Tenascin-R (J1 160/180) inhibits fibronectin-mediated cell adhesion -
functional relatedness to tenascin-C

Penka Pesheva1,*,†, Rainer Probstmeier1, Amy P. N. Skubitz2, James B. McCarthy2, Leo T. Furcht2 and Melitta Schachner1

1Department of Neurobiology, Swiss Federal Institute of Technology, Hönggerberg, 8093 Zürich, Switzerland
2Department of Laboratory Medicine and Pathology, and Biomedical Engineering Center, University of Minnesota, Medical School, 420 Delaware Street SE, Minneapolis, Minnesota 55455, USA
*Present address: Laboratory of Biochemistry, Swiss Federal Institute of Technology, 8092 Zürich, Switzerland
†Author for correspondence

SUMMARY

Cell adhesion and neurite outgrowth on fibronectin is a multistep process modulated by different extra- and intracellular signals. Fibronectin-mediated cell attachment and spreading can be affected in a negative way by tenasin-C, an extracellular matrix glycoprotein expressed in a temporally and spatially restricted manner during early morphogenesis. Tenasin-R (J1-160/180), consisting of two major isoforms of 160 kDa (tenasin-R 160) and 180 kDa (tenasin-R 180) in mammals, is an extracellular matrix glycoprotein of the central nervous system that shares high structural homologies with tenasin-C. Here we show that in relation to fibronectin-mediated adhesion, the two extracellular matrix molecules are also functionally closely related. When offered as mixed substrata with other extracellular matrix molecules, the two tenasin-R isoforms and tenasin-C derived from mouse brain selectively inhibit fibronectin-dependent cell adhesion and neurite outgrowth, and affect cell morphology of different mesenchymal and neural cells. This effect is partially due to interactions at the substrate level that result in a steric hindrance and/or conformational change of the cell binding sites of the fibronectin molecule. In addition, tenasin-R 180 and tenasin-C interact with cells by an RGD- and β1 integrin-independent mechanism, leading to cell rounding and detachment from such substrata. The expression of tenasin-R and tenasin-C in the nervous system at times and locations where fibronectin-mediated cellular processes take place may be related to the role of inhibitory signals in the extracellular matrix in the regulation of cell migration and differentiation in general.

Key words: cell adhesion, extracellular matrix, fibroblast, fibronectin, β1 integrin, inhibitory substrate, neurite outgrowth, tenasin

INTRODUCTION

Morphogenetic events during normal development are critically dependent on cellular interactions with the surrounding extracellular matrix (ECM), which result in signal transduction via transmembrane glycoproteins, gene regulation and altered cell behavior. These interactions could thus navigate cells through the different stages of proliferation, migration and final differentiation (for review, see Hynes and Lander, 1992). Among the most intensely studied ECM molecules, fibronectin (FN) and tenasin (TN) are two structurally related multidomain glycoproteins that affect cellular adhesiveness and differentiation in a positive and negative way (reviewed by Chiquet-Ehrismann, 1990; Adams and Watt, 1993). During development of the nervous system, their action may influence neuronal cell migration and neurite extension (Reichardt and Tomaselli, 1991; Wehrle and Chiquet, 1990; Lochter et al., 1991; Husmann et al., 1992; Prieto et al., 1992). The two molecules deserved our interest for the following reasons.

First, during pattern formation in the peripheral (PNS) and central (CNS) nervous systems, FN supports cell adhesion and neurite outgrowth mediated by interaction with cell surface integrins (an RGD-dependent process) and proteoglycans (an RGD-independent process), two major groups of FN receptors (FNRs) (reviewed by Reichardt and Tomaselli, 1991). Second, TN interferes with FN-mediated cell adhesion in vitro and may thus modulate cellular morphology and differentiation state (Chiquet-Ehrismann et al., 1988; Chiquet-Ehrismann, 1991; Lightner and Erickson, 1990; Probstmeier et al., 1990b). Third, J1 160/180, an ECM molecule in the CNS of mammals that consists of two major isoforms of 160 and 180 kDa (Pesheva et al., 1989), and its species homolog restrictin in the chicken (Rathjen et al., 1991), are structurally related to TN in that they consist of EGF- and FN type III-like repeats, and a C-terminal region homologous to fibrinogen (Nörenberg et al., 1992; Fuss et al., 1993). Together with the newly identified TN-like ECM protein encoded by a gene in the human major histocompatibility locus, termed TN-X (Bristow et al., 1993), they have
recently been defined as members of the TN multigene family with so far identified three family members: TN-R (restrictin and J1 160/180), TN-C (tenascin/cytotactin) and TN-X (the large TN-like protein in human) (for review, see Erickson, 1993). In contrast to TN-C, TN-R has so far been found expressed only in the CNS by oligodendrocytes and some neuronal subpopulations (Fuss et al., 1993; Pesheva et al., 1989; Rathjen et al., 1991). In the mouse CNS, TN-R and TN-C are expressed in different temporal windows and by different cell types during development (Pesheva et al., 1989; Prieto et al., 1990). As well as their structural relatedness, the two ECM glycoproteins display functional similarities. When applied as substrates for neural cells in vitro, they cause repulsion of CNS neurons and inhibit neurite outgrowth into substrate areas enriched in these molecules (Faisssner and Kruse, 1990; Pesheva et al., 1989, 1993). It was therefore an intriguing question as to whether TN-R would display similar functional properties to TN-C in relation to FN-mediated adhesion of cells and, if so, what are the mechanisms behind it.

