Characterization of Functional Domains of the Tenascin-R (Restrictin) Polypeptide: Cell Attachment Site, Binding with F11, and Enhancement of F11-Mediated Neurite Outgrowth by Tenascin-R

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Abstract. The extracellular matrix glycoprotein tenascin-R (TN-R) is a multidomain protein implicated in neural cell adhesion. To analyze the structure–function relationship of the different domains of TN-R, several recombinant TN-R fragments were expressed in bacterial cells. Two distinct binding regions were localized on the TN-R polypeptide: a region binding the axon-associated immunoglobulin (Ig)–like F11 protein and a cell attachment site. The binding region of the glycosyl-phosphatidylinositol (GPI)–anchored F11 was allocated to the second and third fibronectin type III (FNIII)–like domain within TN-R. By using a mutant polypeptide of F11 containing only Ig-like domains, a direct interaction between the Ig-like domains of F11 and FNIII-like domains 2–3 of TN-R was demonstrated. The interaction of TN-R with F11 in in vitro cultures enhanced F11-mediated neurite outgrowth, suggesting that the combined action of F11 and TN-R might be of regulatory influence on axon extension. A cell attachment region was identified in the FNIII-like domain eight of TN-R by domain-specific antibodies and fusion constructs. This site is distinct from the F11 binding site within TN-R.

ENASCIN-R (TN-R)[1], previously designated restrictin in the chick and J1-160/180 or janusin in the rat, is the smallest member of the tenascin family of extracellular matrix glycoproteins, including tenascin (TN-C) and tenascin-X (TN-X) (Bristow et al., 1993; for review, see Erickson, 1993; Chiquet- Ehrismann et al., 1994). In the chick it was identified by its copurification with the axon-associated Ig-like glycoprotein F11 (Rathjen et al., 1991). Like the two other tenascin family members, it is a modular glycoprotein composed of four structural motifs. The NH₂-terminus contains a cysteine-rich segment unique to members of the tenascin family. Within this segment, a stretch of 20 residues could form three heptad repeats of hydrophobic amino acids in an α-helix that might generate a triple-stranded coiled coil to link three or two TN-R polypeptides to trimers or dimers. In the COOH-terminal direction, the cysteine-rich region is followed by four and a half epidermal growth factor (EGF)–like repeats and then by nine consecutive motifs that are related to fibronectin type III (FNIII)–like domains. At the COOH terminus,

TN-R contains a 220–amino acid segment similar to the β and γ chains of fibrinogen, including a calcium-binding segment (Nörenberg et al., 1992; Fuss et al., 1993). The overall domain arrangement of these domain types is identical in the three proteins; however, the number of the EGF- and FNIII-like modules varies. Two regions of alternative splicing of the pre-mRNA have been described for TN-R. In the chick, an isoform has been identified lacking a 45–amino acid segment close to the NH₂ terminus that might affect the assembly of the TN-R polypeptides into oligomers (Nörenberg et al., 1992), while in the rat the sixth FNIII-like domain is found only in some forms (Fuss et al., 1993). Although TN-R shows considerable sequence similarity with TN-C in the NH₂-terminal portion, including the spacing of cysteines, the predominant forms of TN-R seen in electron microscopy are trimers, dimers, and monomers (Pesheva et al., 1989; Nörenberg et al., 1992), in contrast with TN-C, which forms hexamers (Erickson and Iglesias, 1984).

While TN-C and TN-X have been found in many, although distinct, embryonic tissues (Matsumoto et al., 1994), TN-R exhibits a site-restricted localization in the nervous system. In the chick spinal cord, for example, TN-R is localized around motor neurons and in lower concentration on motor axons; in the embryonic cerebellum, TN-R appears to be expressed primarily in the prospective white matter.

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1. *Abbreviations used in this paper.* COS, African green monkey kidney cells (SV 40 transformed); EGF, epidermal growth factor; FG, fibrinogen; FNIII, fibronectin type III; GPI, glycosyl-phosphatidylinositol; GST, glutathione-S-transferase; TN-C, tenascin-C; TN-R, tenascin-R; TN-X, tenascin-X.

