The Integrin Receptor α₈β₁ Mediates Interactions of Embryonic Chick Motor and Sensory Neurons with Tenascin-C

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Summary

This paper identifies a neuronal receptor for tenascin-C (tenasin/cytotactin), an extracellular matrix protein that has previously been detected in developing sensory and motor neuron pathways and has been shown to regulate cell migration in the developing CNS. Antibodies specific for each subunit of the integrin α₈β₁ are used to demonstrate that α₈β₁ mediates neurite outgrowth of embryonic sensory and motor neurons on this extracellular matrix protein. In addition, expression of α₈ in K562 cells results in surface expression of α₈β₁, heterodimers that are shown to promote attachment of this cell line to tenascin. The major domain in tenasin that mediates neurite outgrowth is shown to be localized to fibronectin type III repeats 6–8.

Introduction

Interactions between neurons and cell- and extracellular matrix (ECM)–associated proteins are essential for regulating development of the nervous system (reviewed in Goodman and Shatz, 1993). Tenasin-C (tenacin/cytotactin) is a member of a small family of ECM-associated glycoproteins that have been proposed to have many distinct functions in neural development (reviewed in Reichardt and Tomaseelli, 1991; Riou et al., 1992). In cell culture, substrate-bound tenasin has been observed to inhibit migration of neural crest cells and axon growth by hippocampal, cerebellar, and mesencephalic neurons (Tan et al., 1987; Halfter et al., 1989; Faisstner and Kruse, 1990). In vivo, its distribution in regions of the central nervous system, such as barrel fields in the somatosensory cortex, is consistent with the possibility that it delineates barriers that restrict axon growth (e.g., Steinlieder et al., 1989; Bartsch et al., 1992). In the peripheral nervous system, tenasin is found in migratory pathways of neural crest cells and along routes followed by motor and sensory neurons, suggesting it might promote and direct neural crest migration and axon outgrowth (e.g., Wehrle and Chiquet, 1990; Riou et al., 1992). Consistent with this, substrate-bound tenasin promotes neurite outgrowth by sensory and motor neurons in vitro (e.g., Wehrle and Chiquet, 1990; Wehrle-Haller and Chiquet, 1993).

The diverse responses of neurons to tenasin seem likely to be mediated by different receptors interacting with separate domains in the tenasin molecule. Tenasin is a hexamere whose individual subunits consist of linear arrays of several epidermal growth factor (EGF)–like repeats, 8–14 fibronectin type III (FNIII) domains, and a C-terminal fibrinogen-like domain (see Figure 3A). Differential splicing of exons encoding FNIII domains generates several isoforms of tenasin. Previous work indicated that several domains of tenasin interact with cells. The third FNIII domain in chicken tenasin contains an RGD sequence, similar to cell binding sites in fibronectin and vitronectin. The RGD-containing domain in tenasin has been shown to mediate attachment by at least some nonneural cells (Prieto et al., 1992). Both monoclonal and polyclonal antibodies to FNIII domains 6–7 strongly inhibit neurite outgrowth, implying that neurons recognize this portion of the molecule (Husmann et al., 1992; Wehrle-Haller and Chiquet, 1993). Other domains of tenasin have also been shown to promote cell attachment (e.g., Husmann et al., 1992; Prieto et al., 1992). Inhibitory or “antiadhesive” domains of tenasin have been localized tentatively to the EGF repeat region and to alternatively spliced FNIII domains (e.g., Spring et al., 1989).

Several proteins, including an immunoglobulin-class cell adhesion molecule named F11, two proteoglycans named syndecan and cytactin-binding proteoglycan, and integrins, have been proposed to function as receptors for tenasin (Zisch et al., 1992; Salmivirta et al., 1991; Hoffman et al., 1988; Bourdon and Ruoslahti, 1989; Sriramamo et al., 1993; Prieto et al., 1993). Both α₈ and β₁ subunit–containing integrins have been implicated as receptors. Inhibitory effects of integrin-specific antibodies implicate α₈β₁ and α₈β₁ as receptors for endothelial cells (Sriramarao et al., 1993). The astrocytoma U-251 MG cell line has been shown to attach to the third, RGD-containing fibronectin repeat of tenasin via the integrin α₈β₁ (Prieto et al., 1993). α₈ associated with another β₁ subunit may also function on other cells as a tenasin receptor (Prieto et al., 1993).

β₁ integrins also appear to be important in mediating cell attachment to tenasin domains (Prieto et al., 1993). In addition, β₁ integrins have recently been implicated as neuronal receptors for tenasin (Wehrle-Haller and Chiquet, 1993). Neurite outgrowth by sensory neurons is strongly inhibited by a monoclonal antibody (MAB), JG22, to the integrin β₁ subunit.

