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Domains of tenascin involved in glioma migration

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SUMMARY

Tenascin (TN) is an extracellular matrix protein found in areas of cell migration during development and expressed at high levels in migratory tumor cells. TN was previously shown to support the attachment and migration of glioma cells in culture. To determine the domains responsible for glioma migration and attachment, we produced recombinant fusion proteins that collectively span the majority of the molecule including its epidermal growth factor-like repeats, fibronectin type III repeats and fibrinogen domain. These domains were tested for their ability to support migration of C6 glioma cells in an aggregate migration assay. A recombinant fusion protein including fibronectin type III (FNIII) repeats 2-6 (TNfn2-6) was the only fragment found to promote migration of C6 glioma cells at levels similar to that promoted by intact TN. Evaluation of smaller segments and individual FNIII repeats revealed that TNfn3 promoted migration and attachment of glioma cells and TNfn6 promoted migration but not attachment. While TNfn3 and TNfn6 promoted migration individually, the presence of both TNfn3 and TNfn6 was required for migration on segments of the FNIII region that included TNfn5. TNfn5 inhibited migration in a dose dependent manner when mixed with TNfn3 and also promoted strong attachment and spreading of C6 glioma cells. TNfn3 and TNfn6 promote cell migration and may function cooperatively to overcome the inhibitory activity of TNfn5. Additional cell attachment studies suggested that both β1 integrins and heparin may differentially influence the attachment of glioma cells to TN fragments. Together, these findings show that C6 glioma cells integrate their response upon binding to at least three domains within TN.

Key words: Extracellular matrix, Type III repeat, Cell attachment, Adhesion, Counter adhesion

INTRODUCTION

Tenascin (TN) is a multidomain extracellular matrix (ECM) protein that is expressed during early embryonic development at sites coinciding with epithelial-mesenchymal transformations and cell migration (reviewed by Crossin et al., 1996). TN expression is decreased in the adult except in regions of secondary neurogenesis and cell migration, such as the lateral subventricular zone (Miragall et al., 1990; Gates et al., 1995; reviewed by Faissner, 1996). The protein is synthesized by astrocytes in the developing CNS (Bourdon et al., 1983; Prieto et al., 1990; Grierson et al., 1990) and its expression is upregulated in reactive astrocytes (Laywell et al., 1992) and in association with high grade astrocytomas (Bourdon et al., 1983; Higuchi et al., 1993; Zagzag et al., 1995). The presence of TN in the ECM produced by astrocytomas and gliomas in vivo may contribute to the high invasive potential of these tumor cells (Kleihues et al., 1995).

The observation that TN is expressed at high levels in astrocytomas (Bourdon et al., 1983; McComb and Bigner, 1985; Higuchi et al., 1993; Zagzag et al., 1995) prompted examination of its effects on the migration of primary astrocytomas or glioma cell lines in vitro in comparison with the effects of other ECM proteins (Giese et al., 1994, 1995; Friedlander et al., 1996; Deryugina and Bourdon, 1996; Giese et al., 1996). Glioma cell lines and cells derived from primary tumors displayed a differential response to various ECM substrates (Giese et al., 1994, 1995; Friedlander et al., 1996). In glioma cell lines, TN stimulated migration at a level comparable to or higher than that on other ECM proteins (Giese et al., 1995; Deryugina and Bourdon, 1996), although TN promoted a low to moderate level of migration of primary tumor cells (Giese et al., 1995; Friedlander et al., 1996). Migration of human glioma cell lines on TN was inhibited by function-blocking antibodies to β1, α2, or αv integrin subunits (Deryugina and Bourdon, 1996; Friedlander et al., 1996; Giese et al., 1996) and stimulated by antibodies to α3 integrin subunits (Giese et al., 1996). The detailed mechanisms by which TN promotes migration remain unknown but, based on these findings, are likely to involve the contribution of several cellular receptors and binding sites within the molecule.

TN is composed of a linear arrangement of domains homologous to epidermal growth factor (EGF), fibronectin type III (FNIII) repeats, and fibrinogen (fg) (Jones et al., 1990). Recombinant domains within the FNIII region tested in previous studies did not support migration (Deryugina and Bourdon, 1996). The third FNIII repeat, which contains an RGD tripeptide sequence, has been shown to interact with cells via a variety of integrins, including αvβ3, αvβ6, α2β1, and α9β1 (Prieto et al., 1992, 1993; Joshi et al., 1993; Sriramarao

et al., 1993; Yokosaki et al., 1994; Phillips et al., 1995; Götz et al., 1996; Dörries et al., 1996). A site in the sixth FNIII repeat was shown to interact with β1 integrins (Phillips et al., 1995) and to promote the extension of neurites from primary cultures of central or peripheral neurons (Phillips et al., 1995; Götz et al., 1996; Dörries et al., 1996). TN binds to a number of other cell surface proteins including the immunoglobulin superfamily member contactin (Zisch et al., 1992; Weber et al., 1995), cell surface annexin II (Chung and Erickson, 1994), a cell surface form of the chondroitin sulfate proteoglycan, phosphacan (Grumet et al., 1994), as well as to ECM proteins such as fibronectin, collagens, and proteoglycans (reviewed by Crossin et al., 1996).

In the present study, we examined recombinant TN domains for their effects on C6 glioma cell migration. We found that, individually, both TNfn3 and TNfn6 promoted cell migration and that TNfn5 in combination with the migration-promoting domains inhibited migration. On segments of TN that contained TNfn5, both TNfn3 and TNfn6 were required to overcome the inhibitory effect on glioma migration of TNfn5. Cell attachment studies suggested that TNfn5 may bind cells through a cell surface heparin sulfate proteoglycan and a β1 integrin. The results from this study demonstrate that TN contains within the FNIII repeat region at least three domains that support attachment or influence migration for C6 glioma cells and that cells integrate their response upon binding to these regions simultaneously.

