Tenascin Promotes Cerebellar Granule Cell Migration and Neurite Outgrowth by Different Domains in the Fibronectin Type III Repeats

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Abstract. The extracellular matrix molecule tenascin has been implicated in neuron-glia recognition in the developing central and peripheral nervous system and in regeneration. In this study, its role in Bergmann glial process–mediated neuronal migration was assayed in vitro using tissue explants of the early postnatal mouse cerebellar cortex. Of the five mAbs reacting with nonoverlapping epitopes on tenascin, mAbs J1/tn1, J1/tn4, and J1/tn5, but not mAbs J1/tn2 and J1/tn3 inhibited granule cell migration. Localization of the immunoreactive domains by EM of rotary shadowed tenascin molecules revealed that the mAbs J1/tn4 and J1/tn5, like the previously described J1/tn1 antibody, bound between the third and fifth fibronectin type III homologous repeats and mAb J1/tn3 bound between the third and fifth EGF-like repeats. mAb J1/tn2 had previously been found to react between fibronectin type III homologous repeats 10 and 11 of the mouse molecule (Lochter, A., L. Vaughan, A. Kaplony, A. Prochiantz, M. Schachner, and A. Faissner. 1991. *J. Cell Biol.* 113:1159–1171). When postnatal granule cell neurons were cultured on tenascin adsorbed to polyornithine, both the percentage of neurite-bearing cells and the length of outgrowing neurites were increased when compared to neurons growing on polyornithine alone. This neurite outgrowth promoting effect of tenascin was abolished only by mAb J1/tn2 or tenascin added to the culture medium in soluble form. The other antibodies did not modify the stimulatory or inhibitory effects of the molecule. These observations indicate that tenascin influences neurite outgrowth and migration of cerebellar granule cells by different domains in the fibronectin type III homologous repeats.

NEURONAL migration is an important morphogenetic feature in the developing nervous system (Purves and Lichtman, 1985; Jacobson, 1991). The exquisite specificity of this process has been studied in the developing mammalian cerebellar cortex, because it is a relatively simply organized structure of the central nervous system and because it contains few and identifiable neuronal and glial cell types arranged in simple geometric arrays (Rakic, 1971; Altman, 1982). In rodents, much of cerebellar histogenesis, including neuronal migration, takes place postnatally, rendering these processes more easily amenable to experimental manipulation (Miale and Sidman, 1961; Fujita, 1967). Thus, the migration of young postmitotic granule cells from the external to the internal granular layer has been studied in detail, since it exemplifies a general principle in neuron-glia interaction, in which migrating neurons are guided by the radial processes of glial cells (Rakic, 1971; Hatten et al., 1984).

To characterize the molecular mechanisms involved in cerebellar morphogenesis, mono- and polyclonal antibodies reacting with particular cell surface and extracellular matrix components have been used in in vitro assay systems to modify particular aspects of neuronal migration. In such studies it could be shown that the neural recognition molecule L1 and its structurally related form in chicken, Ng-CAM (Burgoon et al., 1991), are implicated in cerebellar granule cell migration, possibly by influencing the fasciculation of axons (Lindner et al., 1983, 1985; Fischer et al., 1986; Chuong et al., 1987; Persohn and Schachner, 1987). In addition to L1, the adhesion molecule on glia (AMOG)—a homologue of the β-subunit of the Na/K-ATPase (Antonicek et al., 1987; Gloor et al., 1990), a neural lectin and one of its ligands (Lehmann et al., 1990), the neural proteoglycan astrochondrin (Streit, A., C. Nolte, T. Rásonyi, M. Schachner, manuscript submitted for publication), the tissue plasminogen-activator/plasmin system (Moonen et al., 1982), and a protease inhibitor of the nexin family (Lindner et al., 1986) have all been implicated in granule cell migration in vitro. Two glia-derived extracellular matrix glycoproteins, thrombospondin and tenascin (see Sanes, 1989, for review), have also been shown to influence neuronal migration since antibodies to thrombospondin (Lawler, 1986) and cytotactin (Grumet et al., 1985)—which is related to J1/tenascin (Kruse et al., 1985, Faissner et al., 1988), tenascin (Chiquet-Ehris-

mann et al., 1986), hexabrachion (Erickson and Inglesias, 1984), and glial-mesenchymal extracellular matrix (GMEM) protein (Bourdon et al., 1983)—inhibit granule cell migration in vitro (Chuong et al., 1987, O'Shea et al., 1990).

The tenascin glycoproteins are extracellular matrix molecules with a cysteine-rich amino terminal domain followed by 13.5 EGF type repeats in chicken and 14.5 EGF type repeats in mouse and human (Spring et al., 1989; Jones et al., 1989; Nies et al., 1991; Siri et al., 1991; Weller et al., 1991), eight fibronectin type III homologous repeats and a fibrinogen β and γ homologous domain at the carboxy-terminal end. An increasing number of isoforms have been found, which appear to be generated by insertion of additional, alternatively spliced fibronectin type III homologous repeats between the constitutive fibronectin type III homologous repeats 5 and 6 (Spring et al., 1989; Jones et al., 1989; Nies et al., 1991; Siri et al., 1991; Weller et al., 1991). Tenascin monomers are assembled to hexamers by disulfide bridges at the amino-terminal end and appear as hexabrachia by EM of rotary shadowed molecules (Erickson and Inglesias, 1984). The tenascin glycoproteins are expressed during formation of various tissues and are absent or expressed only at low levels in the adult (see Erickson and Bourdon, 1989, for review). In the developing nervous system, tenascin is transiently expressed by immature astrocytes in boundaries of vibrissae-related barrel fields in the somatosensory cortex during the period of afferent fiber ingrowth (Steindler et al., 1989a,b; Crossin et al., 1989; Jhaveri et al., 1991). Furthermore, these molecules have been suggested to play a crucial role in regeneration in the peripheral nervous system, because the expression of the molecules increased after lesioning the sciatic nerve (Daniloff et al., 1989; Martini et al., 1990).

To appreciate the functional roles of tenascin in neuronal migration and formation of neural connections, the behavior of neural cells towards the purified molecule has been characterized. Tenascin has been implicated in neural crest cell migration (Tan et al., 1987; Bronner-Fraser, 1988; Mackie et al., 1988; Stern et al., 1989) and promotion of neuroblastoma cell migration (Halfter et al., 1989). It inhibited adhesion and spreading of hippocampal and mesencephalic neurons (Lochter et al., 1991) or neural crest cells (Tan et al., 1987; Halfter et al., 1989). Furthermore, tenascin was found to be a repulsive substrate for central nervous system neurons (Faissner and Kruse, 1990) or neurites (Crossin et al., 1990) and to inhibit neurite outgrowth when exposed to cells in a soluble form (Lochter et al., 1991; Crossin et al., 1990). However, tenascin has also been shown to mediate attachment of neurons to astrocyte surfaces in short-term cell binding assays (Kruse et al., 1985; Grumet et al., 1985) and to promote neurite outgrowth when neurons were maintained on tenascin/polyamine substrates (Lochter et al., 1991; Wehrle and Chiquet, 1990).

In view of the observations that a molecular domain between the tenth and eleventh fibronectin type III homologous repeats of the mouse molecule was involved in neurite outgrowth promotion (Lochter et al., 1991), we asked the question which domains on the tenascin glycoprotein could mediate cerebellar granule cell migration. Furthermore, it seemed important to investigate whether these domains are the same as or different from those involved in neurite extension. Here, we report that tenascin influences migration and neurite outgrowth of cerebellar granule cells by different domains in the fibronectin type III homologous repeats.