The aim of the present study was to investigate the functional consequences of the association between the TN-R isoforms or TN-C with FN in FN-mediated cell adhesion and neurite outgrowth, and compare the molecular mechanisms involved.

Here we show that the two TN-R isoforms, TN-R 160 and TN-R 180, and TN-C derived from mouse brain, inhibit FN-dependent cell adhesion and neurite outgrowth, and affect cell morphology. For TN-R 160, this effect is due to interaction with FN, resulting in a covering and/or conformational change in the cell binding sites on the FN molecule. TN-R 180 and TN-C bind to putative cellular receptor(s) different from β1 integrin FNRs, thereby leading to cell rounding and detachment, which imply signal transduction mechanisms that follow the initial recognition event. We propose that TN-R 180 and TN-C are functionally closely related and that their deposition in the ECM can modulate cell migration and differentiation.

MATERIALS AND METHODS

Extracellular matrix proteins

The two TN-R isoforms, TN-R 160 and TN-R 180, were purified from adult mouse brain by immunoaffinity chromatography using 597 and 596 monoclonal antibody columns (Pesheva et al., 1989). TN-C was obtained from early postnatal (P3) mouse brain by immunoaffinity chromatography on a monoclonal TN-C antibody (J1/tm2) column (Faisssner and Kruse, 1990; Loechter et al., 1991). Human plasma FN was purified by sequential ion-exchange and gelatin affinity chromatography (McCarthy et al., 1986). Laminin (LN) from EHS sarcoma was purchased from Sigma. Collagen type IV from mouse EHS sarcoma was isolated after mild pepsin treatment whereby the C-terminal NC1 domain was destroyed (Fahrig et al., 1987).

FN-derived fragments and synthetic peptides

The 75 kDa tryptic fragment containing the central cell-binding domain of FN was generated and purified as described previously (Hayashi and Yamada, 1983; Humphries et al., 1988). The 33/66 kDa heparin binding fragment was purified from tryptic/catheptic digests of FN (McCarthy et al., 1988, 1990). The 45 kDa collagen binding fragment was purchased from Chemicon (La Roche). Peptides from FN were synthesized at the Microchemical Facility of the University of Minnesota using a peptide synthesizer (System 990; Beckman Instruments Co., Fullerton, CA; McCarthy et al., 1990) and conjugated to ovalbumin (Drake et al., 1992). These included the heparin binding peptides FN-C/H-I and FN-C/H-II, containing the sequences YEPGSPPREVVPFRHGGV and KNNQKSEPLRKY, respectively (McCarthy et al., 1988, 1990), and peptide CS1 containing the sequence DLEQVLTPLPHNLGPEILDVPST (Humphries et al., 1987), which does not bind heparin and promotes adhesion of melanoma cells (McCarthy et al., 1990) and neurite extension of dorsal root ganglion (DRG) neurons (Humphries et al., 1988), all located within the 33/66 kDa FN fragment. The molar ratio of each peptide bound to ovalbumin was 1:12 for CS1, 1:4 for FN-C/H-I and 1:5 for FN-C/H-II (Drake et al., 1992). The RGD-containing peptide (GRGDS) was purchased from Boehringer Mannheim.