Bossy et al. (1991) isolated cDNAs encoding the integrin α₂ subunit by virtue of homology with other integrin α subunits. These investigators observed strong expression of the integrin α₂ subunit by subclasses of neurons in both...
the peripheral and central nervous systems, including motor and sensory neurons. Similarities in its distribution with that of tenascin in peripheral tissues has suggested that tenascin is a potential ligand. In addition, the α₁ subunit protein was found to associate with β₁ in fibroblasts.

In the present paper, we examine the role of the integrin α₁β₁, in mediating neuronal interactions with tenascin. We show that neurite outgrowth by motor or sensory neurons requires interactions with the C-terminal FNIII repeats of tenascin, consistent with previous observations using sensory neurons (e.g., Wehrle-Haller and Chiquet, 1993). We demonstrate that α₁ associates with β₁ on sensory neurons. We generate specific, inhibitory antibodies to α₁ and use these to show that antibodies specific for either α₁ or β₁ inhibit neurite outgrowth responses of sensory and motor neurons on tenascin but not on control substrates. The results indicate that this integrin is a tenascin receptor and suggest that it mediates neurite outgrowth by many neurons on this ECM constituent.

Results

Sensory and Motor Neuron Outgrowth on Tenascin

Dissociated neurons from dorsal root ganglia (DRGs) or ventral spinal cord were cultured on nitrocellulose-based substrates coated with tenascin, laminin, or N-cadherin (Figure 1; Figure 2). For sensory neuron cultures, E7–E8 DRGs were dissociated, plated at low density, and cultured for 4–8 hr. On a tenascin substrate, about 20%–40% of the neurons extended processes longer than two cell diameters (Figure 1A). On laminin (Figure 1B) or N-cadherin (data not shown), a larger percentage of the cells (~40%–60%) extended processes. In control wells coated only with bovine serum albumin (BSA), all neurons appeared round, and none were able to extend neurites (data not shown). Therefore, in these experiments all neurite outgrowth depended upon the presence of an adhesion molecule or ECM molecule.

For motor neuron cultures, ventral neural tubes from stage 19 embryos were dissociated and plated on either tenascin- or N-cadherin-coated substrates. After 24 hr on tenascin, ~10%–20% of the cells extended neurites longer than two cell diameters, with some extending much longer neurites (Figure 2A). When plated on N-cadherin, more cells (~30%–40%) extended neurites longer than two cell diameters (Figure 2G). On both tenascin and N-cadherin, the remainder of the cells in the culture remained round and appeared detached from the substrate. Most of the neurons extending processes longer than two cell diameters were expected to be motor neurons on both substrates, because few other neurons are present in the stage 19 ventral spinal cord. To confirm this, cells plated on tenascin were stained with DM1; this MAb recognizes a 95 kDa cell surface protein referred to as DM-GRASP or SC-1 (Burns et al., 1991; Tanaka and Obata, 1984), which is present on motor neurons and floorplate cells of the ventral neural tube at stage 19 in chick development (Tanaka and Obata, 1984). In our cultures, 95%–99% of the neurons extending neurites greater than two cell diameters expressed the DM1 epitope (data not shown).

To characterize further the domain(s) in tenascin mediating neurite outgrowth by sensory and motor neuron outgrowth, effects of antibodies to whole tenascin or to a C-terminal fragment were examined (Figure 3A). Anti-tenascin, AS142, specific for intact tenascin, inhibited >90% of neurite outgrowth by sensory or motor neurons on tenascin.
Integrin αβ1 Is a Tenascin Receptor

Figure 2. Effects of Integrin- and Tenascin-Specific Antibodies on Motor Neuron Cultures Plated on Substrate-Bound Tenascin and N-Cadherin

Neural tubes were dissected from stage 19 chick embryos, dissociated, and plated on substrate-bound tenascin (A-F) or N-cadherin (G-L). The following antibodies were added at the specified concentration to the culture medium: anti-α, MAb CHAV-1 (50 μg/ml; A and G), anti-β, MAb W1B10 (50 μg/ml; B and H), anti-α-EX (300 μg/ml; C and I), anti-α-EX (300 μg/ml; D and J), anti-tenascin AS142 (1:100; E and K), and anti-tenascin AS474 (1:100; F and L). Cultures were incubated for 24 hr, fixed, viewed with phase optics, and photographed. Bar, 30 μm.

Characterization of Antibodies to Integrin Receptor Subunits

To determine whether individual integrin heterodimers function as receptors for tenascin, several integrin subunit-specific antibodies were tested for inhibitory activities. The chick β1 integrin-specific MAb, W1B10, has previously been shown to inhibit cellular interactions with laminin, collagen, and fibronectin (Hayashi et al., 1990). The anti-α, specific MAb, CHAV-1, has been shown to inhibit interactions of α subunit-containing integrins (Neugebauer et al., 1991). Two additional sera to gel-purified fusion proteins containing large portions of the extracellular domains of the chick integrin α or α subunits were also prepared and tested (see Experimental Procedures). Anti-α-EX has been shown to inhibit interactions between ciliary neurons and laminin-1, to immunoprecipitate the integrin α subunit from chicken retinal lysates, and to blot this subunit (Weaver et al., submitted; and data not shown).