Materials and Methods

Animals

For immunization, adult Lou x Sprague Dawley F1 hybrid female rats were used. C57BL/6J mice were used for the migration assay. All other experiments were carried out with NMRI mice.

Antibodies

mAbs to tenascin were prepared by immunization of Lou x Sprague Dawley F1 hybrid female rats (2 to 3 mo old) with glycopeptidase F–treated tenascin from mouse brain. Deglycosylated tenascin (see Deglycosylation of N-linked glycoconjugates) was chosen for immunization to minimize the generation of antibodies against the strongly immunogenic L2/HNK-1 carbohydrate epitope (Kruse et al., 1984, 1985). Rats were immunized for the first time with 50 μg tenascin in 1 ml PBS, pH 7.4, mixed with an equal volume of complete Freund's adjuvant and three or four times subsequently with 50 μg tenascin in incomplete Freund's adjuvant at time intervals of 3 to 5 wk, all subcutaneously. Animals with serum titers between 1:5,000 and 1:10,000 dilution as determined by ELISA (see Analytical procedures) were chosen for fusion. Rats received two final intraperitoneal injections, each with 20 μg of the immunogen in PBS, 4 and 3 d before the fusion. Fusions were carried out with the mouse myeloma clone X-Ag8-653 (Kearney et al., 1979) following established procedures (Lagenaur et al., 1980) with minor modifications (Faissner and Kruse, 1990). Hybridoma culture supernatants were screened by ELISA using purified tenascin and further tested by Western blot analysis. Out of 27 tenascin immunoreactive hybridoma supernatants, three were found by competition ELISA to recognize epitopes different from each other and from the epitopes recognized by the previously described mAbs J1/tn1 and J1/tn2 (Lochter et al., 1991). Corresponding hybridoma cells were then subcloned twice by the method of limiting dilution (Lagenaur et al., 1980) and designated J1/tn3 (clone 630), J1/tn4 (clone 633), and J1/tn5 (clone 635).

The Ig subclass of the mAbs was determined by ELISA using affinity-purified rabbit antibodies to rat Ig isotypes (kind gifts of Dr. H. Bazin, Brussels). mAbs J1/tn3 and J1/tn4 could be assigned to the IgG2a subclass and mAb J1/tn5 to the IgG1 subclass of rat Igs.

Larger quantities of mAbs were obtained by growing the hybridoma clones in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 1% (vol/vol) Nutridoma (Boehringer Mannheim Biochemicals, Indianapolis, IN). Culture supernatants were concentrated by ammonium sulfate precipitation and dialyzed against PBS. By SDS-PAGE, the antibodies revealed a purity of >95%.

The mouse mAb to bromodeoxyuridine (BrdU)[1] was purchased from Dianova (Hamburg, Germany). Rabbit polyclonal antibodies to tenascin from mouse brain (KAF 9(1)) have been described (Faissner and Kruse, 1990).

As secondary antibodies the following reagents were used: FITC-coupled goat anti–mouse IgG and IgM antibodies (Dakopatts, Hamburg, Germany) were used for indirect immunofluorescence staining at a dilution of 1:75. For ELISA, HRP-coupled goat anti-rat IgG and IgM antibodies (Dianova, Hamburg, Germany) were used at a dilution of 1:5,000. Western blot analysis was performed using ^{125}I-labeled sheep anti-rat IgG antibodies (5–20 μCi/μg, 250 nCi/ml; from Amersham International, Amersham, UK) at a dilution of 1:400.

Isolation of Extracellular Matrix and Neural Recognition Molecules

Tenascin was immunoaffinity purified from 1 to 15-d-old mouse brains or supernatants from primary cultures of mouse embryonic fibroblasts using mAb columns (Faissner and Kruse, 1990). In some cases, immunoaffinity-purified tenascin preparations were subjected to gel filtration on a Superose12 (HR 10/50) column using fast performance liquid chromatography (FPLC) (Pharmacia/LKB, Piscataway, NJ). In brief, immunoaffinity-purified tenascin (1.2–2.0 mg/4 ml in PBS) was loaded onto the column and separated in PBS at 0.5–1 ml/min and a fraction size of 1 ml. Fractions were analyzed by ELISA and SDS-PAGE with subsequent silver staining. Fractions containing tenascin were pooled. Immunoaffinity-purified and immunoaffinity and subsequently FPLC-purified tenascin were used in all experiments with identical results. Tenascin from chicken embryonic fibroblast supernatants was a kind gift of A. Kaplony (Zürich, Switzerland; Kaplony et al., 1991). Tenascin from human embryonic fibroblasts supernatants (a kind gift of

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1. *Abbreviation used in this paper*: BrdU, bromodeoxyuridine.

Dr. Wieser, Mainz, Germany) was isolated as described (Faissner and Kruse, 1990).

The neural recognition molecules L1 and myelin-associated glycoprotein (MAG) were prepared as described (Rathjen and Schachner, 1984; Poltorak et al., 1987). Crude membrane fractions from 9-d-old mouse brains were prepared according to Fuss et al. (1991).

Analytical Procedures

Protein determinations were carried out according to Bradford (1976). SDS-PAGE was performed on slab gels (Laemmli, 1970). Linear gels were stained with reducing silver ions as described (Merril et al., 1982). Western blot analysis was performed after SDS-PAGE separation of proteins on 4–10% linear gradient slab gels, transfer to nitrocellulose filters, and detection of proteins with mAbs and ^{125}I-labeled sheep antibodies to rat IgG (Faissner and Kruse, 1990).

For ELISA, wells of micro-test flexible assay plates (Falcon 3912; Becton Dickinson Labware, Oxnard, CA) were coated overnight at 4°C with tenascin (100 μl/well at 0.5 μg/ml 0.1 M NaHCO$_3$). Wells were washed with PBS, incubated for 1 h at 37°C with 0.1 M NaHCO$_3$ containing 5 mg/ml BSA, washed three times with PBS, and incubated for 3 h at 37°C with hybridoma supernatants and mAbs. After three washes with PBS, wells were incubated for 2 h at 37°C with HRP-coupled goat anti-rat IgG and IgM polyclonal antibodies, washed three times, and developed with 1 mg/ml ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate (6)]; Boehringer Mannheim Biochemicals) in 100 mM Na-acetate, 50 mM Na-phosphate (pH 4.2), and 0.01% H$_2$O$_2$. The optical density was measured at 405 nm with an ELISA reader (Titertek Multiskan MKII, Flow).

To determine whether tenascin-recognizing hybridoma supernatants competed with each other or with the previously described mAbs J1/tn1 and J1/tn2 for binding to tenascin, a competition ELISA was carried out (Friguet et al., 1983). Hybridoma supernatants or mAbs to be compared, were incubated together with tenascin coated onto assay plates as described for the ELISA. In parallel, wells were incubated individually with each antibody. Hybridoma supernatants or mAbs indicating an increase in absorbance when incubated together in comparison to being incubated individually were taken to recognize different epitopes on the tenascin molecule. As a positive control, mAbs J1/tn1 and J1/tn2, which recognize different epitopes, were incubated together. As a negative control, a twofold amount of hybridoma supernatants or mAbs was incubated.

Deglycosylation of N-linked Glycoconjugates

Tenascin was treated with glycopeptidase F (Boehringer Mannheim Biochemicals) as described (Faissner et al., 1988). The efficiency of deglycosylation was determined by monitoring a shift to lower apparent molecular weights by SDS-PAGE and subsequent silver staining.