MATERIALS AND METHODS

TN fragment constructs, fusion proteins and ECM proteins

The generation of TNegf, TNfn2-6, TNfn4-6, TNfn3, TNfn5, TNfn6, the alternatively spliced repeats, and TNfn7-8fg GST fusion proteins from the cDNA sequence of chicken TN (Jones et al., 1990) has been described elsewhere (Prieto et al., 1992, 1993; Phillips et al., 1995). TNfn3-6 and TNfn3-5 were generated by PCR amplification of the corresponding FNIII repeats using a common 5′ primer that contained a *Bam*HI restriction site followed by the first 21 nucleotides corresponding to the 5′ end of the coding sequence for the third FNIII repeat in TN. The 3′ primer for the fifth repeat contained an *Eco*RI site followed by the complement of the last 21 nucleotides of the coding sequence for this repeat. The 3′ primer for the sixth repeat corresponded to the last 21 nucleotides of the coding sequence for this repeat. The sixth repeat contains a natural *Eco*RI site 6 base pairs from the 3′ end of the repeat and this site was used in construction of fusion proteins containing the sixth repeat. Amplified DNA fragments were gel purified, digested with *Bam*HI/*Eco*RI, and ligated into pGex4T2 (Pharmacia) vector that had been previously digested with *Bam*HI/*Eco*RI. Fusion protein constructs were verified by DNA sequencing and transformed into *E. coli* strains NM522 or BL21/DE3. Single colonies were grown into one liter cultures, and induction and purification of the fusion proteins was performed as described (Prieto et al., 1992). Lyophilized fusion proteins were dissolved in sterile phosphate buffered saline (PBS), pH 7.4, and stored in small aliquots at −70°C.

To verify the integrity of the fusion protein preparations, 1-5 μg of each fusion protein were electrophoresed on 10% polyacrylamide gels and stained with Coomassie blue, or transferred to nylon membranes and probed with either anti-TN antiserum (Hoffman et al., 1988) or with an antibody to a 35 kDa cyanogen bromide fragment of TN (anti-35 kDa CNBr) (Friedlander et al., 1988). Specific bands were visualized using the appropriate secondary antibodies and chemiluminescence detection (ECL, Amersham).

Substrate preparation

Petri dishes (Falcon #1008) were coated with TN (Gibco/BRL) at a concentration of 100 μg/ml, or fibronectin (FN), laminin (LN), or collagen I (COL) at 40 μg/ml (Collaborative Biomedical Products). Purified glutathione S-transferase (GST) or fusion proteins of GST and the various TN domains were coated at a concentration of 1 or 10 μM as noted. Concentrations of all fusion proteins in solution were determined by the method of Bradford (1976). Proteins were diluted to the appropriate concentration in sterile PBS, pH 7.4. 200 μl of protein solutions were applied in a circular area 1.5 cm in diameter at the center of the culture dishes. Proteins were coated for 30 minutes, removed from the plate by aspiration, and the plates were washed three times in 1% bovine serum albumin (BSA) in PBS (1% BSA/PBS). Non-specific protein binding sites on the substrate were blocked by treatment with 1% BSA/PBS for 30 minutes. After the blocking period, the BSA solution was aspirated and 100 μl of the suspension of cellular aggregates was applied (see below).

To assess the amount of TN GST-fusion proteins bound to plastic substrates, an ELISA assay was performed using an antibody to GST, which therefore recognized an identical site within each fusion protein. Amounts of the TN GST-fusion proteins bound were compared with levels of GST alone bound at identical coating concentrations. Solutions of fusion proteins in PBS were coated on non-tissue culture treated round-bottom 96-well polystyrene plates (Falcon, #3918) at concentrations of 0.1, 0.5, 1.0, and 10.0 μM. The plates were incubated with 1% BSA/PBS for 30 minutes, then with antibody to GST (Pharmacia, 1:5,000) in the same solution for one hour followed by anti-goat IgG coupled to alkaline phosphatase for one hour (Sigma, 1:20,000). The color reaction using *p*-nitrophenyl phosphate (Sigma, N9389; used according to the manufacturers' specifications) was allowed to proceed for 15 minutes at which time the reaction was terminated with 2 M NaOH and the relative amount of reaction product was determined spectrophotometrically at 405 nm. The amount of GST bound to the substrate at 1 μM was 77% of the amount bound at 10 μM. To determine the relative levels of binding of each fusion protein, the amount of GST bound was set to 100% at each concentration. The amount of TN fusion protein bound to the substrate relative to GST was as follows. At a coating concentration of 1 μM: TNfn3-6, 105%; TNfn3-5, 101%; TNfn4-6, 92%; TNfn3, 78%; TNfn4, 72%; TNfn5 116%; TNfn6, 114%. At a coating concentration of 10 μM: TNfn3-6, 126%; TNfn3-5, 115%; TNfn4-6, 114%; TNfn3, 46%; TNfn4, 40%; TNfn5, 103%; TNfn6, 115%. For experiments where mixtures of other FNIII repeats with TNfn3 were used as substrates, a coating concentration of 1 μM for TNfn3 was used to allow additional protein to bind.

Migration assays

The rat glioma cell line C6 (ATCC CCL-107) was grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, and 10 mM L-glutamine and penicillin/streptomycin. Aggregate cultures of the rat glioma cell line C6 were grown as follows: confluent monolayers of C6 glioma were trypsinized and seeded into 50 ml sterile, siliconized flasks at a density of 1×10^6 cells in 10 ml of medium. The flasks were rotated at 100 rpm on a gyratory shaker at 37°C and 5% CO_2 for 4-5 days. After the growth period, the aggregates were placed into a 15 ml tube and allowed to settle by gravity. The medium was aspirated and the aggregates were washed three times by gentle resuspension in 10 ml Opti-mem (Gibco) without additives. After washes, the aggregates were resuspended in 1-3 ml of Opti-mem such that 100 μl containing approximately 30 aggregates was applied to the precoated substrates. The plates were incubated at 37°C and 5% CO_2. Migration was allowed to proceed for 20 hours at which time the cultures were fixed in 1% glutaraldehyde and photographed.

To evaluate the effect on cell migration of pretreatment of the substrates with heparin or the anti-35 kDa CNBr antibody, the protein-coated substrates were first blocked with 1% BSA/PBS for 30 minutes, the BSA solution was aspirated and a solution of either

heparin (Sigma) at a concentration of 100 μg/ml in PBS or anti-35 kDa CNBr fragment antibody (total immunoglobulin fraction) at a concentration of 2.2 mg/ml in PBS was applied over the substrates for 60 minutes. After treatment, the heparin or antibody solution was aspirated, the plates were washed 3 times in 1% BSA/PBS and blocked for an additional 30 minutes in the same solution prior to the addition of the aggregates.