Furthermore, in the developing retina, TN-R is localized in the neurite-containing inner and outer plexiform layers (Rathjen et al., 1991). Immunofluorescence and in situ hybridization studies indicate that TN-R is associated with the surface of neurons, myelinating oligodendrocytes, and type-2 astrocytes (Pesheva et al., 1989; Rathjen et al., 1991; Bartsch et al., 1993; Fuss et al., 1993; Wintergerst et al., 1993). The synthesis of TN-R by oligodendrocytes appears to be modulated by astrocytes and PDGF in in vitro cultures (Jung et al., 1993). In addition to this spatially restricted expression pattern, Northern blot analysis indicates a transient expression pattern. In the chick brain, TN-R mRNA becomes weakly detectable at embryonic day 6, reaches a maximum at E16, and declines in the adult (Nörenberg et al., 1992). This transient occurrence of TN-R in axon-rich regions in parts of the developing nervous system suggests a participation in axonal growth. This assumption is supported by the finding that TN-R undergoes a direct interaction with the axonal surface recognition protein F11 but not with other axon-associated surface glycoproteins such as L1/NgCAM, NrCAM, NCAM, and TAG-1 (Brümmendorf et al., 1993). However, although tectal cells adhere to immobilized TN-R, it was not found sufficient to induce neurite extension, indicating that additional signals provided by other environmental components might be required to generate axons on TN-R (Rathjen et al., 1991). When TN-R is combined with laminin and immobilized on culture dishes, neurites are induced to similar length than on laminin alone (Rathjen et al., 1991). A cell attachment site has been mapped by the use of a monoclonal antibody in the distal portion of the TN-R arms (Nörenberg et al., 1992). This site allows an initial cell binding, which, however, is not followed by a subsequent strengthening response, as seen for typical adhesive proteins. In contrast, adhered cells appear to loosen their attachment and tend to aggregate after a further cultivation period. This behavior of neural cells on TN-R has been interpreted as a repulsive activity and has initiated studies on an inhibitorial activity on neurite extension. Furthermore, the expression of TN-R by oligodendrocytes (Pesheva et al., 1989) that are nonpermissive for axonal growth in vitro (Schwab et al., 1993) suggested that TN-R may help to confine axonal pathways and might prevent axonal regeneration after injury in the central nervous system. In vitro studies support the assumption that TN-R provides a repulsive signal on neurite outgrowth of cerebellar cells and dorsal root ganglion (DRG) neurons, which appears to be mediated by the glycosyl-phosphatidylinositol (GPI)–anchored protein F11 (Pesheva et al., 1993). However, a growth cone collapse activity that seems characteristic for growth cone repulsion in in vitro assays (Kapfhammer and Raper, 1987; Walter et al., 1990; Luo et al., 1993; Luo and Raper, 1994) has not been observed for TN-R. In contrast, the motility of growth cones from DRG neurons contacting a substrate border of TN-R was not affected, indicating that TN-R does not modulate growth cone motility itself (Taylor et al., 1993). Induction of neurites on a pure TN-R substratum has only been reported for hippocampal neurons (Lochter et al., 1994), while retinal, tectal, or cerebellar neurons did not extend neurites (Pesheva et al., 1989; Rathjen et al., 1991). The interactions mediated by TN-R might therefore be complex and may involve both nonpermissive and adhesive properties (Schachner et al., 1994), as has also been discussed for TN-C (Faissner, 1994).

The adhesive function of TN-R and its interaction with the F11 protein in conjunction with the multidomain structure are not well understood. In this study we characterized the cellular and molecular interaction of F11 and TN-R and mapped the cell attachment site within TN-R. We reveal that specific FNIII-like domains of TN-R interact with a mutant F11 polypeptide containing only Ig-like domains. These investigations, therefore, demonstrate for the first time a direct binding between Ig- and FNIII-like domains. The interaction between F11 and TN-R enhanced F11–mediated neurite extension, suggesting a modulatory activity of TN-R on F11.