Antibodies

Monoclonal antibodies 596 and 597 from mouse, each recognizing different epitopes on TN-R 160 and TN-R 180, have been characterized (Pesheva et al., 1989). Polyclonal antibodies to TN-R 160, which react with both TN-R 160 and TN-R 180, but not with TN-C, were produced in rabbits (Pesheva et al., 1991). Rat monoclonal antibody J1/tm2 recognizes all isoforms of TN-C (Faisssner and Kruse, 1990). Polyclonal antibodies specific for TN-C were raised in rabbits by three subcutaneous injections (the first in complete and the following two in incomplete Freund’s adjuvant) at two-week intervals with 40 µg/ml of brain-derived TN-C per animal. Polyclonal antibodies to LN from EHS sarcoma and to human plasma FN (both from Bethesda Research Laboratories) were produced in rabbits (Pesheva et al., 1989). Polyclonal rabbit antibodies directed against the FNR derived from CHO cells (a kind gift from Dr R. L. Juliano; Brown and Juliano, 1986) and recognizing the 140 kDa heterodimeric (ε3β1) integrin complex in the mouse (Pesheva et al., 1988) were also used.

Analytical procedures

Protein determinations were carried out according to Bradford (1976). Protein samples were separated by SDS-PAGE using 7% polyacrylamide slab gels, and protein bands were visualized by the reducing silver staining method (Oakley et al., 1980).

Cell cultures

Mouse L 929 fibroblast cells (L cells; Pantazis and Jensen, 1988) were maintained in Eagle’s basal medium (BME) containing 10% horse serum. CV-1 (monkey kidney epithelial cell line; Schneider-Schaulies et al., 1990), WISH (human amiotic epithelial cell line, a kind gift from Dr G. Keilhauer, Knoll, Ludwigsfahan) and mouse N2A neuroblastoma cells (Rathjen and Schachner, 1984) were maintained in RPMI containing 10% fetal calf serum (FCS, Gibco). TSC, transfected rat Schwann cells (pMT3_SVneo H1, a kind gift from Dr G. I. Tennekoon; Peden et al., 1990), were maintained in RPMI containing 5% FCS and 100 µM ZnCl2. Primary cultures of Schwann cells and DRG neurons were prepared from neonatal ICR mice and maintained in Dulbecco’s modified Eagle’s medium containing 10% FCS and 20 ng/ml nerve growth factor (Boehringer Mannheim; Seilheimer and Schachner, 1987).

Treatment of L cells with glutaraldehyde

L cells from monolayer cultures were gently trypsinized with 10 µg/ml of trypsin in Ca2+- and Mg2+-free Hanks’ balanced salt solution (CMF-HBSS) for 5 minutes at room temperature. To eliminate the integrin complex in the ECM can modulate cell migration and differentiation.
two washing steps with HEPES buffer. Cells were stored at 4°C as a 5% suspension in HEPES buffer containing 0.02% sodium azide. For binding studies, single cell suspensions were used at a final density of 1×10^6 cells/ml as described in the following section.

**Cell-to-substrate adhesion assays**

Cell-to-substrate adhesion assays were performed essentially as described (Pesheva et al., 1989, 1991). Briefly, TN-R and TN-C, alone (40 µg/ml in PBS) or mixed with other ECM molecules or fragments (1:1 (w/w) on a protein basis at a final protein concentration of 20 µg/ml for 1 hour at room temperature), were coated as 2-3 µl droplets onto tissue culture Petri dishes (30 mm in diameter) or into the wells of 24-well plates (Nunclon) for 60 minutes at 37°C. The oligomer status of the purified molecules is as follows: dimers, for TN-R 160 and FN; trimers, for TN-R 180; and hexamers, for TN-C (mainly represented by the 200 and 220 kDa isoforms in an approximate ratio of 1:1). At a final protein concentration of 20 µg/ml in mixed substrates, the calculated molarities of the individual components were: 62 µM, for TN-R 160; 37 µM, for TN-R 180; 16 µM, for TN-C; and 45 µM, for FN. Whether TN-R 160 (31 µM) and TN-R 180 (18.5 µM) were coated in a mixture with FN (45 µM) at a final concentration of 10 µg/ml, the same adhesion patterns were observed. Overlayed substrates were prepared in the following way: BSA (lipid-free bovine serum albumin, from Sigma), TN-R and TN-C (10 or 20 µg/ml in PBS) were coated onto culture dishes for 30 minutes at 37°C. FN and LN (10 or 20 µg/ml) were then coated on top of the first substrates for 2 hours at 37°C. Unlabeled protein binding sites were unoccupied with 1% BSA in PBS for 60 minutes at 37°C and the dishes were subsequently washed three times with CMF-HBSS. Probe cells were obtained from monolayer cultures by gentle trypsinization (10 µg trypsin/ml CMF-HBSS) for 5 minutes at room temperature. Trypsinization was terminated by addition of ice-cold BME containing 10% horse serum. Cells were centrifuged at 600 g for 10 minutes at 4°C and resuspended in the same medium (incubation medium). Alternatively, BME containing 0.1% BSA was used as an incubation medium with identical results. Single cell suspensions were plated into the substrate-coated dishes at densities of 1×10^6 cells/ml and incubated for one or two to three hours at 37°C. Nonadherent cells were removed by gently washing the dishes with CMF-HBSS and cell-to-substrate adhesion was evaluated microscopically. For estimation of the number of cells adhering to the substrate, cells from micrographs were counted in microscope fields corresponding to 800 µm^2.