To characterize anti-α-EX serum, anti-α-EX and anti-α-cyto IgG (specific for the α cytoplasmic tail [Bossy et al., 1991]) were used to immunoprecipitate surface-labeled (Figures 3B and 3C; Figure 2E). AS474, specific for a pronase fragment that includes the last three FNIII repeats, inhibited about 80% of neurite outgrowth by sensory neurons and 90% by motor neurons on tenascin (Figure 3C; Figure 2F). Tn68, a MAb to an epitope in FNIII repeat 7, also blocked about 80% of sensory neuron outgrowth (Figure 3B; Figure 1E). Inhibitory effects were substrate specific, since neurite outgrowth by sensory neurons on laminin and by motor neurons on N-cadherin appeared to be normal in the presence of each antibody tested (Figures 3B and 3C; Figure 1F; Figures 2K and 2L). In conclusion, tenascin-stimulated neurite outgrowth by either sensory or motor neurons requires interactions with a domain in the C-terminal region of substrate-bound tenascin. For sensory neurons, the inhibitory effect of MAb Tn68 makes it probable that this activity requires interaction with a domain in or near FNIII repeat 7 (Figure 3A). These results are consistent with a previous report in which it was shown that sensory neurons respond to a domain in the last three FNIII repeats of tenascin (Wehrle-Haller and Chiquet, 1993).
chick embryo fibroblast extracts. Autoradiograms of immunoprecipitates fractionated by SDS-PAGE in nonreducing conditions are presented in Figure 4. Two bands of Mr 110 and 160 kDa were observed in anti-α8-cyto immunoprecipitates (Figure 4A, lane 2). Bossy et al. (1991) have shown with immunodepletion experiments that the upper and lower bands correspond to the integrin α8 and β1 subunits, respectively. In anti-α8-EX immunoprecipitates, two similar bands of Mr 110 and 160 kDa were seen (Figure 4A, lane 1). Together, these results indicate that anti-α8-EX specifically immunoprecipitates the integrin α8 subunit and its associated β1 subunit.

Expression of Integrin α8 Heterodimers by Sensory and Motor Neurons

In previous experiments, Bossy et al. (1991) detected the integrin α8 subunit on spinal sensory and motor neurons and along the dorsal and ventral roots of E6 chicks. Staining with anti-α8-EX also detects the α8 subunit expression in these areas (data not shown). To test whether DRG sensory neurons expressed the integrin α8 subunit in culture and to identify associated β subunits, extracts from cultured DRG neurons were immunoprecipitated with anti-α8-cyto. Two bands of Mr 110 and 160 kDa were observed (Figure 4B). Therefore, anti-α8-cyto immunoprecipitated proteins with the same apparent Mr from both DRG and fibroblast lysates (compare Figure 4B to Figure 4A, lane 2), making it likely that the upper and lower bands correspond to the integrin α8 and β1 subunits, respectively. To verify that the 110 kDa species was the β1 subunit, unlabeled DRG lysates were immunoprecipitated with anti-α8-cyto, electrophoretically transferred to nitrocellulose, and blotted with the β1-specific MAb W1810. β1 antigen was observed at a Mr of 110 kDa, indicating that the α8 subunit associates with the β1 subunit in chick DRG.

To identify additional integrin receptors expressed by DRG neurons or motor neurons cultured on tenascin, cul-

Figure 4. Nonreducing SDS-PAGE of 125I–Surface Labeled Proteins

(A) Chick embryo fibroblast lysates immunoprecipitated with anti-α8-EX (Ex) or anti-α8-cyto (Cyto).

(B) Dissociated E7.5 chick DRG neurons were cultured, labeled, lysed, and immunoprecipitated with anti-α8-cyto. The positions of standards are indicated. Molecular weights are depicted in kilodaltons.
Figure 5. Normalized Percentages of Neurons Bearing Neurites after Culture in the Presence of Anti-Integrin Subunit-Specific Antibodies

(A) E7 DRG sensory neurons from DRG were cultured for 5 hr on substrate-bound tenascin (closed bars), laminin (striped bars), or N-cadherin (open bars). Cultures were incubated in the presence of the following anti-integrin antibodies at the designated concentrations: anti-β1, MAb W1B10 (50 μg/ml), anti-α5-EX (IgG; 300 μg/ml), anti-α6-EX (affinity-purified; 25 μg/ml), anti-α2-EX (Fab'; 300 μg/ml), anti-α5-EX (IgG; 300 μg/ml), and anti-α6 MAb CHAV-1 (100 μg/ml).

(B) Motor neurons were cultured on substrate-bound tenascin (closed bars) or N-cadherin (open bars) in the presence of the following anti-integrin antibodies: anti-β1, MAb W1B10 (50 μg/ml), anti-as-EX (IgG; 300 μg/ml), anti-as-EX (Fab'; 300 μg/ml), anti-a3-EX (IgG; 300 μg/ml), and anti-αv MAb CHAV-1 (50 μg/ml).