Materials and Methods

Neurite Outgrowth and Cell Adhesion Assays

The attachment of chick retinal cells of embryonic day 9 to substrate of purified TN-R or fusion proteins of TN-R was performed as described (Rathjen et al., 1991; Nörenberg et al., 1992). Proteins were immobilized to nitrocellulose-coated petriperm dishes (Heraeus, Germany; Lagenaur and Lemmon, 1987), with 50 μg/ml in the coating solution. Neurite extension was analyzed using chick tectal cells of embryonic day 6, which were prepared and cultivated on immobilized F11 as described (Brümmendorf et al., 1993; Morales et al., 1993). Purified TN-R (25 μg/ml) or recombinant TN-R proteins (80–150 μg/ml) were added to these cultures at the time of cell plating. In some experiments, immobilized F11 was preincubated for 1 h with TN-R or fusion proteins at 37°C, followed by two washing steps. In both bioassays, mouse antisera to fusion proteins were used at a 1:500 to 1:1000 dilution, while purified mAbs or Fab fragments of IgG fractions of polyclonal antibodies to F11 or TN-R were employed at a concentration of 5–10 μg/ml. In some experiments, TN-R or fusion proteins were preincubated for 2 h with antibodies as specified in Results.

Cloning of Specific TN-R Fragments

PCR from TN-R cDNA clones (Nörenberg et al., 1992) was used to terminate segments of TN-R precisely at domain boundaries. We cloned 15 different constructs containing one or more FNIII domains into the pGEX-2TN vector: each 5′ primer contained a NotI site and the first 15 nucleotides of the generated domains, and each 3′ primer contained an EcoRI site and the last 18 nucleotides of the generated domains, so the resulting PCR product, upon digestion, could be ligated in frame into the NotI/EcoRI site of the pGEX-2TN vector (modified pGEX-2T vector; Pharmacia Biotech Europe, Brussels, Belgium).

Cloning of the Fibrinogen Domain into the pGEX-3X Vector

The 5′ primer contained a BamHI site and the first 18 nucleotides of the fibrinogen (FG) domain. The 3′ primer contained an EcoRI site and the last 19 nucleotides of the domain. The resulting PCR product was, upon digestion, ligated into the pGEX-3X vector (Pharmacia Biotech).

Cloning of [7]FNIII, [8]FNIII, and FG Domains into pQE-30 Vector

The 5′ primers of [7]FNIII and FG domains contained a BamHI site and the first 18 nucleotides of the generated domains. The 5′ primer of [8a]FNIII contained a SacI site and the first 18 nucleotides of the domain. Each 3′ primer of the three domains contained a HindIII site and the last 18 nucleotides of the domains. PCR was performed with 30 cycles of denaturation (94°C for 5 min), annealing (55°C for 2 min), and extension (72°C for 3 min). Fragment [8b]FN III, which contains the [8]FNIII domain plus 13 amino acids of the [7]FNIII domain, was generated by subcloning the HaeIII fragment (3432–3740 bp) of TN-R cDNA clone into the SmaI site of pGEX-3X vector. The numbering of nucleotide positions refers to that

given by Nörenberg at al. (1992). PCR products were not sequenced; instead we analyzed several independently derived PCR clones of TN-R regions and used constructs that were generated by subcloning of fragments of the TN-R cDNA clone obtained by digestions with restriction enzymes (Nörenberg et al., 1992). These independently derived clones gave identical results in the binding studies with wild-type F11 or mutant polypeptides of F11 and in the cell attachment assay.