**RESULTS**

**Selective inhibition of FN-mediated adhesion and neurite outgrowth by TN-R and TN-C**

To study the effect of TN-R on FN-mediated adhesion, we examined the behavior of different cells on substrates containing TN-R 160 or TN-R 180 compared to that on substrates containing TN-C derived from early postnatal mouse brain. First, TN-R 160, TN-R 180 and TN-C were coated onto tissue culture dishes either alone or in a mixture with other ECM molecules and the adhesion of L cells to these substrates was examined 2 hours after plating (Fig. 1A). In a mixture with FN, both TN-R 160 (a) and TN-R 180 (b), as well as TN-C (c), drastically inhibited cell adhesion and spreading. The number of cells on these substrates comprised about one third of those adhering to FN/BSA substrates (d, Fig. 1B). The inhibitory effect of TN-R- and TN-C-containing substrates correlated specifically with FN-mediated cell adhesion, since mixed substrates of TN-R 160/180 and TN-C with LN (Fig. 1A, e-g) or collagen type I (Fig. 1A, i-k) did not produce different adhesion patterns from those on LN (Fig. 1A, h) and collagen type I (Fig. 1A, i) mixed with BSA (Fig. 1B). Like TN-C, TN-R 160 and TN-R 180 substrates did not support the adhesion of L cells after 2 hours of incubation (not shown).

A possible explanation for these observations could be that TN-R and TN-C bring about their inhibitory effect by an interaction with: (i) FN, which leads to a steric hindrance or conformational change of the cell binding sites of the FN molecules and the adhesion of L cells to these substrates was examined 2 hours after plating (Fig. 1A). In a mixture with FN, both TN-R 160 (a) and TN-R 180 (b), as well as TN-C (c), drastically inhibited cell adhesion and spreading. The number of cells on these substrates comprised about one third of those adhering to FN/BSA substrates (d, Fig. 1B). The inhibitory effect of TN-R- and TN-C-containing substrates correlated specifically with FN-mediated cell adhesion, since mixed substrates of TN-R 160/180 and TN-C with LN (Fig. 1A, e-g) or collagen type I (Fig. 1A, i-k) did not produce different adhesion patterns from those on LN (Fig. 1A, h) and collagen type I (Fig. 1A, i) mixed with BSA (Fig. 1B). Like TN-C, TN-R 160 and TN-R 180 substrates did not support the adhesion of L cells after 2 hours of incubation (not shown).

A possible explanation for these observations could be that TN-R and TN-C bring about their inhibitory effect by an interaction with: (i) FN, which leads to a steric hindrance or conformational change of the cell binding sites of the FN molecule; and/or (ii) cell surface receptor(s), which thus prevents cell attachment and spreading on FN. Supporting the second possibility are recent studies on the kinetics of L cell adhesion to TN-R and TN-C demonstrating that cells initially attach to such substrates and are rapidly repelled already after 15 minutes of incubation, suggesting an interference with second signaling pathways (Pesheva and Schachner, unpublished data). For TN-C, it has been shown that the adhesion and spreading of cells of different origin on FN are negatively affected by the molecule, a process in which interference with both FN substrate and cellular receptor(s) has been suggested (reviewed by Chiquet-Ehrismann, 1991). To investigate whether TN-R displays the same inhibitory effect as TN-C, we studied the attachment of different cell lines (L 929, CV-1, WISH, TSC, N2A) and cells from primary cultures with 1% BSA in PBS for 1 hour at room temperature. TN-R and TN-C (5 µg/ml in PBS containing 1 mg/ml BSA), with or without additives, were added to each well (100 µl/well) and incubated for 1 hour at 37°C. Plates were washed three times with PBS and specific binding was determined by using monoclonal antibodies 596 and J1/112, or polyclonal rabbit antibodies to TN-R and TN-C, respectively, and secondary antibodies coupled to horseradish peroxidase (HRP, Promega). Each value was determined in at least three independent experiments carried out in triplicate.