Quantitation of migration

Aggregates were judged as suitable for quantitation if the cells migrating from them did not come into contact with cells from any other aggregate. In most cases, aggregates were of roughly the same size (approximately 300 μm) and migration from each aggregate on a given substrate was relatively uniform. All aggregates suitable for quantitation were photographed with a 10× objective and an enlarged photographic print was produced. The average distance of cell migration from each aggregate was derived from measurement at eight equidistant axes that were established at random. Migration along each axis was measured as the distance from the edge of the aggregate to the leading edge of the farthest cell from the edge along the particular axis. The eight measurements for each aggregate were averaged and migration on a particular substrate was determined from multiple aggregates. Results shown are representative of two to three independent experiments.

Attachment assays

Short term attachment to fusion protein and ECM protein substrates was evaluated as described (Prieto et al., 1993; Phillips et al., 1995). The coating concentration for fusion proteins was 10 μM. To evaluate the effect of inhibitors to β1 integrin subunits on attachment, a monoclonal antibody to rat β1 integrin subunits (Mendrick and Kelly, 1993), purchased from Pharmingen, was added to the cell suspension at a concentration of 40 μg/ml. Cells were incubated in suspension with or without the antibody for 20 minutes with occasional mixing before adding to the substrates. The substrates were also treated with heparin as described above for the migration assays.

RESULTS

Glioma migration on purified ECM proteins and major TN domains

The ability of TN to support the migration of the rat C6 glioma cell line was evaluated and compared to the activity of other purified ECM proteins and poly-l-lysine (PLL) (Fig. 1A). C6 glioma cells were grown in rotational suspension cultures for a period of 4-5 days at which time they formed uniform aggregates of approximately 200-300 μm in diameter. These aggregates were harvested, gently washed and plated in Opti-Mem medium on non-tissue culture plastic substrates coated with either TN, fibronectin (FN), collagen I (COL), laminin (LN), PLL (Fig. 1A) or BSA (not shown). All of the ECM proteins promoted the migration of cells away from the aggregates after 20 hours in culture, although the extent of migration on the different substrates varied. PLL did not support a significant amount of cell migration even though the aggregates remained attached. Substrates treated with BSA did not support attachment of the aggregates (not shown). A quantitative assessment of migration on the different substrates (Fig. 1B) revealed that TN promoted C6 glioma migration at a level similar to FN but less than COL or LN. The average distance of migration for each aggregate ranged from 85±4.4 μm on FN to 193±5.5 μm on LN.

In an initial attempt to define regions within TN that promote glioma migration, we tested a series of non-overlapping recombinant proteins corresponding to the majority of the TN

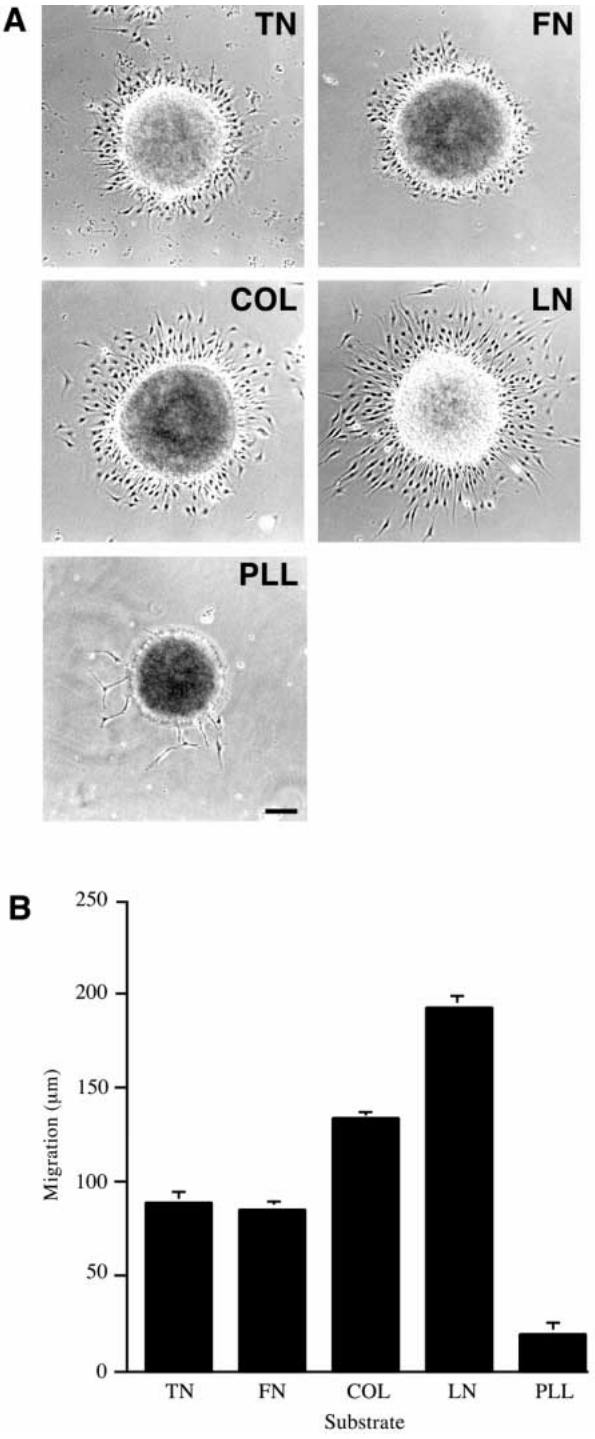

Fig. 1. (A) Migration of C6 glioma cells from aggregate cultures plated on ECM protein substrates. Tenascin (TN), fibronectin (FN), laminin (LN) and Collagen type I (COL) promoted migration of cells away from aggregates but poly-l-lysine (PLL) did not promote significant levels of migration. Bar, 100 μm. (B) Quantitative analysis of migration on different substrates. TN promoted levels of migration similar to FN but less that COL and LN. Values in B were determined from 9-13 aggregates per substrates and are expressed as average distance of migration per aggregate ± s.e.m.