Expression and Purification of Recombinant Proteins

Expression and protein product purification from glutathione-S-transferase (GST) fusion constructs has been described by Smith and Johnson (1988). *Escherichia coli* NM 522 were transformed with either pGEX-2TN or pGEX-3X recombinants. Overnight cultures were diluted 1:10 in Luria-Bertani medium containing ampicillin (100 μg/ml) and incubated for 4 h at 37°C with shaking. After 1 h of growth, isopropyl-β-D-thiogalactopyranoside (BIOMOL Research Labs, Plymouth Meeting, PA) was added to a final concentration of 2 mM. Bacterial cultures were pelleted by centrifugation at 5,000 g for 10 min at 20°C and resuspended in 1/10 vol 50 mM Tris, pH 8. The cells were lysed with 1 mg/ml lysozyme (Sigma Chemical Co., St. Louis, MO) in the presence of 1 mM PMSF (Sigma Chemical Co.) for 20 min on ice, followed by treatment with 1% Triton X- 100 for an additional 10 min. After sonication (2 × 20 s) on ice, the disrupted cells were pelleted at 12,000 g for 20 min at 4°C. All proteins comprising one or more FNIII domains were completely soluble in the bacterial supernatant. The FG domain was not obtained as soluble protein in the bacterial supernatant. The FG domain was therefore solubilized by suspending the pellet of the bacterial lysate in 8 M urea containing 50 mM Tris-HCl, pH 8, and incubated for 1 h at room temperature. The solution was dialyzed successively against 6 M urea, 2 M urea, and 50 mM Tris-HCl, pH 8 (each of the dialyses buffer contained 1 mM DTT), followed by extensive dialysis against 50 mM Tris-HCl, pH 8, without DTT. The solution was centrifugated at 12,000 g for 20 min. Domains expressed as GST fusion proteins by pGEX vectors were applied to glutathione Sepharose 4B columns (Pharmacia Biotech), previously equilibrated with PBS. After washing the matrix with 10 bed volumes of PBS, the bound fusion proteins were eluted with 5 mM glutathione in 50 mM Tris-HCl, pH 8.

E. coli M 15(pREP4) were transformed with pQE-30 recombinants. Growth and harvesting of pQE-30 recombinants were performed as described earlier, except that the growth medium contained kanamycin (25 μg/ml) in addition to ampicillin (100 μg/ml). In cases where the expression

proteins remained soluble, the cell pellet was resuspended in 50 mM Na-phosphate, 300 mM NaCl, pH 8. The cells were lysed with 1 mg/ml lysozyme (Sigma) in the presence of 1 mM PMSF (Sigma) for 20 min on ice. After sonication (2 × 20 s) on ice, the disrupted cells were pelleted at 12,000 g for 20 min at 4°C. The proteins with an affinity tag consisting of six consecutive histidine residues were isolated by affinity chromatography on Ni-NTA resin according to instructions of the manufacturer (QIAGEN, Chatsworth, CA). Bound proteins were eluted with 150 mM imidazole in 50 mM Na-phosphate, 300 mM NaCl, 10% glycerol, pH 6. The FG domain was insoluble in the bacterial supernatant. According to the instructions of the manufacturer (Qiagen), the pelleted cells were therefore resuspended in 8 M urea, 0.1 M Na-phosphate, 0.01 M Tris-HCl, pH 8, and incubated for 1 h at room temperature. The lysates were cleared at 10,000 g for 15 min at 4°C. The supernatant was then applied to an Ni-NTA column as described previously, except that the column was run in the presence of 8 M urea, 0.1 M Na-phosphate, 0.01 M Tris-HCl, pH 8.0. After washing the matrix first with 10 bed volumes of 8 M urea, 0.1 M Na-phosphate, 0.01 M Tris-HCl, pH 8.0, and then with 10 bed volumes of 8 M urea, 0.1 M Na-phosphate, 0.01 M Tris-HCl, pH 6.3, the bound protein was eluted with 250 mM imidazole in 8 M urea, 0.1 M Na-phosphate, 0.01 M Tris-HCl, pH 6.3. Dialysis of eluted proteins was performed as described earlier. Purity of isolates was assessed by SDS-PAGE and Western blotting.

Isolation of TN-R, TN-C, and F11

TN-R, TN-C, and F11 were purified from adult chicken brains by immunoaffinity chromatography as previously described (Chiquet and Fambrough, 1984; Rathjen et al., 1987). Purity of isolates was analyzed by SDS-PAGE.

Antibodies

Generation and specificity of polyclonal and monoclonal antibodies to F11 and TN-R (restrictin) have been described elsewhere (Rathjen et al. 1987, 1991; Nörenberg et al. 1992; Brümmendorf et al., 1993). Polyclonal antibodies to fusion proteins of TN-R expressed in pGEX vector were produced by injecting Balb/c mice or rabbits intraperitoneally, in complete Freund's adjuvant, with 40 μg of purified fragments. Booster injections were given with the same amount of antigen in Freund's incomplete adjuvant 2 and 4 wk later. The specificity of antibodies was analyzed in im-
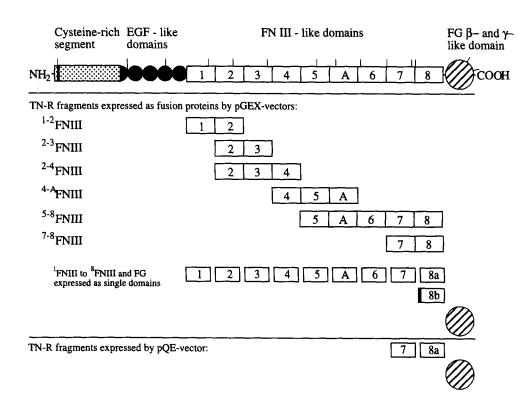