Rotary Shadowing and EM

The binding sites of the mAbs J1/tn3, J1/tn4, and J1/tn5 were localized on tenascin molecules as described (Vaughan et al., 1987; Lochter et al., 1991) with minor modifications. In brief, tenascin and mAbs to tenascin were dialyzed against 0.1 M ammonium bicarbonate and mixed to yield final concentrations of 40–80 μg/ml tenascin and 10–40 μg/ml mAbs. They were incubated for 2 h at room temperature or overnight at 4°C. Samples were mixed with an equal volume of glycerol and sprayed onto the surface of freshly cleaved mica chips. The chips were dried in a freeze-fracture unit (model BAF300; Balzers AG, Liechtenstein) under vacuum (lower than 10^{-7}T) for at least 4 h and rotary shadowed with platinum/carbon at an angle of 8° followed by a carbon backing film at 90°. Electron micrographs were obtained with a Zeiss EM10C electron microscope. The curvilinear lengths of the arms from the T-junction and the center of the distal knob to the antibody binding sites were measured with a graphics tablet interfaced with the Kontron IBAS graphics system (Kontron Instruments, Zürich, Switzerland).

Cell Migration Assay

Organotypic explants of cerebellar folia from 10-d-old mice were maintained in culture by a procedure modified from a previously described method (Lindner et al., 1983). Pieces of folia from the cerebellum were incubated in a 25-ml Erlenmayer flask with 10 μM BrdU (Sigma Chemical

Co., St. Louis, MO) at 70 rpm and 37°C for 90 min in 3 ml serum-free defined culture medium (Fischer, 1982). The tissue pieces were washed thoroughly with defined medium and five to seven pieces were incubated in a 25-ml Erlenmayer flask or 2 h at 37°C in a CO$_2$ incubator in 3 ml defined medium in the presence or absence of antibodies. The flasks were then closed air-tight and maintained under constant agitation at 70 rpm and 37°C for 3 d. The tissue pieces were embedded in Tissue-Tek (Miles Laboratories, Elkhart, Indiana) and fresh-frozen for sectioning in a cryostat (Jung 2700; Reichert & Jung, Nussloch, Germany). 12-μm-thick sections were cut in an orientation perpendicular to the length of the folia and used for immunofluorescence staining with antibodies to BrdU.

Indirect immunofluorescence staining of BrdU was performed using a procedure modified from Houck and Loken (1985). Sections were fixed for 30 min on ice with 0.5% paraformaldehyde in PBS. DNA was denatured in 4 N HCl containing 0.5% polyoxyethylene sorbitan monolaurate (Tween 20) for 20 min at room temperature followed by neutralization with 0.1 M borate buffer, pH 8.5. Sections were washed for 3 min with PBS and incubated with the mAb to BrdU (10 μg/ml in PBS containing 0.5% Tween 20) for 30 min at room temperature. After washing to PBS and blocking of unspecific protein binding sites with 10% (vol/vol) horse serum and 1% (wt/vol) BSA in PBS, sections were incubated for 20 min with FITC-coupled secondary antibodies.

To allow detection of the different cerebellar layers, consecutive sections of each folium were stained with hematoxylin and eosin. To this aim, sections were fixed with 4% ice-cold paraformaldehyde in PBS for 5 min and washed in PBS for 2 min before staining with hematoxylin (1 g hematoxylin, 0.2 g NaIO$_3$, 50 g KAl(SO$_4$)$_2$, 50 g Chloralhydrate, 1 g citric acid in 1 liter water) for 5 min and eosin (1:10[vol/vol] diluted with H$_2$O; Riedel-deHaen, Hannover, Germany) for another 5 min. Sections were briefly washed in water and dehydrated by incubation in an ascending alcohol series.

The extent of cell migration was determined by counting the BrdU-labeled cells in the external granular, molecular, and internal granular layers. The dimensions of the counting fields taken for microscopic evaluation were 280–400 μm $\times$ 280 μm (width, parallel to the pial surface $\times$ length, perpendicular to the pial surface). At least six fields were analyzed for each experiment and ~300 labeled cells per field were counted in average in the presence or absence of antibodies. Setting the total number of BrdU-labeled cells in each field as 100%, the percentage of cells in each layer was then calculated. The mean values $\pm$ SD of the percentages from at least three independent experiments for each experimental condition were determined and compared for statistical significance by the Mann-Whitney U test (Clauss and Ebner, 1977).

Neurite Outgrowth Assay

Glass coverslips (15 mm in diameter) were coated for 1–2 h at 37°C with 10 μg/ml poly-DL-ornithine (Sigma Chemical Co.) in 0.1 M borate buffer, pH 8.5 (Collins, 1978), washed twice with distilled water, air dried, and incubated overnight at 37°C with PBS or PBS containing 75 μg/ml tenascin, sterilized by passage through 0.22-μm filters (Millex GV4; Millipore). Finally, coverslips were washed twice with PBS before cell plating.

Small cerebellar neurons from 6–8-d-old mice were purified by centrifugation through a Percoll gradient (Keilhauer et al., 1985). Cells were resuspended in culture medium (basal medium Eagle's (BME) containing 10% [vol/vol] horse serum) and plated at a density of 35,000 cells/350 μl culture medium per coverslip in wells of four-well plastic multidishes (A/S Nunc, Raskilde, Denmark). Cultures were maintained for 48 h without change of culture medium.

When cultures were maintained in the presence of soluble tenascin or antibodies to tenascin, the protein solutions were dialyzed against BME and sterilized by filtration (Millex GV4; Millipore) before addition to the cultures 2 h after cell plating at final concentrations of 15, 60, or 250 μg/ml. When antibodies were tested for their influence on soluble tenascin, antibodies and soluble tenascin were preincubated together for 2 h at room temperature and added to the cultures 2 h after cell plating at the final concentrations indicated for the individual components.

Morphometric measurements were carried out on cultures stained with toluidine blue (Lochter et al., 1991). In each experiment, the longest and the other, shorter neurites of 50 randomly selected, single neurons were measured. The fraction of process-bearing neurons was determined by counting at least 100 randomly selected, single neurons. The statistical significance of differences between experimental values was determined by the Mann-Whitney U test (Clauss and Ebner, 1977).

Results

Immunochemical Analysis of Three Novel mAbs to Tenascin from Mouse Brain

From five fusions, 27 hybridoma supernatants were found to react with tenascin from mouse brain by ELISA. Out of these, three hybridoma supernatants were selected, since they did not interfere with each other or with the previously generated mAbs J1/tn1 and J1/tn2 (Faissner and Kruse, 1990) in binding to tenascin as assayed by the competition ELISA (not shown). The resultant clones were designated J1/tn3, J1/tn4, and J1/tn5.