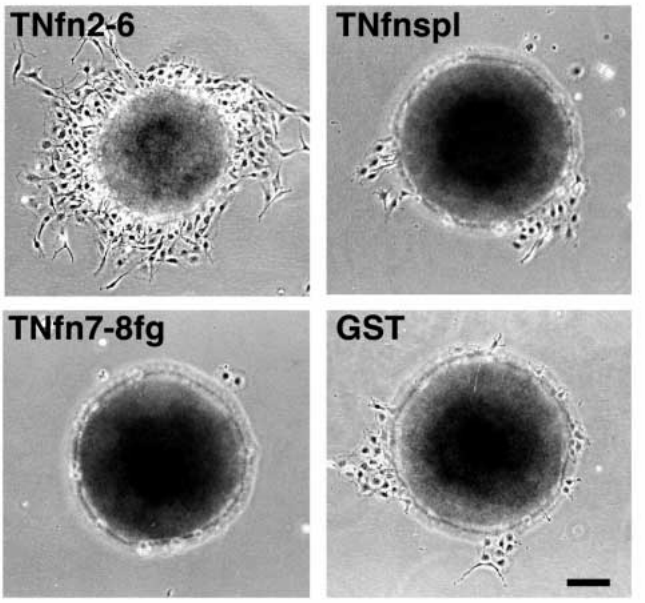

Fig. 2. Glioma migration on TN fusion proteins. Cells migrated away from aggregates plated on substrates coated with TNfn2-6 but few migrating cells were observed from aggregates on substrates coated with TNspl, TNfn7-8fg or GST. Cells did not attach to TNegf (not shown). Bar, 100 μm.

protein (Prieto et al., 1992). The fusion proteins tested span the EGF like repeats (TNegf), TNfn2-6, the alternatively spliced FN repeats (TNspl), and the distal two FN repeats plus the fibrinogen domain (TNfn7-8fg) (see Fig. 3A). The ability of these non-overlapping fusion proteins to support or inhibit short term cell attachment of a variety of cell types has been previously described (Prieto et al., 1992, 1993). Of these fusion proteins, only TNfn2-6 was able to stimulate the migration of cells from C6 glioma aggregates (Fig. 2). Aggregates attached on TNspl or TNfn7-8fg but few cells migrated; aggregates did not attach to substrates coated with TNegf (not shown). Substrates coated with GST alone supported attachment of aggregates but did not promote migration.

TNfn fragment constructs

To characterize further the FNIII repeats that mediate glioma migration, smaller segments were generated within the region spanning TNfn2-6 (Fig. 3). A segment spanning FNIII repeats 3-6 (TNfn3-6), repeats 4-6 or repeats 3-5 (TNfn4-6 and TNfn3-5) were generated (Fig. 3B), as well as the individual FNIII repeats TNfn3, TNfn4, TNfn5, and TNfn6 (Fig. 3C). A fusion protein containing the third FNIII repeat followed by the sixth FNIII repeat (TNfn3/6) was also generated to evaluate the effect of deletion of TNfn4 and TNfn5 (Fig. 3D) as described below. The fusion proteins were expressed in bacteria, purified, and analyzed by SDS-polyacrylamide gel electrophoresis. Gels were stained with Coomassie blue (Fig. 4, top panels) or transferred to nylon membranes and probed either with polyclonal antibodies against TN (Hoffman et al., 1988) (Fig. 4, middle panels) or with an antibody against a 35 kDa CNBr fragment of TN (anti-35 kDa CNBr) (Fig. 4, bottom panels). This antibody has been shown to block the binding of neurons and glia to intact TN in short term adhesion assays (Friedlander et al., 1988). All TN fusion proteins, but not GST alone, reacted strongly with polyclonal antibodies to purified TN, however, only fragments containing either TNfn6 or TNspl reacted with anti-35 kDa CNBr (Fig. 4, bottom panels). The domain-specific reactivity of this polyclonal antibody was utilized in experiments to block the function of TNfn6 (see below).

Migration on TNfn fragments

The deletion fragments and individual FNIII repeats were compared for their ability to support glioma migration. Cells migrated away from aggregates plated on TNfn3-6, TNfn3, and TNfn6, but not from aggregates plated on TNfn3-5, TNfn4-6, TNfn4, TNfn5 and GST (Fig. 5A). The extent of migration on these fusion proteins was evaluated quantitatively at coating concentrations of 1 μM and 10 μM (Fig. 5B, see Materials and

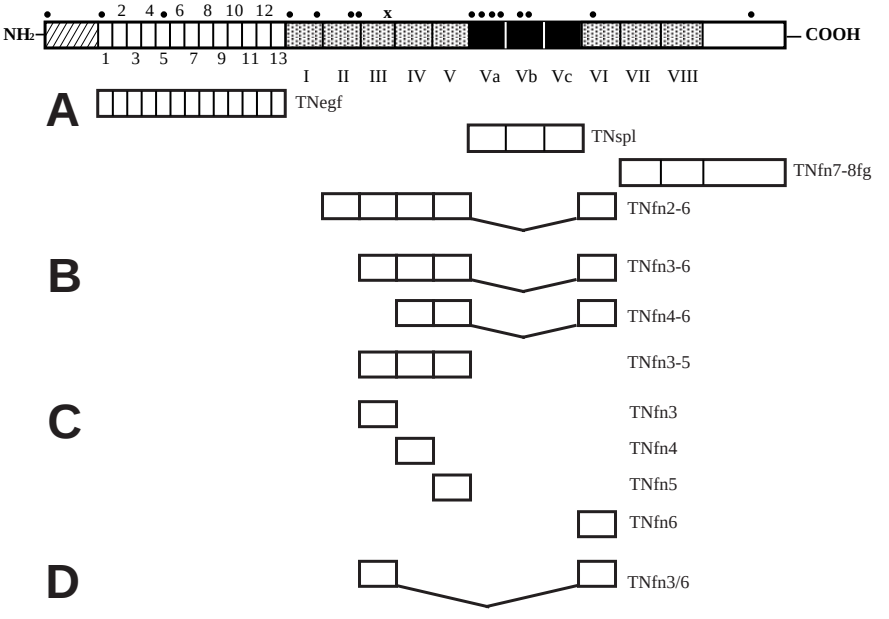

Fig. 3. The recombinant fusion proteins used to map the FNIII repeats of TN that mediate the migration of glioma cells. Schematic representation of TN fusion protein constructs based on the linear cDNA model for chicken TN (Jones et al., 1990). Fragments of TN were generated as described in Materials and Methods corresponding to the indicated domains of TN. Some of the proteins have been previously described (Prieto et al., 1992, 1993; Phillips et al., 1995). (A) Multiple repeat fragments spanning TN. (B) Multiple repeat fragments within TNfn2-6. (C) Individual FNIII repeats. (D) A fusion protein containing TNfn3 immediately adjacent to TNfn6 (TNfn3/6).