Figure 1. Schematic model of the TN-R polypeptide and recombinant proteins. The primary structure is shown at the top of the figure. The rectangle (*left*) represents the cysteine-rich segment that might link monomers into trimers by disulphide bridges. Epidermal growth factor (EGF)–like repeats are drawn as filled circles. The rectangles numbered 1–8 represent the FNIII-related domains, and the alternatively spliced FNIII unit is labeled with an A. The fibrinogen (FG)–like segment is indicated by an open circle on the right. The various TN-R protein fragments expressed as GST fusion protein by the pGEX vector or with an affinity tag consisting of six consecutive histidine residues by the pQE vector are shown below the primary structure and are labeled as presented in the text.
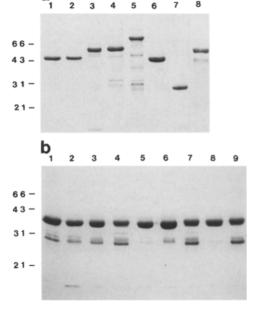
Figure 2. Analysis of recombinant TN-R fragments. TN-R fragments have been produced by PCR and the pGEX expression vector, analyzed by 12% SDS-PAGE, and visualized by Coomassie blue staining. (*a*) The fragments are as follows: $^{1-2}$FNIII domains (lane *1*); $^{2-3}$FNIII domains (lane *2*); $^{2-4}$FNIII domains (lane *3*); $^{4-A}$FNIII domains (lane *4*); $^{5-8}$FNIII domains, including the alternatively spliced domain A (lane *5*); $^{7-8}$FNIII domains (lane *6*); glutathione-S-transferase (lane *7*); FG domain (lane *8*). (*b*) Single FNII domains are as follows: ^{1}FNIII domain (lane *1*); ^{2}FNIII domain (lane *2*); ^{3}FNIII domain (lane *3*); ^{4}FNIII domain (lane *4*); ^{5}FNIII domain (lane *5*); ^{A}FNIII domain (lane *6*); ^{6}FNIII domain (lane *7*); ^{7}FNIII domain (lane *8*); ^{8}FNIII domain (lane *9*). Several of the single FNIII-like domains appeared less stable, and therefore degradation products are observed in SDS-PAGE. Molecular mass markers are indicated in kD to the left of each panel.

munotransfers against TN-R and TN-R fragments expressed in pQE vector.

Protein Analytical Procedures

Protein concentrations were determined according to Peterson (1977). SDS-PAGE (Laemmli, 1970) was performed with 12% acrylamide under reducing conditions followed by Coomassie staining.

F11 Deletion Mutants and Transfection of COS Cells

The F11 deletion mutants used in this study have been characterized in detail elsewhere (Brümmendorf et al., 1993). In brief, all mutant polypeptides comprise the signal sequence and the hydrophobic carboxyterminal sequence involved in posttranslational attachment of the GPI anchor. Plasmids could be transfected in COS cells with equivalent transfection efficiencies, and the expressed polypeptides reveal the expected molecular masses in SDS–polyacrylamide gel electrophoresis. Maintenance and transfection of COS cells were done as outlined previously (Brümmendorf et al., 1993).