In Western blot analysis, the three antibodies reacted with two closely migrating, strong bands of 225 and 240 kD and with two faint bands of 190 and 200 kD apparent molecular masses in preparations of tenascin from mouse brain (Fig. 1, lanes *1, 6,* and *11*), as described for J1/tn1 and J1/tn2 (Faissner and Kruse, 1990). In preparations of tenascin from mouse fibroblasts, the antibodies recognized two bands of 200 and 235 kD apparent molecular masses (Fig. 1, lanes *3, 8,* and *13*), as expected from previous observations (Aufderheide and Ekblom, 1988; Weller et al., 1991). When tenascin from mouse brain was deglycosylated using glycopeptidase F, the binding of the mAbs was not affected (Fig. 1, lanes *2, 7,* and *12*), indicating that N-glycosidically linked carbohydrate chains are not involved in the binding of the mAbs. The three mAbs did not react with the neural recognition molecules L1 or MAG (Fig. 1, lanes *4, 5, 9, 10, 14,* and *15*). When the mAbs were subjected to Western blot analysis with extracts of crude membrane fractions of 9-d-old mouse brain, the same four immunoreactive bands with 240, 225, 200, and 190 kD apparent molecular masses were seen as with purified tenascin from mouse brain (Fig. 1, lanes *16–18*). In ELISA experiments the mAbs J1/tn3–J1/tn5 bound to tenascin from human fibroblasts, but not to tenascin from chicken fibroblasts (not shown). This was also described for the mAb J1/tn1, but not for the mAb, J1/tn2, which recognized tenascin from both human and chicken fibroblasts (Faissner and Kruse, 1990).

Localization of Antibody Binding Sites on Tenascin by EM

The binding sites for the mAbs J1/tn3, J1/tn4, and J1/tn5 were localized by EM of rotary shadowed tenascin molecules from mouse brain. Tenascin molecules showed the typical hexameric configuration, with the T-junction at the amino terminus in the center, the fine thread of the proximal portion (EGF-like repeats), the thickened distal region (fibronectin type III homologous repeats), and the distal knob (fibrinogen-like domain) at the carboxy terminus (Fig. 2 a). The distribution of the binding sites for the antibodies J1/tn3, J1/tn4, and J1/tn5 were measured as distances from both the T-junction and the distal end (Fig. 3) and are compiled in Table I. Only those antibodies that were clearly visible alongside and touching an arm were chosen for evaluation. The epitope for the J1/tn3 antibody was located 9 ± 2 nm from the T-junction and 66 ± 7 nm ($n = 55$) from the distal knob. The antibody J1/tn4 bound 41 ± 5 nm from the T-junction and 35 ± 5 nm ($n = 61$) from the distal knob, whereas the distances of the epitope of J1/tn5 were 45 ± 5 nm from the T-junction and 29 ± 4 nm ($n = 66$) from the distal knob. The three to four peaks in the distributions of the distances measured between antibody binding site and distal knob (Fig. 3, *b, d,* and *f*), but not between antibody binding site and T-junction (Fig. 3, *a, c,* and *e*), are most likely due to the presence of different isoforms in the tenascin preparations (see Fig. 1, lanes *1, 6,* and *11*), with molecules containing the differently spliced fibronectin type III homologous repeats (see Lochter et al., 1991). Thus, the localization of the immunoreactive epitopes can be assigned to the following domains. The mAbs J1/tn4 and J1/tn5 localize to the thickened distal portion of tenascin in the region of the binding site for J1/tn1 (Lochter et al., 1991), with J1/tn4 binding between the third and fourth fibronectin type III homologous repeats (Figs. 2 c, 3, c and d, and 5) and J1/tn5 binding between the fourth and fifth fibronectin type III homologous repeats (Figs. 2 d, 3, e and f, and 5). mAb J1/tn3 reacts with the proximal thin portion of the molecule between the third and fifth EGF-like repeats (Figs. 2 b, 3, a and b, and 5).

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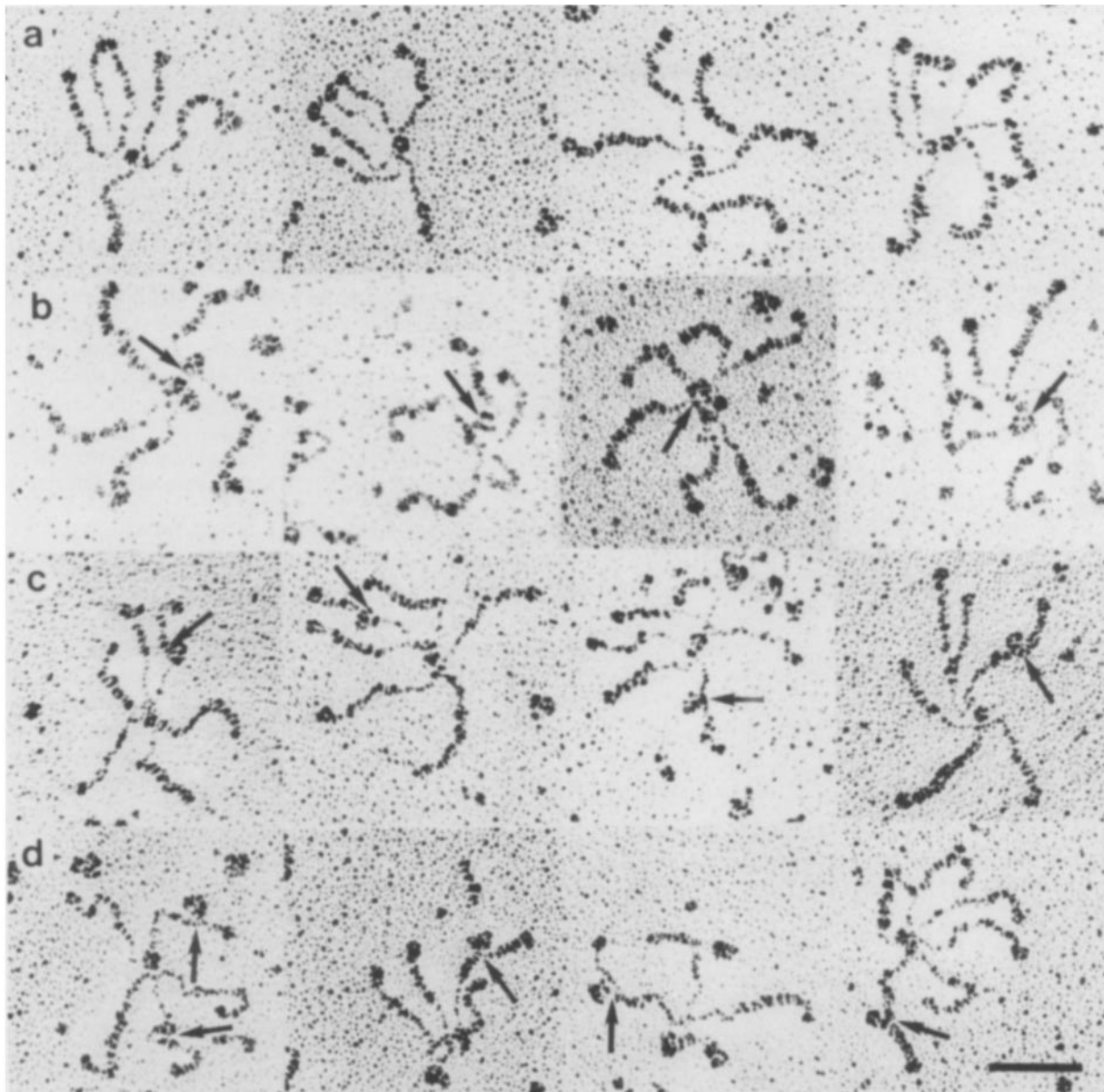
Figure 2. Electron microscopic analysis of mAb binding domains on tenascin from mouse brain after rotary shadowing. Electron micrographs of rotary-shadowed tenascin from mouse brain (*a*) after preincubation with the mAbs J1/tn3 (*b*), J1/tn4 (*c*), and J1/tn5 (*d*) are shown. Each panel consists of four micrographs. Arrows in *b-d* indicate the binding sites of the mAbs. Bar in the last micrograph of *d* represents 50 nm (for all micrographs).