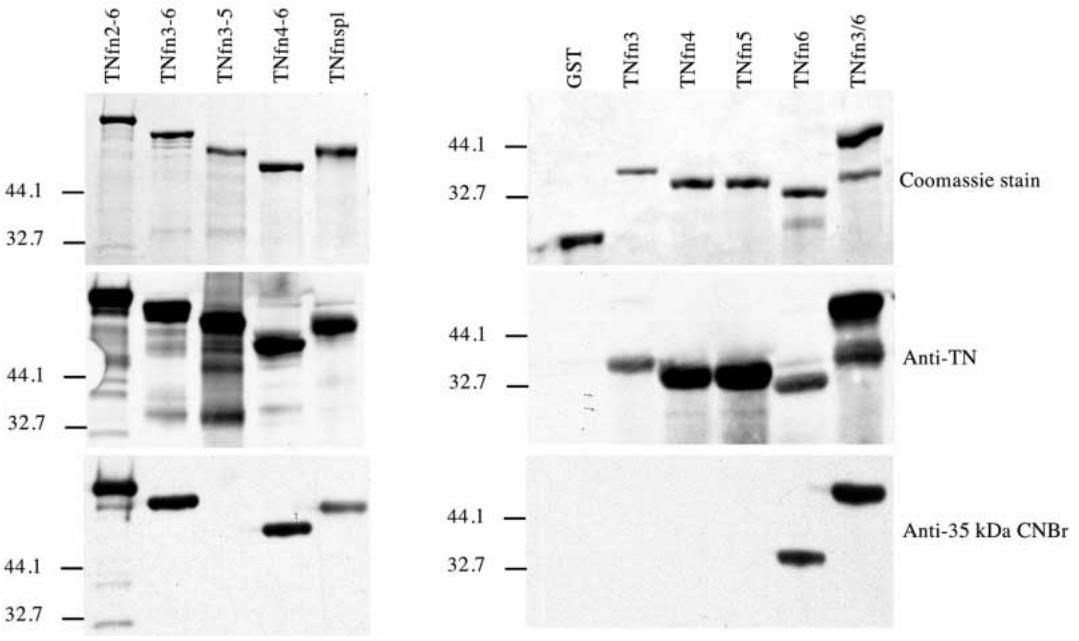

Fig. 4. Polyacrylamide gel electrophoresis of TN fragments. 3 mg of purified fusion proteins were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and stained with Coomassie blue (top panels) or probed with either a polyclonal antibody to TN (middle panels) or an antibody to a 35 kDa cyanogen bromide fragment of TN (anti-35 kDa CNBr; bottom panels). The anti-35 kDa CNBr antibody shows strong reactivity with fusion proteins containing TNfn6 and also shows reactivity to TNspl. Molecular mass standards (in kDa) are shown at right.

Methods). When coated at 1 μM TNfn3-6, TNfn3, and TNfn6 promoted an average migration per aggregate of 132±3.8, 130±5.5, and 98.7±4.4 μm, respectively, over the 20 hour time period (Fig. 5B). Increasing the coating concentration to 10 μM increased the amount of protein bound to the plastic (see Materials and Methods) but did not enhance migration further. We conclude that although TNfn3 and TNfn6 alone promote migration, the activity of both of these repeats is required to promote migration on larger segments of the molecule that included repeats 4 and 5.

To provide further support for the idea that both TNfn3 and TNfn6 are required for migration in the presence of TNfn5, we prepared a chimeric fusion protein containing only the migration-promoting fragments TNfn3 and TNfn6 (TNfn3/6). We compared the activity of this chimeric protein with that of TNfn3-6 in the presence or absence of the anti-35 kDa CNBr antibody to block the function of TNfn6 (Table 1). Treatment of TNfn3-6 and TNfn3/6 with the anti-35 kDa CNBr blocked migration on these substrates by 100% and 46%, respectively. The complete inhibition of migration on TNfn3-6 when the function of TNfn6 is blocked is consistent with the observed lack of migration on the TNfn3-5 protein (Fig. 5). The partial inhibition of migration on TNfn3/6 may reflect a steric effect of the antibody partially to block TNfn3 when it is immediately adjacent to TNfn6 as it is in the chimeric protein. In contrast, the anti-35 kDa CNBr did not reduce the ability of FN to promote migration (Table 1).

These results suggested that an inhibitory activity for migration was present within FNIII repeats 4 or 5. To address this possibility, mixed substrates of the individual FNIII repeats were used. A fixed concentration of TNfn3 (1 μM) was mixed with the fusion proteins TNfn3, TNfn4, TNfn5, or TNfn6, at concentrations of 0.1 μM, 0.7 μM or 1.2 μM and used as substrates for glioma migration (Fig. 6). Addition of TNfn3, TNfn4, or TNfn6 did not substantially affect migration at any concentration tested. In contrast, addition of TNfn5 caused a dose-dependent inhibition of migration. At a coating concentration of 1.2 μM of TNfn5, migration was decreased to 32% of control values (Fig. 6). The combined results suggest that TNfn5 specifically inhibits cell migration and that TNfn3 and TNfn6, together, overcome this inhibition.

Heparin does not block the migration inhibitory activity of TNfn5

Previous studies have demonstrated that TNfn5 binds to heparin (Weber et al., 1995) and this binding activity may be important for modulating the cellular effects of TN (Wehrle-Haller and Chiquet, 1993). To determine whether heparin

Table 1. Glioma migration on TN fragments in the presence and absence of the anti-35 kDa CNBr antibody

Substrate	Control	Anti 35 kDa CNBr Ab	Percentage change
TNfn3-6	110±6	8±6	−100
TNfn3/6	130±7	70±8	−46
FN	140±12	160±7	+14

Distance of migration (μm) was calculated as described in Materials and Methods. Values ± s.e.m. are derived from 6-21 aggregates per condition.

Table 2. Glioma migration on TN fragments in the presence and absence of heparin

Substrate	Control	Heparin	Percentage change
TNfn3-6	95±7	21±4	−78
TNfn3/6	112±6	125±10	+12
TNfn3-5	10±6	4±3	–
TNfn4-6	1±1	2±2	–
TN	132±4	136±6	+3
FN	108±5	131±7	+21

Distance of migration (μm) was calculated as described in Materials and Methods. Values ± s.e.m. are derived from 6-21 aggregates per condition.

