the presence of various concentrations of Shh for either 6 or 12 h. As shown in Fig. 7A, vitronectin mRNA expression could not be detected by PCR amplification in the freshly dissected tissue nor was it found after 6 or 12 hours in culture in the absence of Shh. In contrast, when neural tube explants were cultured in the presence of Shh, vitronectin mRNA-derived PCR product was detected after 12 hours in a Shh concentration-dependent manner. The fact that no signal was detected up to 6 hours incubation suggests that intermediate steps exist between Shh induction and vitronectin mRNA expression. However, as shown in Fig. 7B, Shh induces vitronectin mRNA expression before that of HNF-3 β , a gene

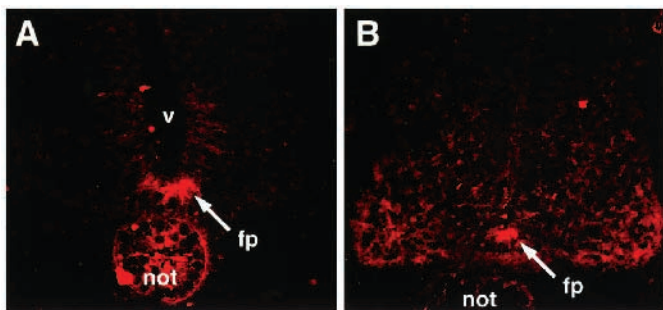


Fig. 3. Confocal micrographs of stage 16 (A) and stage 27 (B) neural tube sections (brachial level), showing vitronectin immunoreactivity (v, ventricle; not, notochord; fp, floor plate).

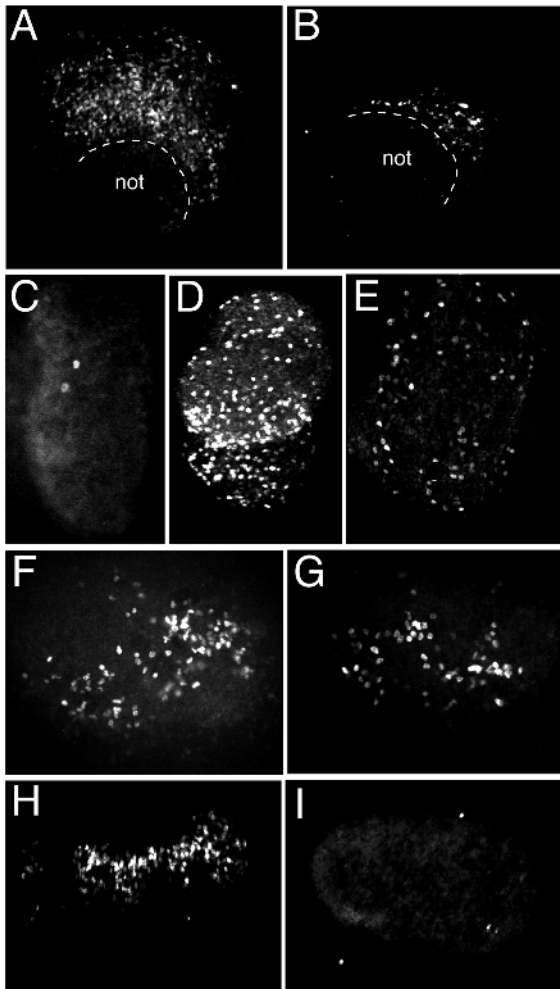


Fig. 4. Effects of vitronectin and anti-vitronectin antibodies on the differentiation of motor neurons in cultured neural tube explants. (A,B) Stage 9 neural tube/notochord co-explants grown in the absence (A) or in the presence (B) of anti-vitronectin antibodies (0.1 $\mu\text{g/ml}$). (C) Lack of motor neuron induction in a control stage 9 explant. (D,E) Stage 9 neural tube explants grown in the presence of N-Shh (1 $\mu\text{g/ml}$) and in the absence (D) or in the presence (E) of anti-vitronectin antibodies, which reduced the number of Islet-1/2-positive cells by $91\pm 2\%$ ($\pm\text{s.d.}$, $n=3$). (F,G) Stage 9 neural tube explants grown in the presence of vitronectin (10 $\mu\text{g/ml}$), and without (F) and with (G) anti-Shh blocking antibodies; vitronectin increased the number of Islet-1/2-positive cells from 2 ± 4 ($\pm\text{s.d.}$, $n=4$) to 74 ± 18 ($\pm\text{s.d.}$, $n=4$) and its activity was not reduced by application of anti-Shh antibodies (72 ± 17 ; $\pm\text{s.d.}$, $n=4$). (H,I) Stage 13 neural tube explants grown in the absence (H) or in the presence (I) of anti-vitronectin antibodies (0.1 mg/ml), which reduced the number of Islet-1/2-positive cells by $96\pm 4\%$ ($\pm\text{s.d.}$, $n=3$). The incubation time for the cultures was 3 days. The data show representative experiments. Each experiment was performed at least four times.