Fibronectin Type III Homologous Repeats Three to Five of Tenascin Are Involved in Cerebellar Granule Cell Migration

To investigate the involvement of different domains of tenascin in cerebellar granule cell migration, an in vitro assay system was used in which the migration of neuronal precursor cells can be quantitatively analyzed (Lindner et al., 1983). As a modification of this assay system, neuronal precursor cells were pulse labeled before migration with BrdU instead of ³H-thymidine. The BrdU-labeled granule cells were visualized by indirect immunofluorescence and their migration distance was quantitatively analyzed by counting the relative frequency of BrdU-labeled cells in the different layers of the cerebellar cortex which could be visualized by staining adjacent sections with hematoxylin/eosin (Fig. 4). After 3 d

in culture in the absence of antibodies, 52 ± 3.7% of the BrdU-labeled neuronal precursor cells migrated into the internal granular layer, while the external granular layer contained 12.7 ± 2.3% and the molecular layer 34.9 ± 3.8% of all BrdU-labeled cells (Table II; see also Lindner et al., 1983). During these 3 d, the size of the external granular layer decreased and the size of the molecular layer increased in the absence of antibodies (Fig. 4, *a* and *b*).

When explants were maintained for 3 d in vitro in the presence of mAbs J1/tn2 or J1/tn3, a similar distribution of BrdU-labeled cells was seen (Table II; compare Fig. 4 *g* to Fig. 4 *f*). The IgG fraction of polyclonal antibodies to tenascin from mouse brain revealed a significant inhibition of migration of BrdU-labeled cells (Table II). The relative distribution of labeled cells after 3 d in vitro was 21.8 ± 4.2% in the external granular layer, 53 ± 4.5% in the molecular layer

Ab → T-junction   **Ab → distal knob**

J1/tn3

J1/tn4

J1/tn5
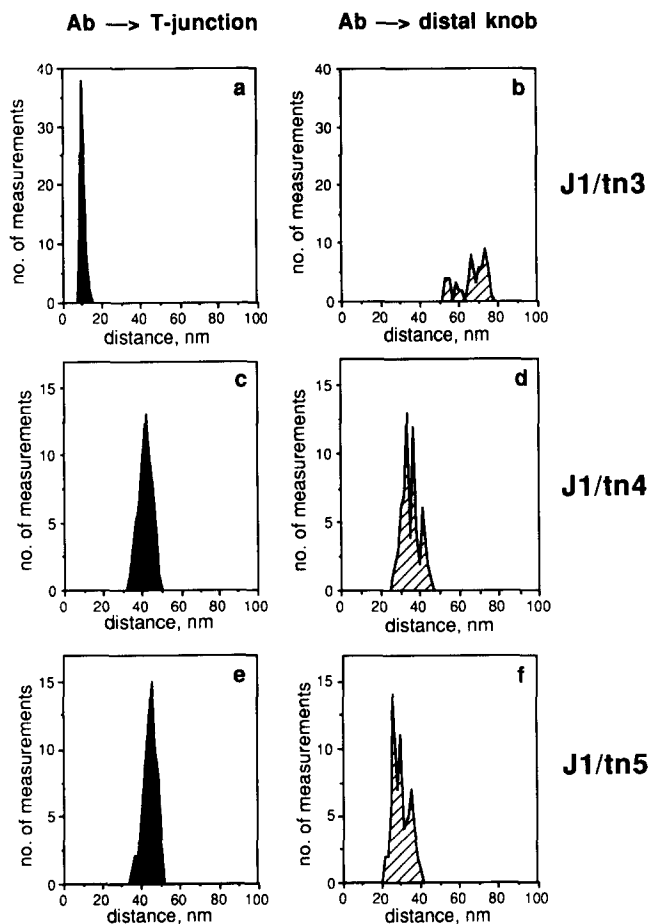
Figure 3. Histograms of the distribution of the antibody binding sites on tenascin. The number of determinations (*ordinate*) is plotted against the curvilinear distance (*abscissa*, in nanometers) of the antibody binding sites (*Ab*) on tenascin from mouse brain starting from the T-junction (*a, c,* and *e*) or from the center of the distal knob (*b, d,* and *f*). Since the tenascin preparations contain a mixture of isoforms (see Fig. 1, lanes *1, 6,* and *11*), the values of the distances encompassing the variable spliced region of the fibronectin type III homologous repeats (*b, d,* and *f*) showed three to four peaks in contrast to the values for the distances from the T-junction to the antibody binding sites (*a, c,* and *e*) where only one peak is visible.

*Table I. Morphometric Analysis of mAb Binding Sites on Tenascin from Mouse Brain by EM of Rotary Shadowed Molecules*

Antibody	Distance	
	T-junction → Ab	Distal knob → Ab
	nm	*nm*
J1/tn1*	47 ± 3	23 ± 3
J1/tn2*	57 ± 4	14 ± 2
J1/tn3	9 ± 2	66 ± 7
J1/tn4	41 ± 5	33 ± 5
J1/tn5	45 ± 5	29 ± 4

The distances (mean values ± SD) from the T-junction and the center of the distal knob to the binding sites on tenascin of the different mAbs are given. The total number of measurements for the different antibodies were *n* = 55 for J1/tn3, *n* = 61 for J1/tn4, and *n* = 65 for J1/tn5. Ab, antibody binding site. * Results from Lochter et al. (1991).

and only 25.2 ± 4.5% in the internal granular layer. The mAbs J1/tn1, J1/tn4, and J1/tn5 inhibited cerebellar granule cell migration to a similar extent at antibody concentrations of 0.2, 0.5, and 1 mg/ml (Table II; for J1/tn4 at 0.2 mg/ml; see Fig. 4 *h*). Only mAb Jt/tn1 displayed reduced inhibitory effects when assayed at a concentration of 0.2 mg/ml (Table II). The inhibitory effects of the mAbs J1/tn1, 4, and 5 and polyclonal tenascin antibodies are not due to interference of antibodies with granule cell proliferation or survival, since the numbers of BrdU-incorporating cells determined under control and experimental conditions were comparable. In addition, the distribution of tenascin in the different layers of the tissue slice after 3 d in culture is the same in the absence or presence of mAbs J1/tn3 and J1/tn4 as evaluated by indirect immunofluorescence staining with a polyclonal antibody to tenascin (not shown). In rare cases, the inhibitory antibodies caused an increase in the size of the molecular layer, but the effects were smaller than those reported in the chicken (Chuong et al., 1987) and often hardly detectable.