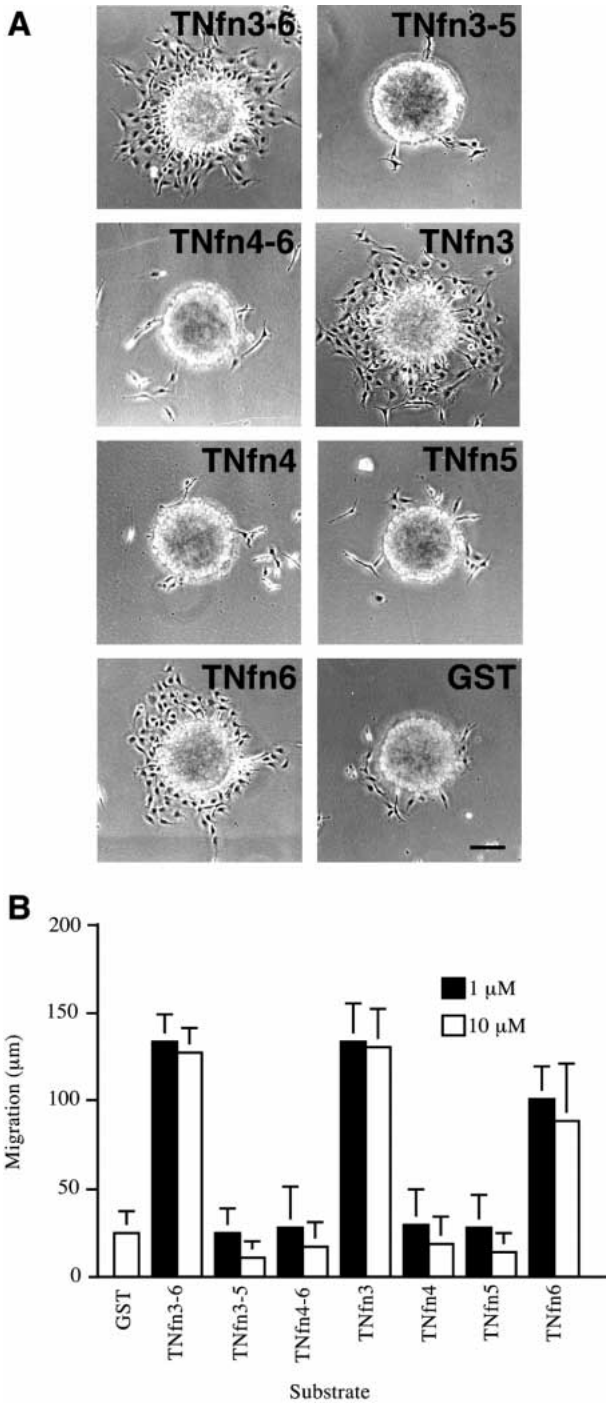

Fig. 5. (A) Migration of C6 glioma cells is promoted by both TNfn3 and TNfn6. Migrating cells were observed on TNfn3-6-coated substrates but few cells were observed from aggregates on TN3-5 or TNfn4-6-coated substrates. Glioma cell migration was also observed on substrates coated with TNfn3 or TNfn6, but not on TNfn4, TNfn5, or GST. Bar, 100 μm. (B) Quantitative analysis of migration on TN fragments. TNfn3-6, TNfn3 and TNfn6 all support robust migration while migration on TNfn3-5, TNfn4-6, TNfn4, TNfn5 was low and similar to GST substrates. Values shown ± s.e.m. are derived from 9-20 aggregates per substrate.

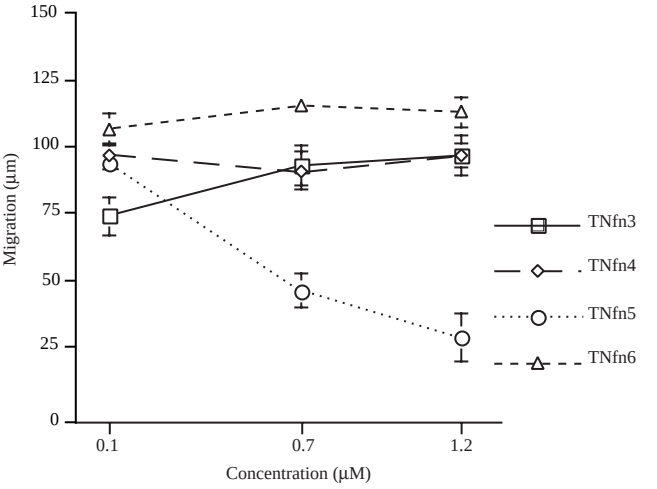

Fig. 6. Glioma migration on substrates of TNfn3 mixed with increasing concentrations of other FNIII repeats. Migration was not substantially affected by addition of TNfn3, TNfn4, or TNfn6. In contrast, addition of TNfn5 caused a dose-dependent decrease in migration that reached 68% inhibition relative to TNfn3 control values at a concentration of 1.2 μM. Values shown ± s.e.m. are derived from 8-21 aggregates per substrate condition.

binding to TNfn5 influenced migration, glioma cell migration on different substrates was evaluated with or without pretreatment of the substrate with heparin (Table 2). Treatment of TNfn3-6 with heparin reduced migration by 78% but heparin treatment did not reduce migration on TNfn3/6, TN, or FN substrates. These results show that heparin treatment reduces cell migration on TNfn3-6 and that removal of TNfn4 and TNfn5 abolishes the inhibition by heparin. There was little migration on TNfn3-5 and TNfn4-6 and this was not altered by heparin pretreatment. Taken together with studies that have shown that TNfn5 specifically binds heparin (Weber et al., 1995), these results suggest that binding of soluble heparin to TNfn5 does not block the migration inhibitory site within TNfn5. Inasmuch as heparin blocked migration on TNfn3-6, it may when bound to TNfn5 sterically block the migration promoting sites within TNfn3 or TNfn6.

C6 glioma cell attachment to TN fragments

To determine whether the ability of the TN domains to influence cell migration was related to their ability to mediate attachment of cells to the substrate, we compared the ability of the various segments of TN to promote short term attachment of C6 glioma cells (Friedlander et al., 1988; Prieto et al., 1992) (Fig. 7). In contrast to their differential effects on migration, the fusion proteins TNfn3-6, TNfn3-5, and TNfn5 all promoted abundant cell attachment and spreading of C6 glioma cells. TNfn4-6 promoted attachment but the cells displayed less spreading than the other multiple domain substrates (Fig. 7). Cells also bound to TNfn3 and TNfn3/6 but did not spread. The fusion proteins TNfn4 and TNfn6 (Fig. 7) did not support attachment above that observed on control substrates of GST (not shown). In this assay, intact TN, FN, LN and COL all supported robust attachment and spreading.