Binding of TN-R to F11-Expressing COS Cells

TN-R at a concentration of 30–60 μg/ml was incubated with F11-expressing COS cells obtained by transfection for 1 h at room temperature. Cells were washed three times with PBS, fixed for 5 min at room temperature with 1% formaldehyde in PBS, again washed three times with short incubation cycles with TBS and 1% BSA and once for 15 min with TBS and 1% BSA, at room temperature. Immunofluorescence detection of bound TN-R made use of a mixture of four mAbs (23-4, 23-9, 23-13, 23-14; di-

rected to TN-R; each at 10 μg/ml) detected by polyclonal goat anti-mouse antibodies conjugated with the Cy3 chromophore (Dianova, Hamburg, Germany). No background signals were observed if only TN-R or primary antibodies were used in the procedure or if both TN-R and the primary antibodies were omitted (data not shown). Comparable signals were obtained if the TN-R specific mAbs were used alone instead of in combinations in the former procedure. Comparable labeling could also be observed if polyclonal antibodies directed to TN-R were used as primary antibodies and were detected by Cy3-conjugated goat anti–rabbit antibodies (Dianova).

Binding of TN-R Fusion Proteins to F11-Expressing COS Cells

Bacterially expressed fusion proteins comprising fragments of TN-R and glutathione-S-transferase at concentrations of 30–250 μg/ml were sonicated for 5 min in an ice-cooled sonifier bath (Sonorex TK52; Bandelin Electronic, Berlin, Germany) and incubated with F11-transfected COS cells for 1 h at room temperature. Slides were processed as outlined above for TN-R using goat anti–glutathione-S-transferase serum (Pharmacia Biotech) as primary antibody and Cy3-conjugated polyclonal rabbit anti-goat antibodies (Dianova) for detection.

Coupling of TN-R or Fusion Proteins to Microspheres and Flow Cytometric Analysis

Affinity-purified TN-R or fusion proteins were coupled to red or blue fluorescing microspheres with a diameter of 0.5 μm (Covaspheres; Duke Scientific Corp., Palto Alto, CA) and incubated for binding as detailed previously (Kuhn et al., 1991; Brümmendorf et al., 1993; Morales et al., 1993). In blocking experiments, protein-coated beads were preincubated for 15 min with mAbs to F11 at a concentration of 1–5 μg/ml. Analysis of aggregated beads by flow cytometry has been outlined previously (Brümmendorf et al., 1993). Flow cytometric data were analyzed with the DAS software package (a kind gift of Dr. Wolfgang Beisker; Beisker, 1994).

Results

Localization of the F11 Binding Region within the TN-R Polypeptide

We have shown previously that the axon-associated and GPI-anchored F11 binds to TN-R (Rathjen et al., 1991; Brümmendorf et al., 1993). To identify the region within the TN-R polypeptide which binds F11, a series of overlapping protein fragments expressed in pGEX or pQE vectors that span the complete fibronectin type III (FNIII) and fibrinogen region of TN-R was isolated as described in Materials and Methods (see Fig. 1). The purity of the fusion proteins was assessed by SDS-PAGE and immunotransfer analysis. All fragments generated were highly pure; however, some preparations contained small amounts of degradation products (Fig. 2). The calculated molecular masses of the fusion proteins differed only slightly from the apparent molecular masses observed in SDS-PAGE. Purified TN-R fusion proteins and the F11 protein isolated from adult chicken brains were covalently coupled to red- or blue-colored fluorescent microspheres. Mixtures of beads were then incubated for 1 h, followed by an analysis with flow cytometry. In dual combinations, only fragments containing FNIII-like domains 2 and 3, including fragments $^{2-3}$FNIII and $^{2-4}$FNIII, were found to bind strongly to F11-coated microspheres (Fig. 3, *b* and *c*). Other TN-R fragments containing two or more FNIII-like modules did not bind above the background values (Fig. 3, *a, d–f, i*). The heterophilic interaction between F11 and fusion protein $^{2-3}$FNIII could be blocked by polyclonal antibodies to TN-R or F11 (not shown), demonstrating additional speci-
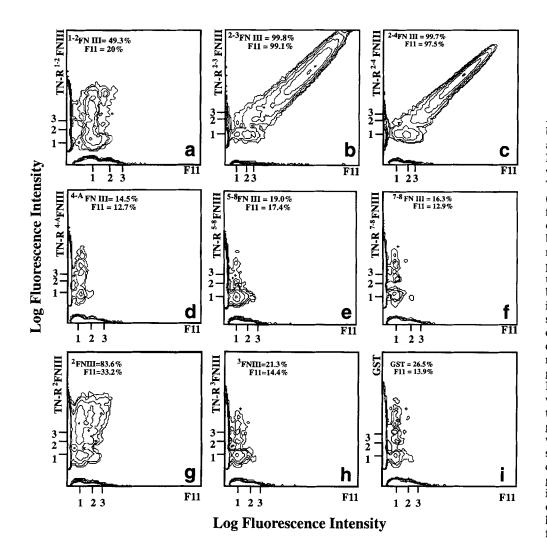
Figure 3. Binding of recombinant TN-R domains to F11 analyzed by flow cytometry. Fluorescent microspheres were covalently coupled with TN-R domains (*blue*) or F11 (*red*), mixed, and incubated for 1 h at a 1:1 ratio. Aliquots of the beads were analyzed by flow cytometry. Two parametric measurements are presented as contour plots using a logarithmic scale for both axes. The fluorescence intensity corresponds to the size of aggregates. Only beads coated with TN-R fragments containing the $^{2-3}$FNIII domain formed large mixed aggregates with F11 (*b* and *c*). In contrast, beads coupled with ^{2}FNIII domain, which tends to form homophilic aggregates (*g*) or beads coated with ^{3}FNIII domain (*h*) as single units, did not bind F11-coated beads above the background values. The numbers in the upper lefthand corner of each square give the calculated percentages of beads found in mixed aggregates.