Effects of mAbs and Soluble Tenascin on Neurite Outgrowth on Substrate-bound Tenascin

Since it had previously been shown that tenascin promotes neurite outgrowth of embryonic mesencephalic and hippocampal neurons through a domain recognized by the mAb J1/tn2 (Lochter et al., 1991), we investigated whether the same domain supported neurite outgrowth of small cerebellar neurons, 90% of which are granule cells. These neurons were obtained from 6–8-d-old mice, a developmental stage when granule cell migration is reaching its peak. Neurons were maintained in vitro for 48 h on tenascin from mouse brain adsorbed to polyornithine (substrate-bound tenascin) to allow attachment of cell bodies (Lochter et al., 1991). When plated onto polyornithine alone, only 11% of all small cerebellar neurons developed neurites after 48 h of maintenance in vitro (Table III). However, when these neurons were maintained on substrate-bound tenascin, the percentage of process-bearing cells increased to ~28% (Table III). The percentage of cells with neurites was similar under all conditions of this study: neurons grown in the presence of mAbs to tenascin, soluble tenascin from mouse brain or mouse fibroblasts, or soluble tenascin in the presence of mAb J1/tn5 (Table III). The lengths of the longest neurites of process-bearing cells on substrate-bound tenascin increased by ~50% when compared to neurite lengths of process-bearing cells maintained on polyornithine alone, whereas the lengths of the other, shorter neurites were increased by ~33% (Table III). The number of neurites per process-bearing cell was 1.4–1.7 in all experiments (not shown). Thus, the longest neurite contributed most to the total neurite length (~85%) (Table III). When the five mAbs to tenascin (J1/tn1 to 5) were tested for their effects on neurite outgrowth of small cerebellar neurons on substrate-bound tenascin, only J1/tn2 strongly inhibited neurite outgrowth at 60 μg/ml (Table III; and see Lochter et al., 1991, for hippocampal and mesencephalic neurons). Inhibition of neurite outgrowth by mAb was the same for the longest and the other, shorter neurites (Table III). None of the other antibodies showed a significant reduction in neurite outgrowth under the conditions of this study (Table III), even at concentrations of 250 μg/ml (not shown).

IGL   ML   EGL   IGL   ML   EGL

IGL   ML   EGL   IGL   ML   EGL
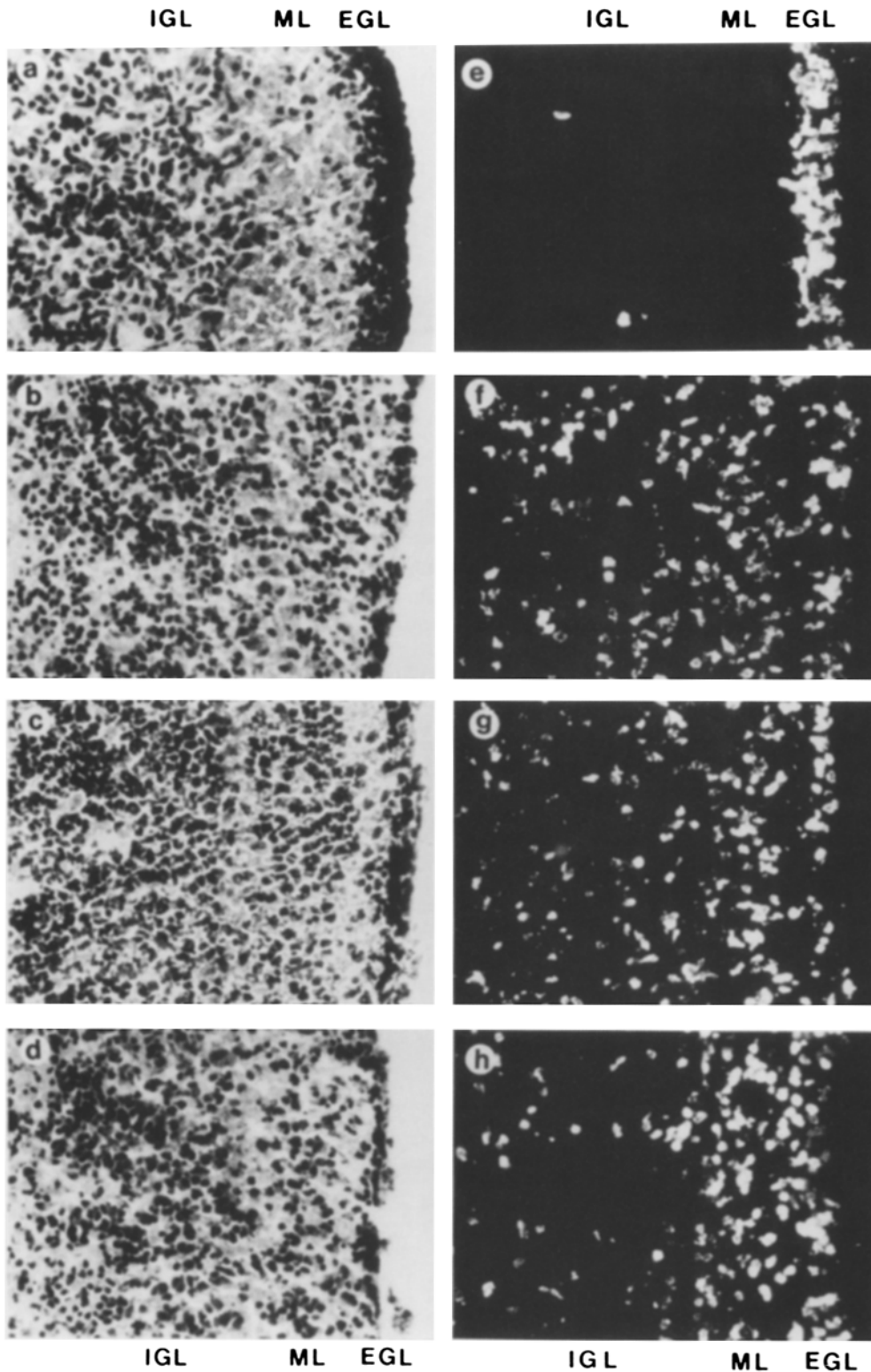
Figure 4. Influence of monoclonal tenascin antibodies on cerebellar granule cell migration. The distribution of BrdU pulse-labeled cells in sections of cerebellar folium explants is shown by indirect immunofluorescence (*e–h*) directly after pulse labeling (*e*), after 3 d in vitro in the absence of antibodies (*f*), after 3 d in vitro in the presence of mAb J1/tn3 at 1 mg/ml (*g*), or after 3 d in vitro in the presence of mAb J1/tn4 at 0.2 mg/ml (*h*). Adjacent sections, stained with hematoxylin/eosin corresponding to the fluorescence images *e–h* are shown in *a–d*, respectively. EGL, external granular layer; ML, molecular layer; IGL, internal granular layer. Bar, 50 μm.

When small cerebellar neurons were maintained on substrate-bound tenascin and tenascin from mouse brain or mouse fibroblasts was added at a concentration of 15 μg/ml to the culture medium (soluble tenascin), neurite outgrowth was reduced to levels seen on polyornithine alone (Table III). This inhibitory effect of soluble tenascin could not be neutralized by any of the mAbs to tenascin (Table III; only shown for J1/tn5), even at concentrations of 250 μg/ml (not shown). Furthermore, the percentage of process-bearing cells and the number of neurites per cell were not significantly different between cultures maintained in the presence of soluble tenascin and cultures maintained in the combined presence of soluble tenascin and mAb J1/tn5 (Table III) as described for hippocampal neurons (Lochter et al., 1991).