Table values for 2 wells from each experiment were normalized to mean values for control wells (wells to which no antibody was added). Values in bar graph represent means ± SD (for n = 3–6) or means ± range (for n = 2); n is indicated below each bar.

Integrin α5β1 Is a Tenascin Receptor

Integrin α5β1 subunits were stained with several different integrin subunit-specific antibodies. Most motor and sensory neurons (~90%) expressed integrin subunits α5, α6, and αv (data not shown). When stained with preimmune serum or in the absence of primary antibody, neurons remained unstained. Cultured embryonic DRG neurons have previously been shown to express several additional integrin subunits, including α1, α6, and αv (Tomaselli et al., 1993).

Identification of Integrin Receptors That Mediate Interactions between Neurons and Tenascin

To identify receptors that mediate neurite outgrowth-promoting interactions with tenascin, neurons were cultured in the presence of function-inhibiting antibodies to several integrin subunits. A MAb to the β1 integrin subunit (W1B10) completely inhibited neurite outgrowth by sensory or motor neurons on tenascin or laminin (Figures 5A and 5B; Figure 2B). In the presence of this MAb, all neurons remained round and appeared totally detached from the substrate (see Figure 2B). In striking contrast, when anti-β1 MAb was added to neuronal cultures plated on N-cadherin, neurite outgrowth was not detectably inhibited and appeared identical to cultures to which no antibody was added (Figures 5A and 5B; Figure 2H). These results suggest that a β1 subunit-containing integrin heterodimer mediates the neurite outgrowth-promoting interactions of motor neurons and sensory neurons with tenascin and laminin.

To identify possible integrin α5β1 heterodimers that mediate this response to tenascin, antibodies specific for several integrin α subunits known to associate with β1 (α5, α6, and αv) were tested for possible inhibitory effects. An integrin α subunit-specific antibody, anti-α5-EX, strongly, but not completely, inhibited neurite outgrowth by sensory neurons on tenascin (see Figure 1C) but not on laminin (see Figure 1D). The same antibodies inhibited neurite outgrowth by motor neurons on tenascin (see Figure 2C) but not on N-cadherin (see Figure 2I). On tenascin, only ~50% of the normal number of sensory neurons (Figure 5A) and ~33% of the normal number of motor neurons (Figure 5B) extended neurites in the presence of this antibody.

For both sensory and motor neurons, Fab' fragments of anti-α5-EX inhibited neurite outgrowth to almost the same extent as intact IgG, indicating that inhibition was not due to IgG-induced aggregation of α5 subunit receptors on the cell surface (Figure 5). Furthermore, affinity-purified IgG blocked neurite outgrowth by sensory neurons to the same extent as total IgG, indicating that the block by intact IgG is due to specific antibodies raised to the integrin α5 subunit fusion protein (Figure 5A). As one control, effects of anti-α6-EX IgG or Fab' fragments were shown to be substrate specific (Figure 5). Inhibitory effects were not seen on neurite outgrowth on laminin or N-cadherin. As a second control, adsorption with a control bacterial extract was shown not to reduce the inhibitory effects of anti-α5-EX IgG (data not shown). As a third control, IgG prepared from the anti-α5-EX preimmune serum did not inhibit outgrowth by either sensory or motor neurons (data not shown). These controls demonstrate that inhibition does not result from nonspecific toxic effects or from the presence of nonspecific antibodies. They indicate that α5 integrins mediate, in part, interactions of these neurons with tenascin.

Antibodies specific for α5 (anti-α5-EX) or αv (MAb CHAV-1), when tested individually, had no detectable inhibitory effect on neurite outgrowth by either sensory or motor neurons on tenascin (Figure 5; Figures 2A and 2D) or N-cadherin (see Figures 2G and 2J). Therefore, heterodimers containing either of these integrin subunits are not strong candidates for mediating neuronal interactions with tenascin.

The presence of anti-α5-EX IgG or Fab' fragments also significantly reduced the distribution of neurite length on tenascin compared with controls. The mean neurite length in sensory or motor neurons was reduced by ~40% compared with cultures incubated with the anti-α5 MAb CHAV-1 or anti-α5-EX (Figure 6); these differences were significant (p < .001, ANOVA; Sokol and Rohlf, 1989). Cumulative frequency distribution plots show an apparently continuous reduction in neurite length when cultures were
incubated with anti-α4-EX compared with anti-αv, or no antibody (Figure 6). Consistent with previous results demonstrating that neurite outgrowth on tenascin by the majority of neurons and DRG sensory neurons, this suggests that neurites are inhibited by anti-α4-EX IgG or Fab' fragments in these cultures.