regulating the differentiation of the floor plate (Ruiz i Altaba and Jessell, 1992) and before that of Islet-1, necessary for motor neuron differentiation (Pfaff et al., 1996). These results show that Shh induces the expression of vitronectin mRNA and, combined with previous results suggest that the motor neuron inducing activity of Shh is at least partially modulated by vitronectin.

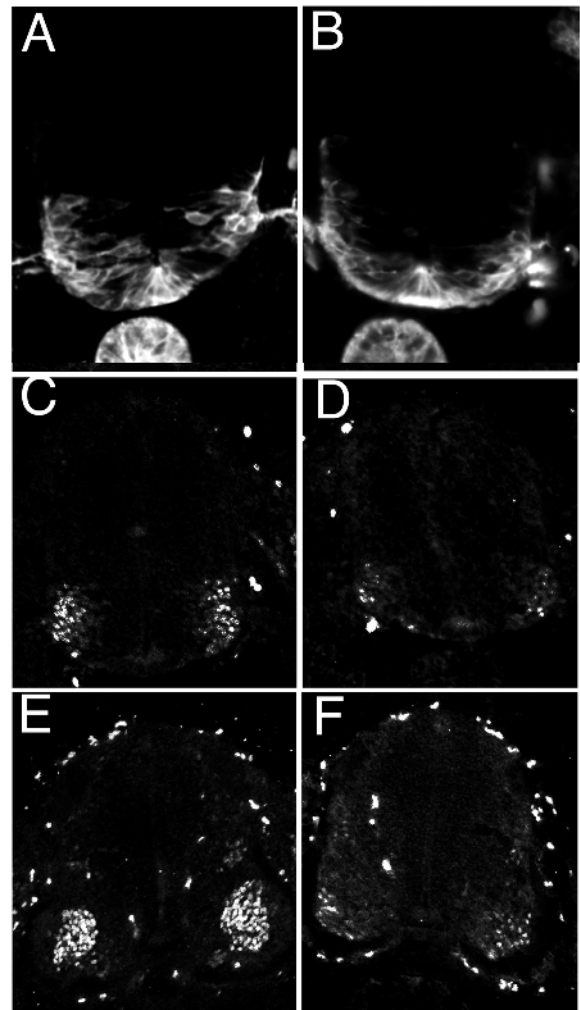


Fig. 5. Effects of vitronectin neutralisation on motor neuron differentiation in chick embryos. Sections of neural tube from the brachial region of stage 18 control (A,C) and anti-vitronectin treated embryos (B,D), and from stage 27 control (E) and anti-vitronectin treated embryos (F) were immunostained with BEN mAb, which stains floor plate and motor neurons (A,B), and anti-Islet-1/2 mAb, which labels motor neurons in the ventral horn (C-F). Observe the decrease in motor neurons demonstrated by both BEN and anti-Islet-1/2 mAbs in the treated embryos.

DISCUSSION

This study shows that vitronectin expression in the ventral neural tube is induced by Shh at the time of motor neuron differentiation and also that vitronectin promotes that differentiation. Thus, our results indicate that vitronectin could be either a mediator of motor neuron differentiation operating down-stream of Shh, or alternatively, vitronectin may cooperate synergistically with the Shh inducer to promote the differentiation of these neurons.