Table II. Quantification of the Effects of mAbs to Tenascin on Cerebellar Granule Cell Migration

Antibody	Concentration	% Cells in EGL	in ML	in IGL
	mg/ml			
t = 0		93.7 ± 2.9	2.2 ± 2.2	4.2 ± 2.8 (4)
Without antibody		12.7 ± 2.3	34.9 ± 3.8	52.4 ± 3.7 (4)
J1/tn2	1.0	12.5 ± 3.5	37.5 ± 2.9	50.2 ± 4.1 (3)
J1/tn3	1.0	13.2 ± 2.3	40.1 ± 4.4	46.8 ± 5.4 (4)
Polyclonal antibody	1.0	21.8 ± 4.2	53.0 ± 4.5	25.2 ± 4.5 (3)
J1/tn1	1.0	22.5 ± 3.4	51.6 ± 3.6	25.9 ± 4.8 (3)
J1/tn1	0.5	17.3 ± 4.4	52.4 ± 2.7	30.3 ± 3.9 (3)
J1/tn1	0.2	16.7 ± 2.6	43.0 ± 4.1	40.4 ± 4.3 (3)
J1/tn4	1.0	15.8 ± 5.2	52.8 ± 3.6	31.0 ± 4.7 (3)
J1/tn4	0.5	18.3 ± 3.7	54.4 ± 3.4	27.4 ± 2.4 (3)
J1/tn4	0.2	19.3 ± 2.8	52.5 ± 2.8	28.0 ± 4.7 (3)
J1/tn5	1.0	17.6 ± 3.9	54.8 ± 4.2	27.5 ± 5.0 (3)
J1/tn5	0.5	17.8 ± 4.1	55.5 ± 4.1	26.8 ± 5.0 (3)
J1/tn5	0.2	18.2 ± 5.5	53.5 ± 3.3	28.4 ± 6.7 (3)

The percentage of the relative distribution of BrdU-labeled cells (mean value ± SD) in the different cerebellar layers are shown directly after pulse labeling (t = 0) or after 3 d in vitro in the absence (without antibody) or presence of antibodies as indicated. Numbers in brackets represent the number of independent experiments for each value. Significance levels for the values of the ML and the IGL (compared to the values in the absence of antibodies [without antibody]) were $P \leqslant 0.03$ for the mAbs J1/tn1, J1/tn4, J1/tn5 and the polyclonal antibody and not significant for the mAbs J1/tn2 and J1/tn3. EGL, external granular layer; ML, molecular layer; IGL, internal granular layer.

Discussion

An important first step towards identifying the functional domains of tenascin glycoproteins depends on the availability of mAbs directed against distinct epitopes of the molecule. Several mAbs have been generated to tenascin from mouse, chicken, and human (Bourdon et al., 1983; Chiquet and Fambrough, 1984a,b; Chiquet-Ehrismann et al., 1988; Faissner and Kruse, 1990; Kaplony et al., 1991), but only the mAbs Tn68 (Chiquet-Ehrismann et al., 1988) and J1/tn2 (Faissner and Kruse, 1990; Lochter et al., 1991) identify two distinct functional domains on the tenascin glycoproteins: Tn68 recognizes a cell binding domain for fibroblasts which localizes to fibronectin type III homologous repeats 10 or 11 (corresponding to repeats 12 or 13 in mouse) in all isoforms

of chicken tenascin (Chiquet-Ehrismann et al., 1986; Spring et al., 1989) and J1/tn2 recognizes a neurite outgrowth promoting domain for mesencephalic and hippocampal neurons which localizes to a short region of transition between fibronectin type III homologous repeats 10 and 11 of mouse tenascin (corresponding to repeats 8 or 9 in chicken). This is not conserved in the chicken, since J1/tn2 reacts with all known isoforms of mouse tenascin (Faissner and Kruse, 1990), but does not recognize the 190-kD isoform of chicken tenascin (L. Vaughan, unpublished observations).
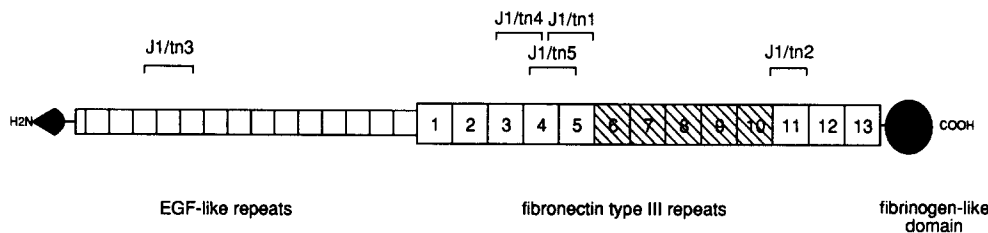
To extend the repertoire of mAbs directed against functionally important molecular domains, three novel mAbs, J1/tn3, J1/tn4, and J1/tn5, have been generated against mouse tenascin. The immunoreactive domains, determined by electron microscopic analysis of the antibody binding sites on ro-

Table III. Quantification of the Influence of mAbs to Tenascin and Soluble Tenascin on Neurite Outgrowth of Cultured Cerebellar Granule Cells on Substrate-bound Tenascin

		Summed neurite lengths from 50 neurons of Longest neurite	Other neurites	Fraction of process bearing cells
		μm	*μm*	*%*
PORN	(10)	2,370 ± 281*	431 ± 80*	11 ± 2.3*
bTN	(10)	3,692 ± 357	574 ± 112	28 ± 5.1
bTN+J1/tn1	(3)	3,699 ± 60	600 ± 125	30 ± 2.9
bTN+J1/tn2	(5)	2,380 ± 206*	442 ± 101‡	23 ± 4.6
bTN+J1/tn3	(6)	3,489 ± 352	620 ± 184	24 ± 3.6
bTN+J1/tn4	(5)	3,851 ± 461	579 ± 189	27 ± 4.1
bTN+J1/tn5	(4)	3,730 ± 322	523 ± 235	28 ± 2.4
bTN+sTN	(7)	2,715 ± 93*	483 ± 104‡	25 ± 6.2
bTN+sTN(mes)	(3)	2,475 ± 287*	480 ± 159	26 ± 5.2
bTN+sTN+J1/tn5	(3)	2,620 ± 115*	512 ± 61	26 ± 3.5

The summed neurite length (mean values ± SD) of 50 randomly selected neurite-bearing cells from one coverslip are shown for the longest neurite and for the other, shorter neurites of these cells. The fraction of neurite bearing cells per coverslip (mean value ± SD) was determined by counting at least 100 randomly selected, single cells. Concentrations of additives in the experiments were 60 μg/ml for each antibody and 15 μg/ml for soluble tenascin from mouse brain (sTN) or from mouse fibroblasts (sTN[mes]). Significance levels (compared to neurons cultured on substrate-bound tenascin [bTN]) are indicated by the following symbols: *, $0.001 < P \leqslant 0.01$; ‡, $0.01 < P \leqslant 0.05$. Numbers in parentheses represent the number of independent experiments for each culture condition.

tary shadowed molecules, could be assigned to the EGF-like repeats 3–5 for J1/tn3, to fibronectin type III homologous repeats 3 or 4 for J1/tn4 and to fibronectin type III homologous repeats 4 or 5 for J1/tn5 (Fig. 5). These domains do not belong, like the previously described mAbs J1/tn1 and J1/tn2 (Lochter et al., 1991) to the alternatively spliced parts of the molecule, since mAbs J1/tn1 to 5 react with all tenascin isoforms known in the mouse by Western blot analysis. The epitopes involved are resistant to glycopeptidase F treatment, thus, either localizing these to the protein backbone or to O-glycosidically linked carbohydrates.

By the use of this panel of mAbs to mouse tenascin, we could show that migration and neurite outgrowth of cerebellar granule cells is influenced by different domains in the fibronectin type III homologous repeats of tenascin: migration is influenced by the third to fifth fibronectin type III homologous repeats, while promotion of neurite outgrowth, not only of cerebellar granule cells, but also of mesencephalic and hippocampal neurons (Lochter et al., 1991), depends on a domain of the eleventh fibronectin type III homologous repeat. The specificity of the assignment of the domain for migration is underscored by previous observations on the effects of polyclonal antibodies raised against J1-160/180, a tenascin-related extracellular matrix glycoprotein of oligodendrocytes (Pesheva et al., 1989). These antibodies show some crossreactivity with the mouse tenascin isoforms (Kruse et al., 1985; Faissner et al., 1988), but did not reduce neuron migration in the cerebellar explant system (Antonicek et al., 1987).