The coating efficiencies of the 45 kDa collagen binding, 75 kDa FN cell binding, and 33/66 kDa heparin binding fragments of FN on microtiter plates were determined by using 125I-labeled FN fragments as described above. At equimolar concentrations, the coating efficiency of the 75 kDa fragment was the highest observed (about 20% of the input). Compared to this value, the coating efficiencies of the 45 and 33/66 kDa fragments comprised about 95% and 65%, respectively.
Schwann cells and DRG neurons) using the same cell-substrate adhesion assay as described above. FN-mediated adhesion of all cell types was strongly reduced (more than 50%) by the presence of substrate-bound TN-R 160, TN-R 180 or TN-C, while the molecules did not interfere with the LN-mediated adhesion of these cells (not shown). The same was true for the adhesion of DRG neurons to mixed substrates containing TN-R or TN-C (Fig. 2). As for the cell lines, neuron attachment was reduced only on mixed FN substrates (a-c), but not on mixed LN (e-h) or type I collagen substrates (i-l). In a substrate mixture with FN, TN-R and TN-C almost completely inhibited neurite outgrowth, but had no effect on the process formation mediated by LN or type I collagen.

Thus, TN-R and TN-C display inhibitory substrate properties towards FN-mediated adhesion independently of the cell type capable of binding and spreading on FN.

Fig. 1. Selective substrate inhibition of FN-mediated adhesion of L cells by TN-R 160, TN-R 180 and TN-C. (A) L cell adhesion to substrate mixtures of TN-R or TN-C with FN, LN or type I collagen (coll). Substrate mixtures were coated onto plastic and L cell adhesion and spreading were examined after 2 hours in vitro. (a) FN/TN-R 160; (b) FN/TN-R 180; (c) FN/TN-C; (d) FN/BSA; (e) LN/TN-R 160; (f) LN/TN-R 180; (g) LN/TN-C; (h) LN/BSA; (i) coll/TN-R 160; (j) coll/TN-R 180; (k) coll/TN-C; (l) coll/BSA. Bar, 50 µm. (B) Quantitative data on the adhesion of L cells to the different substrate mixtures shown in (A). The number of cells adherent on the substrate mixtures with BSA was set as 100%. Mean values of three independent experiments ± s.d. are shown.
Binding of TN-R and TN-C to FN and FN-derived fragments correlates with inhibition of adhesion

The results described above suggest that in mixed substrates TN-R and TN-C inhibit FN-mediated cell adhesion by an interaction with and/or steric blocking of the cell binding domain(s) on the FN molecule. We therefore examined the interaction of TN-R and TN-C with FN and other ECM molecules by a solid phase binding assay (Fig. 3). TN-C, TN-R 160 and TN-R 180 bound to FN, but not to LN or BSA. TN-R 160, TN-R 180 and TN-C also bound to type I collagen, but not to type IV collagen, which is in agreement with previous data (Faissner et al., 1990; Probstmeier et al., 1990a).

Further, we asked whether TN-R and TN-C interact with domains on the FN molecule involved in the interaction of FN with its cellular receptors. Cells bind to FN mainly by two different mechanisms in which interactions of cell surface integrins with the RGDS sequence (RGD-dependent) or of glycosaminoglycans (membrane-bound proteoglycans) with the heparin binding sites (RGD-independent) on the FN molecule are involved (for review see Reichardt and Tomaselli, 1991). Therefore, we examined the binding of TN-R and TN-C to FN-derived proteolytic fragments: (1) the 33/66 kDa heparin binding fragment, which contains two heparin binding sites (FN-C/H-I and FN-C/H-II; McCarthy et al., 1988, 1990), and...
The results described above suggest that TN-R and TN-C binding to these substrates (Fig. 6B). Similar results were obtained by using live cells for the adhesion assays, suggesting that the GA-L cells expressed intact cell surface receptors for FN (not shown).