Vitronectin expression in the neural tube

Transient expression of vitronectin mRNA has been shown previously in the notochord and floor plate of the mouse embryo (Seiffert et al., 1995). Here, we show that vitronectin mRNA

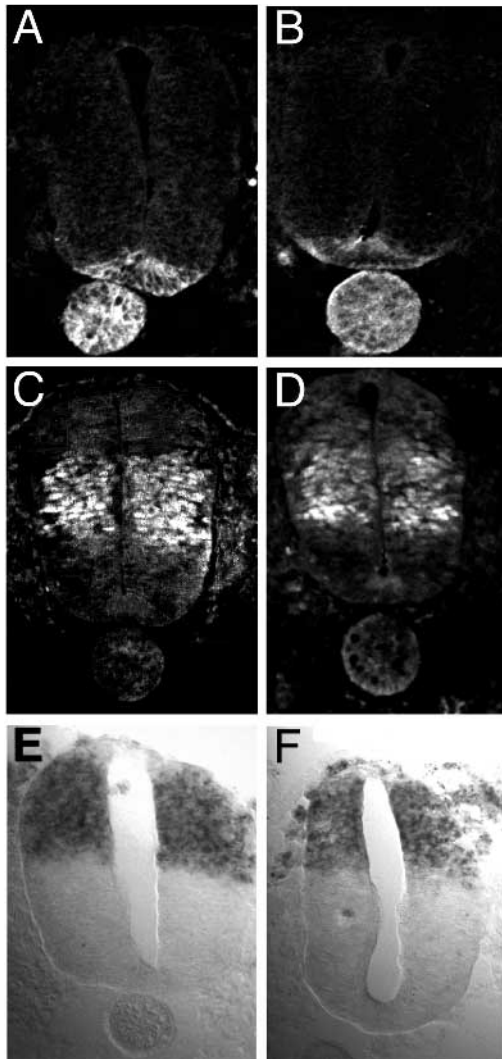


Fig. 6. Micrographs of brachial neural tube of control (A,C,E) and anti-vitronectin treated (B,D,F) stage 16 embryos. (A,B) Immunoreactivity of Sonic hedgehog. (C,D) Immunoreactivity of Pax 6. (E,F) In situ hybridisation pattern of Pax 3 mRNA.

is also expressed in the ventral neural tube of the chick, albeit at lower levels than in notochord and floor plate. Although vitronectin mRNA expression becomes ubiquitous as development proceeds (results not shown), early in development (stage 14-18) its expression is restricted to the ventral regions of the neural tube at the time and position of motor neuron differentiation. Highest vitronectin mRNA levels were first seen in the notochord and floor plate of stage 14 chick embryos, and decreased in the notochord as development proceeds. Notochord-derived vitronectin is likely deposited in the perinotochordal sheath, where vitronectin immunoreactivity remains high until late in development. Expression of vitronectin mRNA persists in the floor plate until later in development and immunohistochemical analysis revealed vitronectin surrounding all floor plate cells (Fig.3). Together, these data are consistent with a role for vitronectin in the development of notochord and floor plate.

The expression pattern of vitronectin mRNA in the

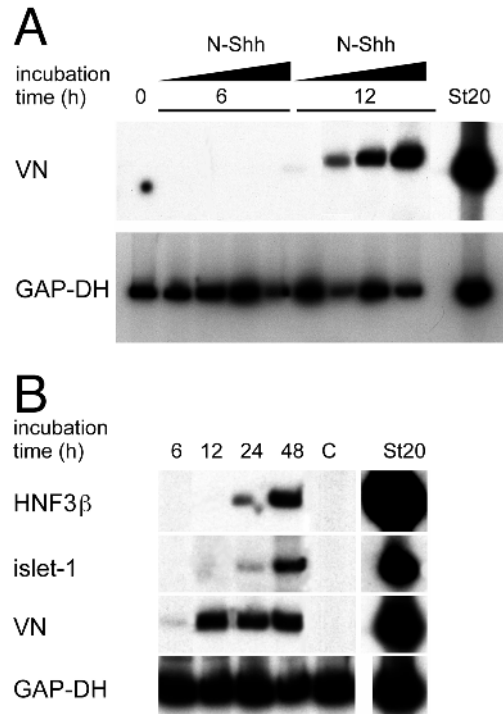


Fig. 7. (A) Shh induces vitronectin mRNA expression in neural tube explants. Intermediate neural tube explants were dissected from stage 9 chick embryos and cultured in the absence of Shh or in the presence of increasing concentrations of Shh (10, 50 and 100 ng/ml). RT-PCR was performed either after 6 hours in culture or after 12 hours. RT-PCR was also done using neural explants at the time of dissection of stage 9 neural tubes (0) and in stage 20 whole spinal cords. Similar results were obtained in three independent experiments. (B) Shh-induced expression of HNF-3 β , Islet-1 and vitronectin mRNA. Expression of vitronectin mRNA is faster than that of HNF-3 β , suggesting that vitronectin-mediated motor neuron induction is independent of floor plate differentiation. GAP-DH was also amplified as described in the Materials and Methods.