A K562 Cell Line Expressing the Integrin αv Subunit Exhibits Increased Attachment to Tenascin

To provide additional evidence that the αvβ1 heterodimer functions as a tenascin receptor, K562 cells (a human myelogenous leukemia cell line) were transfected with a full-length chicken αv cDNA in the vector pAW, which directs transcription from a CMV promoter. K562 cell lines have been shown to express predominantly αvβ5 and, to a lesser extent, αvβ6 integrins (Pasqualini et al., 1993). Integrin αv transfectants were screened for expression of the integrin αv subunit, and those cell lines expressing maximal amounts were chosen for further attachment assays (for details, see Muller et al., 1995). A centrifugation assay was used to measure cell attachment to a tenascin substrate (see Experimental Procedures). K562 cells expressing the integrin αv subunit attached to tenascin with about twice the apparent efficiency of the control K562 cells (Figure 7A). The increased attachment by K562 cells expressing the integrin αv subunit was reduced by anti-αv-EX. This antibody had no apparent effect on attachment by the parental K562 cell line (Figure 7A). In addition, an inhibitory MAb, A2BII, specific for the endogenous human integrin β1 subunit present in these cells (Hall et al., 1990) reduced attachment to tenascin by the same approximate amount as anti-αv-EX. A polyclonal antibody to the human integrin αv subunit (rabbit anti-αvβ5; Telios Pharmaceuticals) did not significantly reduce attachment to tenascin (Figure 7B). Muller et al. (1995) have shown that chick αv associates with human β1 to form heterodimers expressed on the surface of these cells. The results in the present report indicate that these heterodimers promote cellular attachment to tenascin.

Discussion

The results presented in this report support the following conclusions: tenascin-C promotes outgrowth by E7–E8 DRG neurons and E3 motor neurons, a region of tenascin that includes the last three FNIII repeats is responsible for...
most neurite outgrowth-promoting effects, and the integrin receptor αβ₁ functions as a tenascin receptor on these neurons.

**Sensory and Motor Neuron Outgrowth on Tenascin**

At stage 19 of chick development, tenascin is found associated with the tips of motor and sensory neuron axons as they emerge from the neural tube and DRG, respectively, and grow through the anterior region of each somite (Wehrle and Chiquet, 1990). In this report, we show that neurite outgrowth by sensory and motor neurons is promoted by substrate-bound tenascin. Previously, it was reported that tenascin supports neurite outgrowth in spinal cord cultures from day 3 (stage 19) embryos and in sensory neurons from embryos at both day 4 (stage 20-22) and later developmental stages (Wehrle and Chiquet, 1990; Wehrle-Haller and Chiquet, 1993). Therefore, our results and those of other investigators show that in vitro tenascin has the potential to promote outgrowth from a subset of neurons through the anterior region of each somite, at stage 19 as well as later stages of the chick embryo development. The distribution of tenascin in vivo is consistent with the possibility that it functions similarly during embryogenesis.

Neurite outgrowth by sensory or motor neurons requires interactions with a domain of tenascin in the last three FNIII repeats. Antibodies raised to a pronase fragment of tenascin containing FNIII repeats 6-8 inhibited neurite outgrowth by motor (~90%) or sensory (~80%) neurons on substrate-bound tenascin. In addition, MAB Tn68, which recognizes an epitope in FNIII repeat 7, inhibited ~80% of neurite outgrowth. These results are consistent with previous reports in which an antibody raised to the C-terminal fragment inhibited neurite outgrowth on tenascin from intact E6 DRG (Wehrle-Haller and Chiquet, 1993). Other investigators have also found that a MAB to this region inhibited neurite outgrowth on substrate-bound tenascin by dissociated cerebellar, hippocampal, or mesencephalic neurons (Husmann et al., 1992; Lochter et al., 1991). It is possible that an additional region in tenascin is also required for neurite outgrowth, since neurons from intact DRGs did not extend neurites on the 60 kDa C-terminal fragment of tenascin used to generate AS474 (Wehrle-Haller and Chiquet, 1993).

**The Integrin αβ₁ as a Receptor for Tenascin**

Experiments presented in this and previous reports indicate that neuronal outgrowth on a tenascin substrate requires β₁-containing integrin heterodimers. In the present report, we show that an integrin β₁-specific MAB W1B10 completely blocks neurite outgrowth by dissociated sensory or motor neurons on this substrate. This result corroborates a recently published report in which a different integrin β₁-specific MAB (JG22) blocked neurite outgrowth from cultures of intact DRGs (Wehrle-Haller and Chiquet, 1993). Other investigations have demonstrated that an integrin containing the β₁ subunit is a receptor for attachment of nonneuronal cell lines to non-RGD-containing regions of tenascin (Sriramarao et al., 1993; Prieto et al., 1993).