Two modes of inhibition of FN-mediated adhesion by TN-R and TN-C

The results described above suggest that TN-R and TN-C inhibit FN-mediated adhesion of cells by steric hindrance of the cell binding sites and/or a conformational change in the FN molecule. However, our experiments do not exclude the possibility that TN-R and TN-C may interfere with the activity of the β1 integrin FN receptor by either binding to it or to a cellular receptor that in turn regulates the activity of this integrin. For hexabrachion, integrin (αvβ1 and αvβ3) and non-integrin (heparan sulfate proteoglycans) receptors have already been postulated (Aukhil et al., 1993; Bourdon and Ruoslahti, 1989; Mendler et al., 1991; Prieto et al., 1992; for review, see Erickson, 1993). To distinguish between these two modes of inhibitory action, we performed experiments that address this issue.

First, if the interference with FN-mediated adhesion by substrate-bound TN-R and TN-C is due to steric inhibition of the substrate, then less cells should initially bind to mixed FN/TN-R and FN/TN-C substrates. By eliminating intracellular response mechanisms, one should be able to prevent a possible repulsion of cells and thus register only the initial recognition at the cell surface. Therefore, we studied the binding of L cells that had been gently treated with trypsin (allowing the preservation of an intact cell surface membrane) and subsequently with glutaraldehyde in a way that preserves the activity of the integrin receptors to different ECM molecules (Fig. 6A). Glutaraldehyde-treated L (GA-L) cells bound to mixed FN/BSA, FN/TN-R 180 and FN/TN-C substrates equally well, while the number of cells binding to FN/TN-R 160 was strongly reduced by approximately 60%. Binding to LN, by contrast, was not affected by either TN-R form or TN-C. GA-L cells did not bind to TN-R 160, but bound better to TN-R 180 and TN-C than to FN alone or to the mixed FN/BSA substrate (Fig. 6A).

GA-L cells attached to FN via a β1 integrin, since in the presence of the RGD-containing peptide or polyclonal antibodies to the αvβ1 integrin (anti-FNR) cell binding was strongly inhibited (Fig. 6B). These antibodies did not interfere with L cell attachment to LN, TN-R 180 and TN-C, nor did the presence of the RGD-containing peptide reduce cell binding to these substrates (Fig. 6B). Similar results were obtained by using live cells for the adhesion assays, suggesting that the GA-L cells expressed intact cell surface receptors for FN (not shown).
These results suggest that TN-R 160 interferes with FN-mediated adhesion by a steric hindrance of and/or a conformational change in the cell binding sites of FN. For TN-R 180 and TN-C, an interaction with a cellular receptor different from \( \beta_1 \) integrins may influence cell adhesion to the FN molecule. To investigate this possibility, we examined L cell adhesion to overlayed substrates produced by coating FN or LN on top of TN-R 160, TN-R 180 and TN-C substrates (Fig. 7). This coating procedure permits FN to be present as a co-substrate with TN-R or TN-C, but reduces the possibility of interaction between the molecules. After a 2 hour incubation, L cells adhered and spread on TN-R 160 + FN as well as on BSA + FN substrates, i.e. the inhibitory effect of TN-R 160 was completely abolished. By contrast, overlayed substrates containing TN-R 180 and TN-C were still inhibitory. As already observed for the mixed substrates, TN-R and TN-C did not interfere with L cell adhesion to LN-overlayed substrates (Fig. 7).

From the data obtained, it becomes clear that TN-R 160 inhibits FN-mediated adhesion by a steric hindrance of and/or a conformational change in the cell binding sites of FN when the two molecules are present as mixed substrates. TN-R 180 and TN-C bring about their inhibitory effect by an interaction with putative cellular receptor(s) different from the FNRI (\( \beta_1 \) integrin) but probably associated with it.

**DISCUSSION**

Our present study demonstrates that TN-R 160 and TN-R 180 exhibit inhibitory effects on FN-mediated adhesion. We have shown that: (1) the two TN-R isoforms, TN-R 160 and TN-R 180, presented as substrates in a mixture with the adhesive ECM glycoproteins FN, LN and collagen type I selectively inhibit attachment of different cell types and neurite outgrowth on FN; (2) the inhibition of cell attachment and spreading by TN-R 160 results from its interaction with FN, leading to a
steric masking of and/or a conformational change in the cell binding sites on FN; (3) TN-R 180, in addition, can interact with putative cell surface receptor(s) in a RGD- and β1 integrin-independent way, which may regulate the activity of the FNR. We have compared these effects of TN-R with those elicited by TN-C from early postnatal mouse brain and confirmed the functional relatedness between the two ECM glycoproteins, whereby TN-R 180 is more related to TN-C than TN-R 160.