ficity of the binding. Furthermore, three specific mAbs against the F11 protein, numbers 0, 25, and 26 (Fig. 4, *a, b,* and *d*), were found to neutralize binding between F11 and the $^{2-3}$FNIII domains of TN-R. These mAbs also reduced binding between intact F11 and TN-R coupled to microspheres (data not shown). In contrast, several other anti-F11 monoclonals, including mAb 11 and 24 (Fig. 4, *c* and *d,* and data not shown), had no effect on the interaction. When ^{2}FNIII or ^{3}FNIII as single units were coupled to microspheres, followed by incubation with F11-coated beads, the ^{2}FNIII domain showed a high tendency to form homophilic aggregates, but the binding of ^{2}FNIII or ^{3}FNIII to F11 beads was close to background values (Fig. 3, *g* and *h*), suggesting that the tandem of domains 2–3 is required, while domains 2 or 3 alone are not sufficient. Because none of the currently available mAbs to TN-R are bound to these FNIII domains, they also did not interfere with the binding of TN-R to F11. However, polyclonal antibodies to FNIII domains 2–3 were found to reduce binding between intact F11 and TN-R (data not shown).

To further validate independently the result that the TN-R fragment containing domains 2–3 carries the F11 binding domains, an approach analyzing interaction of the TN-R polypeptide with membrane-bound F11 protein was chosen. Soluble TN-R isolated from chick brain was incubated with F11-expressing COS cells, and, after washing and fixation, bound TN-R was detected by immunofluorescence analysis using specific antibodies. TN-R was found to bind to F11 transfected cells but not to untransfected or mock transfected cells (Fig. 5 *a,* and data not shown), as detailed previously for microspheres coupled to TN-R (Brümmendorf et al., 1993). Bacterially expressed soluble TN-R fragments fused to glutathione-S-transferase were also analyzed with respect to binding to F11 transfected cells. Because all fusion proteins share the glutathione-S-transferase, polyclonal antibodies directed to the enzyme were used for immunofluorescence detection of cell-bound fusion proteins. Recombinant TN-R fragment containing FNIII domains 2–3 clearly bound to F11 transfected cells and not to untransfected or mock transfected cells (Fig. 5 *b,* and data not shown). Other fragments of TN-R were not found to bind specifically to transfected cells (Fig. 5, *c–e,* and data not shown). The gluthathione-S-transferase bound very weakly to COS cells in general (Fig. 5 *f*). In conclusion, the two types of binding experiments demonstrated that TN-R fragments containing FNIII domains 2–3 specifically bound to F11 expressed on cells or coupled to microspheres.
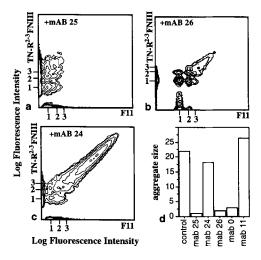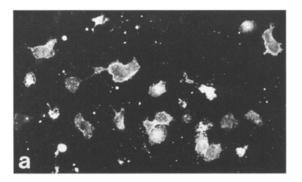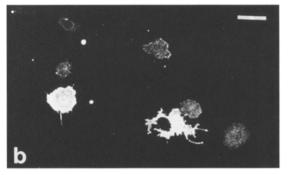
Figure 4. Inhibiton of heterophilic binding of the $^{2-3}$FNIII domains of TN-R to F11. The aggregation of the $^{2-3}$FNIII–coated beads with F11-coated beads was inhibited by specific mAbs directed to F11 (for microsphere aggregation studies, see the legend to Fig. 3). F11-coated beads were preincubated for 15 min with mAbs, mixed with $^{2-3}$FNIII domains–coated beads, and further incubated for 1 h. Preincubation of F11 beads with mAb 25 (*a* and *d*), mAb 26 (*b* and *d*), or mAb 0 (*d*) reduces the size of mixed aggregates by about 90%, whereas mAb 24 (*c* and *d*) or mAb 11 (*d*) has no effect. The epitopes of mAb 0 and mAb 26 have been localized on Ig domain 2–4, those of mAb 24 and mAb 25 on Ig domain 1 or 2, and that of mAb 11 on FNIII domain 3–4 of F11 (Brümmendorf et al., 1993; and data not shown). The mean size of the aggregates is shown in *d*.
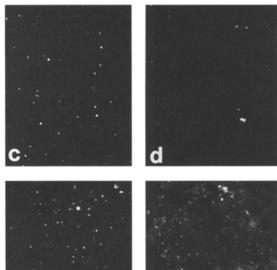
TN-R Fragment Containing FNIII Domains 2–3 Binds within the First Four Ig-like Domains of F11