To identify integrin α subunits that mediate interactions with tenascin, we developed and tested several α subunit-specific antisera. The antisera generated, anti-α5-EX and anti-α6-EX, efficiently immunoprecipitated their respective α subunits and blocked ligand-receptor interactions. Anti-α5-EX inhibited significantly, but not completely, neurite outgrowth by sensory and motor neurons on tenascin. The inhibitory effect was specific to tenascin and was not observed on laminin or N-cadherin. In addition, affinity-purified IgG and monovalent anti-α5-EX Fab′ were effective. These results strongly implicate αβ₁ as a tenascin receptor.

Since the block with anti-α5-EX was incomplete, an additional αβ₁ heterodimer may also help mediate neurite outgrowth-promoting interactions. Alternatively, anti-α5-EX may not inhibit αβ₁-mediated interactions completely. Data in the present report suggest that the α5 and the α6 subunit-containing heterodimers are not strong candidates to be tenascin receptors on these neurons, even though an α5 heterodimer has been shown to mediate interactions by nonneuronal cells with this protein (Priedo et al., 1993; Sriramarao et al., 1993). Neither α5-specific nor α6-specific antibodies detectably reduced neurite outgrowth on tenascin, even though each has been shown to be inhibitory on other ECM substrates, e.g., laminin and vitronectin, respectively (Neugebauer et al., 1991; Weaver et al., submitted). αβ₁ has been shown to mediate some interactions with tenascin by nonneuronal cells (Sriramarao et al., 1993) and is therefore a candidate to function as an additional tenascin receptor on motor and sensory neurons.

Experiments assaying cellular interactions with tenascin have produced a confusing literature. Substrate-bound or soluble tenascin has promoted or inhibited cell attachment or neurite outgrowth, depending on the cell type and/or method of plating tenascin. In immunohistochemical studies on the expression of α5 in developing chicks, Bossy et al. (1991) detected α5 on subsets of neurons and axon tracts within the central and peripheral nervous systems. It seems likely that the presence or absence of α5β₁ heterodimers is a major determinant of these strikingly different responses of neurons to tenascin.

**Experimental Procedures**

**Purification of Substrate Proteins**

A tenascin purification was developed based on previously described methods (Huber et al., 1986; Vaughan et al., 1987). Sterna (40-60) were dissected from E17 chicks and immediately frozen with liquid N₂. Sterna were weighed, and 1.0 ml of extraction buffer was added for each 0.1 g of sterna (1 M NaCl, 0.05 M Tris·HCl [pH 8.0], plus chymostatin, leupeptin, aproatin, pepstatin [each at 5 μg/ml], and 1 mM phenylmethylsulfonyl fluoride). Sterna were homogenized (Polytron) and extracted for 48 hr at 4°C. Extracted sterna were then spun at 12,000 x g for 15 min, and the supernatant was precipitated with 50% saturated ammonium sulfate for 1 hr. After spinning at 12,000 x g for 10 min, the pellet was resuspended in PBS (8 mM Na₂HPO₄, 2 mM KH₃PO₄, 0.136 mM NaCl [pH 7.4]) containing protease inhibitors (see above) and dialyzed against PBS overnight. Tenascin was purified by affinity chromatography using the tenascin-specific MAB M1 IgG coupled to CNBr Sepharose 4B (Pharmacia, Piscataway, NJ). The dialyzed protein solution was applied to the column. After extensive washing with PBS and PBS containing 0.1% Triton X-100,
tenascin was eluted with 80 mM diethylamine (pH 11.0). Eluted fractions were immediately neutralized with 1 M Na2-HEPES (pH 7.0). Eluted material was pooled, dialyzed against PBS, and analyzed on polyacrylamide gels. The predominant species of tenascin in these preparations had a M. of 190 kDa, indicating that it lacks the alternatively spliced FNIII repeats present in larger tenascin isoforms (depicted in Figure 3). Purified tenascin was not reactive with an antibody to chicken fibronectin (see Lewitt et al., 1992).

The extracellular domain of N-cadherin was expressed in COS cells and purified as described elsewhere (Kypa et al., unpublished data). Substrate-bound N-cadherin from these preparations promoted outgrowth from retinal neurons, which was inhibited with the anti-N-cadherin MAb NCD2.

Laminin was purified from Engelbreth-Holm-Swarm (EHS) sarcoma tumors using published procedures (Kleinman et al., 1982; Timpl et al., 1982).

Substrate Preparation
Linbro Titertek 96 well plastic dishes (Flow Laboratories, McLean, VA) were coated with nitrocellulose as described previously (Lagenauer and Lemmon, 1987). Purified tenascin (30–40 gg/ml in PBS), N-cadherin (30–70 gg/ml in PBS plus 1 mM of CaCl2, 1 mM MgCl2), or laminin (10 gg/ml in PBS) was added and incubated either for 15 min at room temperature or overnight at 4°C.