TN-R 160 does not display the same functional properties towards FN-mediated cell adhesion as TN-R 180, although the two isoforms are structurally and functionally very similar (Pesheva et al., 1989, 1991, 1993; Fuss et al., 1993). The actual structural difference between the two TN-R components is not known. The existence of one known alternatively spliced FN type III-like domain (Fuss et al., 1993) and the appearance of TN-R 160 in electron micrographs as a di- but never as a tri-brachion (Pesheva et al., 1989), suggesting a lack of N-terminal sequences involved in the formation of disulfide bonds, might contribute to the different structural organization and functional properties in relation to FN-mediated adhesion.

The finding that the two TN-R isoforms and TN-C selectively inhibit FN-dependent adhesion of different cells to mixed substrates and can interact with at least two sites on the FN molecule suggested to us that the anti-adhesive effect resulted either from a substrate-mediated inhibition of cell attachment through an interaction with FN or from interfering with the activity of one or several FN receptors, or both. To examine the first possibility, we made use of the knowledge of the functional domains of FN. As a multifunctional protein, FN has been found to contain an increasing number of interactive sites for cells, ECM components or assembly of fibrillar matrix (Schwarzbauer, 1991). For binding studies, we used synthetic peptides derived from FN and proteolytic fragments of 45, 75 and 33/66 kDa (from N to C terminus) containing the collagen-binding site (Pierschbacher et al., 1981), the cell-binding site...
for integrins (Humphries et al., 1988) and binding sites for heparin, proteoglycans and RGD-independent integrins (Drake et al., 1992), respectively. The two TN-R isoforms and TN-C bound to FN and to the 75 and 33/66 kDa fragments, but not to the 45 kDa fragment. Interestingly, this binding profile correlated with inhibition of cell adhesion to FN, when the fragments were offered as mixed substrates with TN-R or TN-C. This substrate-induced inhibition could be interpreted in two ways: as a specific interaction with the cell-binding sites of FN that antagonizes binding of the cellular receptors; or, as TN-R and TN-C binding to parts of the FN molecule different from the cell-binding sites, leading to a steric block and/or conformational change affecting the correct exposure of the cell-binding sites of FN. It appears that TN-R and TN-C do not act as antagonists of the cell-binding sites of FN, since binding of the two molecules to the FN fragments could not be inhibited by RGD or other synthetic peptides (FN-C/H-I, FN-C/H-II and CS1) representing the sequences within the 33/66 kDa fragment that are active in cell binding (see also Chiquet-Ehrismann et al., 1988). Rather, TN-R 160, when binding to the fragments containing distinct cell-binding sites stericly blocks and/or conformationally changes these sites on the FN molecule. In contrast to TN-R 160, TN-R 180 and TN-C induce cell repulsion by binding to an RGD-independent cellular receptor and thereby modulating the RGD-dependent β1 integrin-mediated attachment to FN. Thus, TN-R 180 and TN-C influence cellular behavior by an active cellular response specifically to the FN substrate. It is likely, therefore, that the cell surface receptors for TN-R 180 and TN-C are coupled to the β1 integrin receptor for FN by signal transduction, thereby modifying it by ‘inside-out’ mechanisms (Ginsberg et al., 1992). We have recently shown that disialogangliosides most likely represent these TN-R 180 and TN-C receptors (Pesheva and Schachner, unpublished data).

The repellent effect of TN-R and TN-C on FN-dependent adhesion is different from that on CNS neurons. TN-C and both TN-R 160 and TN-R 180 inhibit neuronal cell adhesion and neurite outgrowth independently of the adhesive substrate present (Pesheva et al., 1989, 1991, 1993; Faissern and Kruse, 1990). Moreover, the F3/11 cell adhesion molecule (Brümmendorf et al., 1989; Gennarini et al., 1989), which mediates the repellent effect of TN-R 160, TN-R 180 (Pesheva et al., 1993) and probably the 190 kDa TN-C isoform (Zisch et al., 1992), is not expressed by fibroblasts, Schwann cells and DRG neurons derived from early postnatal mice (Gennarini et al., 1989). It is thus very likely that the selective inhibition of FN-dependent adhesion is due to an interference with the activity of the β1 integrin, since the cell types used in our study all adhere to FN in an RGD-dependent manner (shown only for L cells, Fig. 6B).