notochord and floor plate of the embryonic neural tube is similar to that of genes with ventralising activity, including the Shh and the HNF families (Martí et al., 1995b; Ruiz i Altaba et al., 1995). However, despite this similarity, we found no indication that vitronectin is involved in dorsoventral patterning because treatment of the embryos with anti-vitronectin antibodies did not result in an alteration of Pax 3 mRNA expression pattern, whereas both ectopic notochord implants and ectopic application of Shh downregulate and restrict the regional expression of Pax 3 (Goulding et al., 1993; Liem et al., 1995). Furthermore, anti-vitronectin treatment did not alter the immunoreactivity pattern of Shh in the floor plate, nor that of Pax 6 in the mid-lateral region of the neural tube. Thus, any effects of vitronectin on motor neuron generation appear to be specific and not a consequence of a general ventralising activity. However, the possibility that vitronectin influences the development of other ventrally located neurons along the neural tube cannot be excluded.

Vitronectin promotes motor neuron generation

A large number of studies has shown that Shh promotes the differentiation of both floor plate cells and motor neurons

(reviewed by Placzek, 1995). However, such observations do not indicate unambiguously whether motor neuron differentiation is induced by a direct interaction of Shh with progenitor cells, or if it results from an indirect cascade of inductions triggered by Shh (discussed in Johnson and Tabin, 1995). The latter possibility is supported by the observation that antibodies against the amino terminal part of Shh, which displays motor neuron inducing-activity did not detect Shh outside the notochord and the floor plate (Martí et al., 1995b). This is in contrast to the localisation of vitronectin which is also found outside the floor plate in the ventral neural tube. Since many motor neurons are not born in close contact with the floor plate (Ramón y Cajal, 1899; Altmann and Bayer, 1984) then the induction of motor neurons may be mediated by inducers downstream of Shh. However, it should be noted that a recent study has shown that Shh mRNA, although undetectable in stage 12 neural tube, did appear after 24 hours culture of these explants (Ericson et al., 1996). The possibility therefore exists that at least in culture, Shh may be in direct contact with motor neuron precursor cells. Moreover, since anti-Shh blocking antibodies impaired the appearance of motor neurons in stage 12 neural tube explant cultures devoid of floor plate, Ericson et al. (1996) concluded that motor neuron differentiation is induced by the direct interaction of Shh with motor neuron progenitors. Our results do not rule out this possibility, but rather they point to a role of vitronectin in the stimulation of motor neuron differentiation in response to Shh.

The neutralisation of vitronectin blocked the appearance of most, but not all, Islet-1/2-positive motor neuron induced by either Shh or notochord in stage 9 neural tube explants. Moreover, in stage 13 explant cultures, when neither exogenous Shh nor notochord are needed for motor neuron generation (Ericson et al., 1996), the blockade of endogenous vitronectin also impaired the appearance of most motor neurons. These observations, coupled with the ability of vitronectin to induce motor neurons in the absence of both notochord and added Shh and in the presence of anti-Shh blocking antibodies, point to vitronectin as an important element for the generation of these neurons. However, in the experiments presented here, neither the anti-vitronectin antibody, nor the anti-Shh antibody totally inhibited motor neuron appearance. Therefore, the data are also consistent with the possibility that vitronectin acts with (or synergistically with) Shh to potentiate motor neuron production. Thus, vitronectin may act by increasing the generation rate of Shh-induced motor neurons rather than having phenotype specification properties itself.

Because vitronectin neutralisation did not change the expression of Pax 3, Pax 6 and Shh, we may conclude both that vitronectin does not influence Shh expression and that Shh evokes at least some of its effects in ventralisation of the neural tube by a pathway independent of vitronectin. In this respect, Tanabe et al. (1995) and Ericson et al. (1996) have shown that the pathway of motor neuron differentiation is independent of floor plate induction. Our data support this idea by showing that vitronectin mRNA is induced by Shh faster than that of HNF-3 β , thus suggesting that vitronectin intervenes in motor neuron differentiation independently of floor plate differentiation.