The short term attachment to TN domains was evaluated quantitatively in the presence or absence of anti-β1 integrin antibody or after heparin pretreatment of the substrate (Table 3). Attachment to all of these substrates was inhibited to a large

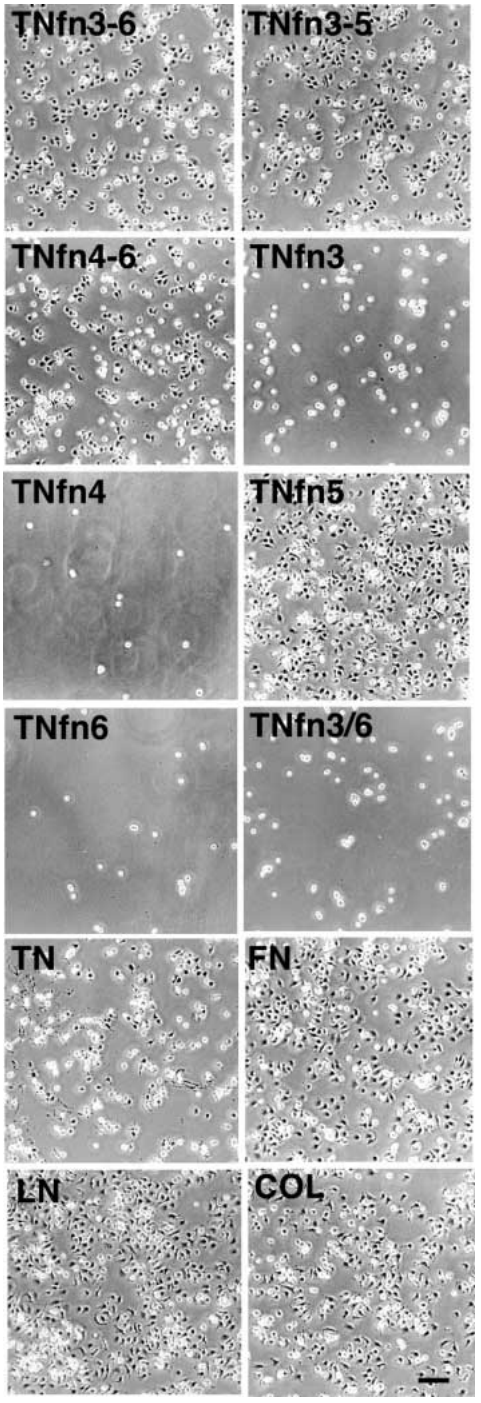

Fig. 7. Short term attachment of dissociated C6 glioma cells to TN substrates and ECM proteins. Cell attachment and spreading was observed on TNfn3-6, TNfn3-5, and TNfn5. Slightly less spreading was observed on TNfn4-6. Lower attachment, but not spreading was observed on TNfn3. TNfn4 and TNfn6 did not support a significant level of attachment. The ECM proteins TN, FN, LN and COL all supported both attachment and spreading. Bar, 100 μm.

extent when the cells were incubated with the anti-β1 integrin antibody. Pretreatment of the substrate with heparin dramatically reduced attachment to TNfn3-6, TNfn3-5 and TNfn4-6, TNfn5, TN, and COL. In contrast, attachment was reduced to a minor extent on TNfn3/6 and attachment was not affected on TNfn3 and LN substrates. C6 glioma cells pretreated with heparitinase bound to all substrates including the BSA-blocked control substrate (data not shown). This finding precluded the assessment of whether heparan sulfate proteoglycans on these cells may serve as TN receptors. These results suggest that β1 integrin subunits may be involved in mediating attachment to TNfn3 and TNfn5 and that β1 integrins may function in cooperation with a cell surface heparin sulfate proteoglycan in mediating the effects of TNfn5.

DISCUSSION

We have identified repeats in TN that mediate its previously described ability to support glioma cell migration (Deryugina and Bourdon, 1996; Giese et al., 1996; Friedlander et al., 1996). Recombinant domains encompassing the majority of the TN molecule were assayed and it was found that a recombinant fusion protein containing TNfn2-6 supported the migration of individual cells from C6 glioma aggregate cultures at a level comparable to that supported by intact TN. TNfn7-8fg and TNspl did not support migration and the EGF domain, which is counteradhesive (Prieto et al., 1992; Götz et al., 1996; Dörries et al., 1996), did not support attachment and therefore could not be evaluated in the aggregate migration assay. Evaluation of individual FNIII repeats revealed that TNfn3 promoted migration and attachment of glioma cells and TNfn6 promoted migration but not attachment. While both TNfn3 and TNfn6 promoted migration individually, the presence of both TNfn3 and TNfn6 was required for migration on larger segments of the FNIII region that included TNfn5. TNfn5 inhibited migration in a dose dependent manner when mixed with TNfn3 and also promoted strong attachment and spreading of C6 glioma cells. Additional studies suggested that heparin and β1 integrins may be involved in C6 glioma cell attachment to TNfn5. The results suggest that glioma cells integrate the signals generated upon simultaneous binding to distinct migration- and attachment-promoting domains within TN.

Previous studies have demonstrated that a variety of glioma-derived cell lines migrate well on TN substrates (Giese et al., 1995, 1996; Deryugina and Bourdon, 1996; Friedlander et al., 1996). Both the migration and attachment of a subclone of the U251-MG glioma cell line were supported by TN substrates but not by fusion proteins that span either TNfn1-5, TNfn 4-5 plus the first 4 alternatively spliced domains, or the distal 3 alternatively spliced repeats plus the distal three constant repeats (fn6-8) of human TN (Deryugina and Bourdon, 1996). These results are consistent with our finding that the inhibition of migration is the predominant effect of TNfn fusion proteins containing TNfn5 unless both TNfn3 and TNfn6 are present. Our preliminary studies indicate that U251-MG glioma cells bound more to TNfn3-6 than to any other segment or individual repeat (data not shown), whereas C6 glioma cells bound equally well to substrates coated with TNfn3-6, TNfn3-5, TNfn4-6 and TNfn5. It is possible that C6 glioma and U251-MG glioma cells express different sets of TN receptors accounting for their differential responses.