The observation that migration and neurite outgrowth by cerebellar granule cells depend on different domains on the tenascin molecule begs the question as to the functional interrelationships and distinctions between the two phenomena. Granule cell migration is the result of a complex series of individual events among which neurite extension is an outstanding feature. Neuronal precursor cells in the external granular layer, the germinal zone of the early postnatal cerebellar cortex, withdraw from the cell cycle and associate with the radial Bergmann glial fibers (Rakic, 1971; Altman, 1982). The postmitotic granule neurons then extend two axonal processes alongside other axons of granule cells that have preceded them to form the dense fascicle bundles of parallel fibers in the molecular layer. The granule cell bodies, after residing in the premigratory zone for ∼28 h, then transverse the molecular layer in close cell surface contact

with Bergmann glial processes from the external to the internal granular layer within a time period of 4 h (Fujita, 1969). This translocation is guided by the leading process of the granule cell which remains in close apposition to the radial glial process throughout its course of migration until it leaves the glial process to take up its final position in the internal granular layer (Rakic, 1990; Sitt and Hatten, 1990). Thus, the phenomenon of neurite outgrowth is an integral part of granule cell migration, first as extension of axons and then as elaboration of the leading process.

It is tempting to allocate a functional role for tenascin to a distinct cellular event in granule cell migration on the basis of our knowledge about the molecule's expression and distribution during the period of granule cell migration at early postnatal stages. Tenascin is synthesized by and detectable at the surface of Bergmann glial cells in all cerebellar layers (Bartsch et al., 1991). However, although granule cells do not synthesize the molecule, their axonal surfaces, but not their cell bodies show highest levels of tenascin immunoreactivity at times coincident with neurite outgrowth. Tenascin has not been detected at the contact sites between Bergmann glial processes and migrating granule cell bodies, while it is amply present at the sites of contact between parallel fibers and migrating granule cells. Thus, the site of action of tenascin in granule cell migration is difficult to deduce from its cellular localization. Since tenascin does not appear to promote granule cell migration by allowing neurite extension to occur, its function is likely to be otherwise. It cannot be as simple as enhancing the motility of cell bodies as it has been observed for neural crest cells on substrate-bound tenascin (Halfter et al., 1989), since tenascin is not present at the site of contact between the migrating granule cell body and Bergmann glial process, where such interactions could be assumed to be most effective. However, tenascin could be instrumental at the sites of contact between the premigratory or migratory granule cell body with the tenascin-positive parallel fibers. Also, tenascin may be active in the detachment of the leading process from the Bergmann glial fiber when entering the internal granular layer.

The distribution of granule cell bodies in the different layers of the cerebellar cortex was determined under influence of the migration inhibitory antibodies, to assess at which migratory stage the functional impact of tenascin could be crucial. As reported previously for antibodies to cytotactin (Chuong et al., 1987), granule cell bodies accu-

mulated throughout the molecular layer, thus opening the possibility that translocation of granule cell bodies on Bergmann glial processes may depend on tenascin. It is interesting in this context to compare the sites of inhibition by other agents. Antibodies to the astrocyte-derived extracellular matrix glycoprotein thrombospondin or a neural lectin and one of its ligands abolish granule cell migration at the outset, possibly by inhibition of attachment of granule cell bodies to Bergmann glial processes, such that granule cells do not leave the external granular layer (O'Shea et al., 1990; Lehman et al., 1990). The neural recognition molecule L1 in the mouse and its related molecule in the chicken, Ng-CAM, have both been implicated in early phases of granule cell migration (Lindner et al., 1983, 1986; Chuong et al., 1987). Since L1 has not been detected at sites of contact between migrating granule cell bodies and Bergmann glial processes (Persohn and Schachner, 1987), since L1 does not promote recognition between granule cells and mature, glial fibrillary acidic protein–positive cerebellar astrocytes (Keilhauer et al., 1985), and since L1 plays an important role in axon fasciculation (Fischer et al., 1986; Rathjen, 1988, for review), we have suggested that disturbance of fasciculation of granule cell axons leads to a reduced migratory activity of granule cells. The adhesion molecule on glia, the tissue plasminogen-activator/plasmin system, and a protease inhibitor of the nexin family have also been implicated in granule cell migration, possibly at the stage of entrance of granule cells into the molecular layer (Moonen et al., 1982; Lindner et al., 1986; Antonicek et al., 1987). Thus, granule cell migration is a composite of distinct cellular interactions which can be perturbed at different sites and times during the migration process and implicates several recognition molecules. Tenascin appears to be the latest acting of all molecular components so far recognized to mediate granule cell migration.

Since tenascin does not mediate granule cell migration by the same molecular domain as that which promotes neurite extension, the question is, which of the multifaceted functional properties of the molecule may be compatible with its role in granule cell migration. The cell binding domain for fibroblasts recognized by mAb Tn68 (Chiquet-Ehrismann et al., 1988; Spring et al., 1989) may be a prerequisite for any tenascin-mediated cell interaction (Lotz et al., 1989), yet is not sufficient for cell migration which depends on the J1/tn1, 4, and 5 domains. Whether these domains would be involved in the stimulation of cell motility (Halfter et al., 1989) is presently unknown. It is also not known to what extent the inhibitory or repulsive properties of tenascin are involved in granule cell migration. Under certain circumstances, tenascin interferes with cell-to-substrate adhesion of most cell types investigated so far, including neural crest cells (Tan et al., 1987; Mackie et al., 1988; Epperlein et al., 1988; Halfter et al., 1989) and mesodermal cells from gastrula stage embryos (Riou et al., 1990). Inhibitory effects can also be seen, when neurons are confronted with a choice of a more favorable and less favorable substrate, where cell body attachment to (Faissner and Kruse, 1990) or growth cone movement into the less favorable substrate tenascin (Taylor, J., P. Pesheva, and M. Schachner, manuscript submitted for publication) is inhibited. Such inhibitory properties have been suggested to play a role in the stabilization of barrel field boundaries in the developing somatosensory cortex (Steindler et al., 1990a,b; Crossin et al., 1990; Jhaveri et al., 1991) and patch-

matrix compartments in the developing striatum (O'Brien et al., 1991). It is conceivable that tenascin, by virtue of its cell repulsive properties, participates in the de-adhesion of neurons from radial glial processes at the transition from the molecular to the internal granular layer, thus slowing down the migrating successor neurons on the same guidepost.

In summary, our study defines a new functional domain of tenascin localizing between the third and fifth fibronectin type III homologous repeats, which is distinct from the neurite outgrowth promoting domain, the cell binding domain, and the presumed inhibitory domain (Chiquet-Ehrismann et al., 1986; Spring et al., 1989; Lochter et al., 1991). It is conceivable that these distinct domains employ distinct receptor systems by which they control different cellular signalling systems, as shown, for example, for the neural recognition molecules L1 and N-CAM (Schuch et al., 1989; Atashi et al., 1991; Von Bohlen und Halbach, J. Taylor, and M. Schachner, manuscript submitted for publication). Identification of the receptors involved and characterization of the transduction mechanisms in complex cellular assemblies (Steindler et al., 1989a,b; 1990) are major challenges for future investigations.

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