Functional relation between vitronectin and Shh

Our results might be explained if vitronectin acts either as a carrier or an activator of Shh. In this respect it is known that the amino-terminal fragment of Shh binds to extracellular matrix, most probably via its heparin binding domain (Bumcrot et al., 1995). Furthermore, vitronectin has the capacity to bind and stabilise a number of proteins including heparan sulfate proteoglycans, the thrombin-antithrombin complex, plasminogen activator inhibitor 1 and other ECM components (reviewed by Preissner, 1991). Whatever the functional interactions of Shh and vitronectin, the most obvious novel function of Shh demonstrated here is its ability to induce the expression of vitronectin mRNA. This is not normally expressed in the neural tube of stage 9 embryos nor is it expressed in isolated explants. However, addition of Shh resulted in a concentration-dependent increase of vitronectin mRNA levels. The need for 12 hours in order to detect the appearance of vitronectin mRNA suggests that a number of events take place between Shh stimulation and induction of vitronectin mRNA expression. Thus Shh might also regulate the activity or expression of other factors in addition to vitronectin that influence motor neuron differentiation.

Interactions between Shh, molecules of the ECM and their receptors remain unexplored in vertebrates. However, in *Drosophila*, epithelial morphogenesis during proventriculus organ development requires the activities of *hh*, *wg*, *dpp* and position specific (PS) integrins (Pankratz and Hoch, 1995). According to these authors, *hh* and/or *wg* might modulate the activities of either PS integrins or various unidentified cytosolic components that interact with integrins. Although vitronectin has not been cloned in insects, it should be noted that vertebrate vitronectin proved to be the most effective ligand for *Drosophila* PS integrins in vitro (Hirano et al., 1991; Bunch and Brower, 1992; Gullberg et al., 1994). Further work is clearly needed to elucidate the genetic and molecular mechanisms linking vitronectin and Shh.

Vitronectin and extracellular matrix in neuron differentiation

The nature of the vitronectin receptor mediating vitronectin activity on motor neuron generation remains unknown. However, the integrin (α subunit, which forms part of vitronectin receptors, is expressed in the early CNS (Hirsch et al., 1994) and both its mRNA and immunoreactivity are evenly distributed along the dorsoventral axis of most neuroepithelial cells of the neural tube without any preferential localisation (results not shown). Therefore, the selectivity of vitronectin resides in its restricted expression pattern rather than in that of its receptor.

Knockout of the murine vitronectin gene has been reported recently but the mutant mice displayed an apparently normal phenotype (Zheng et al., 1995). Although results of the vitronectin knockout may indicate that the vitronectin expressed in the developing neural tube has no apparent function (see Erickson, 1993) our experiments show that this ECM protein is not only expressed at the time and place of motor neuron generation, but also affects that generation in vitro and in vivo. Thus, to reconcile the apparent lack of effect of the vitronectin knockout in mice and the results reported here, we hypothesize that vitronectin in the mutant mice is functionally redundant,

its role being taken over by other ECM components. In the case of vitronectin, redundancy is particularly likely because unlike ECM proteins such as fibronectin and laminin, vitronectin does not have exclusive receptors. Indeed, all known tissue receptors for vitronectin also bind other ECM components (Felding-Habermann and Cheresch, 1993). These may therefore substitute for vitronectin in its absence, when the general pattern of gene expression is established early in development. It is noteworthy that the deletion of the gene for another ECM protein, tenascin-C, is not followed by any apparent mutant phenotype (Saga et al., 1992). The receptors for this ECM component are also promiscuous and may bind other ECM ligands including tenascin-C-related proteins (Chiquet-Ehrismann et al., 1994). Thus helping to explain the lack of a mutant phenotype. For discussions of the interpretations of protein function after targeted deletion of their genes, see Erickson (1993) and Crossin (1994).

We have shown previously that vitronectin can promote neuronal differentiation of retinal neurons in vitro (Martínez-Morales et al., 1995). Furthermore, we have recently demonstrated the expression of vitronectin in the embryonic chick neural retina and experiments indicate that vitronectin specifically promotes the appearance of retinal ganglion cells (Martínez-Morales et al., unpublished). It is therefore likely that the position-specific expression of vitronectin in the embryonic nervous system has an important role in the differentiation of long-projection neurons in addition to motor neurons as demonstrated here.

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