Integrins are major cellular receptors for TNfn domains (reviewed by Crossin et al., 1996). TNfn3 contains well

Table 3. Short term cell attachment to TN, TN domains and other ECM proteins in the presence and absence of anti-β1 integrin and heparin

		Inhibitors			
Substrate	Control	Anti-β1	Percentage change	Heparin	Percentage change
TNfn3-6	314±27	145±27	−54	4±1	−99
TNfn3-5	313±23	80±16	−74	21±5	−93
TNfn4-6	315±17	17±5	−95	9±3	−97
TNfn3	100±16	15±3	−85	107±16	+7
TNfn4	9±12	3±1		10±2	
TNfn5	425±16	17±7	−96	59±21	−86
TNfn6	10±3	0		19±6	
TNfn3/6	84±22	0	−100	68±4	−20
TN	187±20	0	−100	97±12	−48
FN	303±23	81±30	−73	137±15	−55
LN	396±30	40±5	−90	517±71	+31
COL	243±12	0	−100	44±8	−82
PLL	662±38	434±21	−34	0	−100

Attachment was determined as the average number of cells bound per 380 mm^2 ± s.e.m. for each condition. Results shown are representative of two independent experiments.

characterized binding sites for integrins (Prieto et al., 1993; Yokosaki et al., 1994) and mediates attachment of a number of cell types including C6 glioma cells. TNfn6 has been shown to promote cell attachment of fibroblasts and neurons (Phillips et al., 1995; Götz et al., 1996; Dörries et al., 1996) that was sensitive to inhibitors of β1 integrins. The results from our short term assays indicate that β1 integrins are involved in the attachment of C6 glioma cells to multiple TN domains including TNfn3 and TNfn5, but we could not determine the role of β1 integrins in promoting C6 glioma migration. Addition of a function-blocking antibody to rat β1 integrins (Mendrick and Kelly, 1993) in the glioma migration assay often resulted in detachment of a large number of the aggregates even when the antibody was added 8 hours after plating the aggregates (data not shown). Other studies using different types of migration assays have shown that glioma migration was blocked in the presence of antibodies to β1 integrins (Deryugina and Bourdon, 1996; Giese et al., 1996; Friedlander et al., 1996) and αv integrins (Friedlander et al., 1996; Deryugina and Bourdon, 1996) and was stimulated by α3 integrin antibodies (Giese et al., 1996).

Receptors in addition to integrins may also influence glioma migration on TNfn domains. TNfn5 has previously been shown to interact with heparin, heparan sulfate, and dermatan sulfate (Weber et al., 1995, 1996) but had not been shown to promote cell attachment. It is therefore significant that TNfn5 supported strong attachment of C6 glioma cells (Fig. 7 and Table 3) as well as of primary rat astrocytes (not shown). In our studies, treatment of the substrate with heparin blocked cell attachment to TNfn5 suggesting that a cell surface heparin sulfate proteoglycan could participate in this interaction. Heparin did not block the ability of TNfn5 to inhibit migration suggesting the possibility of another receptor for this domain. The dissociation between migratory and adhesive domains in TN has also been shown in studies of oligodendrocyte precursors (Kiernan et al., 1996). Heparin has also been shown to block the binding site for the cell adhesion molecule, contactin (Zisch et al., 1992; Weber et al., 1996), however, this molecule was not found in the C6 glioma cell line used in the current study (unpublished observations), precluding it as a receptor in these cells. The transmembrane form of the proteoglycan phosphacan could serve as a receptor for TN since it is expressed by C6 glioma cells (Sakurai et al., 1996) and can bind TN (Grumet et al., 1994; Milev et al., 1997).

The migration assay measures the cumulative effects of prolonged cell substrate interaction. This extended interaction time may lead to alterations in cellular signaling and gene expression mediated by integrins or other TN receptors (reviewed by Meredith et al., 1996; Crossin et al., 1996). Indeed, interaction of cells with TN has been shown in a number of systems (Tremble et al., 1994; Jones et al., 1995; Tranque et al., 1996; Crossin et al., 1996) to result in changes in gene expression. For example, metalloproteinase expression is increased in TN-treated cells (Tremble et al., 1994) and this change may lead to alterations in the ECM which in turn could influence cell morphology, attachment, and gene expression. Controlled degradation of TN itself by matrix metalloproteinases has been observed (Imai et al., 1994; Siri et al., 1995), which could generate TN fragments that have activities independent of the intact TN molecule. For example, proteolysis may produce segments similar to those observed in the current study and alter migration-promoting activity. Alternatively, the presence of different receptors for TN domains could alter the ability of cells to migrate on fragments of TN. These potential mechanisms of modulating cell migratory responses may account for the widely different responses to TN observed in cells isolated from various grades of primary tumors (Friedlander et al., 1996). It will be of interest in future studies to compare various signaling pathways and changes in gene expression affected by TN and naturally occurring fragments.

Detailed studies of cell migration using controlled levels of integrin expression and binding affinity have suggested that cell migration proceeds at the greatest rate at an intermediate level of integrin-mediated cell-substrate adhesion (Palecek et al., 1997). In the current study, there was an inverse correlation between adhesive and migratory properties of the individual TN domains TNfn3, 5 and 6. TNfn3 and TNfn6 supported low levels of adhesion and allowed cell migration. In contrast, TNfn5 was strongly adhesive and inhibited cell migration. The

lack of cell migration on the multidomain substrates TNfn3-5 or TNfn4-6, however, does not appear to be caused solely by the increased or decreased adhesiveness of these substrates since TNfn3-6, TNfn4-6 and TNfn3-5 displayed comparable amounts of short term adhesion in our assay but only TNfn3-6 was able to stimulate migration.

It is likely that the multidomain fragments interact with multiple cell surface receptors. This interpretation is consistent with recent findings (Fischer et al., 1997) demonstrating that the removal of large functional domains within the TN protein results in cellular effects that are not clearly congruent with studies examining the isolated domains. In studies with isolated domains, only the cellular receptors for the particular domains are activated. In contrast, when domains are deleted, their receptors are not involved in cellular responses, but receptors for the remaining domains are potentially activated to contribute to the overall cellular response. Thus the various TN domains exert a series of complex positive and negative influences on multivariable biological processes such as cell morphology, cell migration, and neurite outgrowth. It appears therefore that while the strength of a single receptor-ligand interaction is capable of modulating the levels of cell attachment and cell migration (Palecek et al., 1997), the final effects on attachment and migration in response to TN reflect the summation of multiple domains and their receptors. This example is likely to reflect a more general situation in vivo where a cell with its complement of receptors encounters a diverse network of binding sites contributed by the numerous glycoproteins and proteoglycans within the ECM.

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