Cell Culture
Motor neuron cultures were prepared from stage 19 embryos according to procedures adapted from Henderson et al. (1984) and Wehrle and Chiquet (1990). Embryos were dissected from membranes, washed in PBS, and incubated in pancreatic (GIBCO, Grand Island, NY) for 2 hr. Embryos were then placed in cold L-15 medium containing 5 mg/ml BSA (Sigma, St. Louis, MO), and neural tubes were dissected by isolation. Dorsal and ventral neural tube fragments were dissected with a glass needle. Ventral neural tubes were then placed in 0.25% trypsin for 5 min at 37°C, washed in L-15 containing 10 mg/ml BSA, centrifuged, and resuspended in complete motor neuron growth medium (F12 medium containing 0.5 mg/ml BSA [Serva, Germany]). Brain-derived neurotrophic factor (diluted 1:1000; a generous gift from Dr. Patricia Olson, Chiron Corporation, Emeryville, CA), and 10 ng/ml basic fibroblast growth factor (Boehringer Mannheim, Indianapolis, IN). Neural tubes were gently triturated four times. Dissociated cells were centrifuged, resuspended in growth medium to an appropriate density (1 x 106 cells/well), plated in 100 ml of growth medium, and incubated for 24 hr at 37°C with 5% CO2.

For sensory neuron cultures, E7–E8 chick DRGs were dissected and dissociated into single cells by incubation in 0.05% trypsin in 0.2% versene, 0.1% glucose, 0.02% EDTA, 0.058% NaHCO3 (UCSF Cell Culture Facility) for 10 min at 37°C, followed by trituration. Dissociated cells were resuspended by centrifugation and resuspended in F12 containing 1% fetal bovine serum. To enrich for neuronal cells, cell suspensions were plated onto 60 mm tissue culture dishes (Falcon) for 1–3 hr. Neurons were pipetted from the culture dishes, centrifuged, and resuspended in DRG growth medium (F12 containing 2% BSA [Serva] and 100 mg/ml nerve growth factor) at an appropriate density (1 x 106 cells/well). Fifty microliters of this cell suspension was added to the well of a 96 well culture dish containing antibody diluted to the appropriate concentration in 50 ml of warmed and equilibrated growth medium. Neurons were gently centrifuged onto the dish and incubated for 4–7 hr at 37°C in a 5% CO2 atmosphere.

Quantitation of Neurite Outgrowth
For motor neurons cultured on tenascin or N-cadherin, neurones with neurites were defined as cells extending neurites longer than two cell diameters. For sensory neurons on tenascin, laminin, or N-cadherin, the percentage of neurons with neurites per total number of cells was determined. To measure neurite length of either motor or sensory neurons, cultures were viewed with an inverted Olympus IMT2 microscope using Nomarski optics. Images were collected with a cooled CCD camera (Photometrics, series 200). Processors longer than 20 mm were measured using the Prism program (Chen et al., 1989).

Tenascin and Integrin Antibodies
Tenascin antibodies AS142 (Chiquet and Fambrough, 1984) and MAb Tn68 (Chiquet-Ehrismann et al., 1988) are specific for whole tenascin and an epitope within FNIII repeat 7, respectively. Tenascin antibody AS474 is specific for a prorase-generated C-terminal fragment of tenascin containing FNIII repeats 6–8 plus the C-terminal fibrinogen-like module. These have been described previously (Wehrle-Haller and Chiquet, 1993).

Anti-a2-EX was generated to an SDS-PAGE-purified GST-a2-fusion protein expressed in E. coli. The sequence included amino acids 62–710 of the chick a2 protein (with an internal, in-frame deletion of amino acids 150–185). This antibody is specific for chick integrin a2 as assayed by immunoblot or immunoprecipitation. It is more fully characterized elsewhere (Muller et al., 1995). Anti-a2-EX was generated to an SDS-PAGE-purified β-galactosidase-a2-fusion protein that contained amino acids 17–558 of the chick a2 protein. This antibody is specific for chick integrin a2 as assayed by immunoblot and immunoprecipitation. It inhibits interactions of ciliary neurons with laminin-1 and is more fully characterized elsewhere (Weaver et al., submitted). Anti-αv-β3 was prepared using a synthetic peptide corresponding to the cytoplasmic domain of the chick αv subunit (Bosey et al., 1991).

IgG from rabbit sera or from anti-β1 chick MAb W1B10 ascites (Hayashi et al., 1990) was purified using MAC protein G disks (Amicon, Beverly, MA) according to the manufacturer's instructions. IgG from anti-chick αv MAb CHAV-1 ascites (Neugebauer et al., 1991) was purified from ascites using affinity chromatography on protein A-Sepharose CI-4B according to the manufacturer's instructions (Pharmacia).

All IgG preparations were dialyzed extensively against PBS and sterilized. The a2 and αv IgG preparations were further dialyzed against F12 medium.