Our results raise several important questions as to the role of TN-R, TN-C or other, unknown, related molecules in the modulation of such processes as cell migration and differentiation, tumor growth and invasive behavior of tumor cells. The possible involvement of TN-C in pattern formation during embryogenesis and early postnatal development, as well as in regeneration and oncogenesis, has been stated in a number of studies (Chiquet, 1989; Erickson and Bourdon, 1989; Chiquet-Ehrismann, 1990, 1991). In the PNS, TN-C expression is upregulated during regeneration (Martini et al., 1990), a process that correlates with increased expression of FN and FNR (Lefcort et al., 1992), thus providing the molecular basis for the control of axonal regrowth and Schwann cell migration and differentiation in the sciatic nerve. TN-C can potentiate neurite outgrowth from both PNS and CNS neurons (Welhrle and Chiquet, 1990; Locher et al., 1991) and appears to be involved in the process of epithelial cell shedding in the small intestine by inducing cell detachment from the basal lamina (Probstmeier et al., 1990b). At the neuromuscular junction, the highly localized appearance of TN-C at the denervated subsynaptic site after peripheral nerve lesion may be responsible for attracting regrowing axons to this site and, at the same time, prevent axonal growth beyond this site by the combined influence of TN-C and FN on neurite outgrowth (Sanes et al., 1986; Gatchalian et al., 1989). It is also conceivable that the fibroblasts around the denervated neuromuscular junction that actively secrete TN-C may use their FN substrate-modifying capacity to support proliferation of perisynaptic fibroblasts (Gatchalian et al., 1989). In the CNS, the coexpression of TN-R and TN-C with FN has not been systematically studied during ontogenesis. At developmental stages, when FN expression is restricted to the basal lamina, a concerted action
of TN-R and TN-C with FN may be possible. Such coexpression is found for TN-C in the early postnatal rodent cerebellum, when granule cell precursor cells adjoining the basal lamina in the external granular layer may allow detachment of these actively proliferating cells from the basal lamina and thereby favour their migration along the radial glial processes (Bartsch et al., 1992; see also Hausmann and Sievers, 1985). TN-R 180, the first TN-R component expressed during development (Pesheva et al., 1989; Bartsch et al., 1993), is detectable in the ventricular zone of neonatal mouse brain (P. Pesheva, unpublished observations) and its co-expression with FN and FNR (Stallcup et al., 1989) may fulfill the same function as already proposed for TN-C.

The finding of our study that, in relation to FN-mediated cell adhesion, TN-R is functionally closely related to TN-C raises the possibility of a functional substitution or redundancy. This question is even more pertinent in the light of recently published work on the lack of abnormal development in TN-C-deficient mouse mutants (Saga et al., 1992). TN-R, or unknown molecules structurally or functionally related to TN-C, could thus be instrumental in molecular and functional substitution. Identification of the cellular receptors mediating the repellent action of TN-R 180 and TN-C, and the mechanism underlying a particular cellular response, are topics for further investigation. In fact, we recently showed that the interference of the two molecules with FN-mediated adhesion involves a disialoganglioside-dependent mechanism, as the interaction of both TN-R 180 and TN-C with cell surface disialogangliosides is coupled to the inhibition of RGD-dependent cell attachment and neurite outgrowth on FN substrates (Pesheva and Schachner, unpublished data).

The authors are grateful to Iris Bahnmüller for expert technical help, R. L. Juliano for the kind gift of FNR (α5β1) antibodies, Lloyd Vaughan for critically reading the manuscript, Hermann and Lilly Schilling Stiftung for a fellowship (P.P.), and Kommission zur Förderung der wissenschaftlichen Forschung for support. This work was supported by grants CA29995 and CA21463 from the National Institutes of Health (to L.T.F.).

REFERENCES


Hausmann, B. and Sievers, J. (1985). Cerebellar external granule cells are attached to the basal lamina from the onset of migration up to the end of their proliferative activity. J. Comp. Neurol. 241. 50-62.


Lightner, V. A. and Erickson, H. P. (1990). Binding of hexabrachion
(tenascin) to the extracellular matrix and substratum and its effect on cell adhesion. J. Cell Sci. 95, 263-277.


(Received 17 January 1994 - Accepted 25 April 1994)