To affinity purify anti-α2-EX IgG, 1 liter cultures of IgG-expressing bacteria were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 4 hr. The bacterial pellets were collected, resuspended in 9.0 ml of PBS with protease inhibitors, sonicated, and centrifuged for 10 min at 10,000 x g. The resultant pellets were solubilized in 1.5% N-lauroylsarcosine (Sigma), 2% Triton X-100 (Sigma) in PBS and protease inhibitors. This solution was extracted on ice for 1 hr and centrifuged for 10 min at 10,000 x g. A 10 x 2 cm piece of nitrocellulose membrane (Schleicher & Schuell, Keene, NH) was immersed in the supernatant for 1 hr. Before use for affinity purification of IgG, each membrane was washed extensively with PBS, pre-€luted with 0.1 M glycine (pH 2.7), equilibrated with PBS, and incubated with IgG for 1 hr. The anti-α2-EX IgG preparation was incubated first with a nitrocellulose membrane containing the immobilized control E. coli extract and then with a membrane containing the immobilized a2-EX fusion protein. After washing with PBS, affinity-purified α2-EX was eluted from the α2 fusion protein-containing membrane with 0.1 M glycine (pH 2.7) and was immediately neutralized with 1 M Tris–CI (pH 8.5). This was dialyzed against PBS followed by F12 medium and was sterilized by filtration.

To generate Fab' fragments, IgG was dissolved in PBS and dialyzed against 100 mM Na~acetate (pH 5.2). Papain–agarose (Sigma) was incubated with the dialysate for 1.5 hr at 37°C. The reaction was stopped with 75 mM iodoacetic acid (Sigma) for 30 min at room temperature. The Fab' solution was then dialyzed against PBS and subsequently against F12 medium.

Immunocytochemistry
Cell cultures in 96 well dishes were fixed with 4% paraformaldehyde for 10 min, carefully washed, and incubated for 2 hr with integrin subunit antibodies (anti-αv-EX [16 μg/ml IgG], anti-αv-EX [1500 serum], or anti-αv MAB CHAV-1 [20 μg/ml IgG] diluted in PBS with 5% normal goat or horse serum. Preimmune controls were incubated at the same concentrations. Cultures were carefully washed and stained further according to instructions included in the Vectastain kits (Vector Laboratories).

Immunoprecipitations
Chick embryo fibroblasts were prepared from E9 or E10 embryos according to Rein and Rubin (1986). Cells were resuspended in PBS and surface labeled in suspension with lactoperoxidase, H2O2, and 125I, as described below.

Dissociated DRG neurons were cultured for 24 hr on 100 mm2 Falcon tissue culture dishes previously coated with a mixed solution of vitronectin (1 μg/ml; Gibco) and fibronectin (20 μg/ml; Calbiochem,
All correspondence should be addressed to L. F. R. We thank Dr. Blaise Bossy for assistance in preparing the α2-transfected K562 cell lines and the anti-α2-EX antibody, Dr. Susannah Chang for the DM1 antibodies and the anti-αβ1 MAb W1 B10 (10 μg/ml for 1 hr. After washing, blots were blocked for 1 hr with horseradish peroxidase–rabbit anti-mouse IgG (Zymed Corp.). After washing, the bands were visualized with electrochemical luminescence using an ECL kit and its enclosed instructions (Amersham, Arlington Heights, IL).

Cell Attachment Assay

A centrifugation assay was used to measure interactions of K562 cells with tenascin (Friedlender et al., 1988). Wells of a Linbro Titeratak 96 well round bottom dish (Linbro) were coated with 100 μl of a nitrocellulose solution, washed with PBS, coated with 40 μl of 20–40 μg/ml tenascin in PBS for 2 hr at 37°C. After washing, wells were equilibrated with 24 mM Tris–Cl (pH 7.5), 137 mM NaCl, 2.7 mM KCl, 2 mM glucose, 2 mM CaCl2, 2 mM MgCl2, 2 mg/ml BSA. An appropriate number of cells were then introduced to the wells, left to adhere for 30 min with shaking, and washed with PBS, cultures were surface labeled by adding lacto-para-diaminobenzoic acid (PBD), anti-αβ1 or anti-αβ2 (10 μg/ml) for 1 hr. After washing, blots were blocked for 1 hr with 5% nonfat dry milk in 100 mM Tris–HCl (pH 7.5), 150 mM NaCl. The blots were then incubated with the anti-β1 MAb W1B10 (10 μg/ml) for 1 hr. After washing, blots were incubated for 1 hr with horseradish peroxidase–rabbit anti-mouse IgG (Zymed Corp.). After washing, the bands were visualized with electrochemical luminescence using an ECL kit and its enclosed instructions.

Acknowledgments

All correspondence should be addressed to L. F. R. We thank Dr. Blaise Bossy for assistance in preparing the α2-transfected K562 cell lines and the anti-α2-EX antibody, Dr. Susannam Chang for the DM1 MAB, Dr. Karla Neugebauer for helping to develop the tenascin purification and Ms. Cristina Weaver for reading the manuscript and for helpful discussions. This work was supported by National Institutes of Health grant PO1-16033. L. F. R. is an investigator of the Howard Hughes Medical Institute.

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Received September 16, 1994; revised March 21, 1995.

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Note Added in Proof