To localize the binding site of the TN-R fragment containing FNIII domain 2–3 on the F11 polypeptide, mutant deletion polypeptides of F11 were expressed on the surface of transfected COS cells (Brümmendorf et al., 1993) and examined for their ability to bind the soluble $^{2-3}$FNIII domain of TN-R. F11 is a glycoprotein composing six Ig-like domains of the C2 subcategory and four FNIII-like domains (Brümmendorf et al., 1989). While construct IG, composing only the six Ig-related domains of F11, bound the $^{2-3}$FNIII domains of TN-R like the wild-type F11 polypeptide (Fig. 6, *a* and *e*), construct FN of F11, lacking the Ig-like domains, did not bind the $^{2-3}$FNIII domains of TN-R (Fig. 6 *b*). Mutant Ig26 lacking the NH$_2$-terminal domain of F11 was not found to bind the $^{2-3}$FNIII domains of TN-R (Fig. 6 *c*), nor does this mutant bind intact soluble TN-R, in contrast to microspheres-coupled TN-R (Brümmendorf et al., 1993). Mutant Ig14, encompassing the first four Ig domains and the two membrane proximal FNIII-like domains of F11, however, was able to bind the $^{2-3}$FNIII domains of TN-R (Fig. 6 *d*). This finding, together with the binding data using mutant IG and FN, demonstrates a direct molecular interaction of FNIII-like domains of TN-R

Figure 5. Binding of TN-R and of distinct TN-R fragments by F11-transfected COS cells. TN-R isolated from chick brain by immunoaffinity chromatography and bacterially expressed proteins

comprising TN-R fragments fused to glutathione-S-transferase were tested for binding to F11-transfected COS cells. Confluent cells containing 5–25% cells expressing F11 were incubated with protein samples, and after washing and fixation, bound protein was detected using immunofluorescence analysis. TN-R was visualized by immunofluorescence using a mixture of mAbs directed to TN-R, and fusion proteins were detected using polyclonal antibodies directed to glutathione-S-transferase. (*a*) TN-R bound to F11-expressing COS cells; (*b*) $^{2-3}$FNIII-like domains bound to F11 transfected cells. No specific binding to F11-expressing cells could be observed, with TN-R fragments containing the $^{5-8}$FNIII-like domains, including the alternatively spliced domain A (*c*), the $^{1-2}$FNIII-like domains (*d*), $^{4-A}$FNIII-like domains (*e*), or the glutathione-S-transferase itself (*f*). Bar, 100 μm.
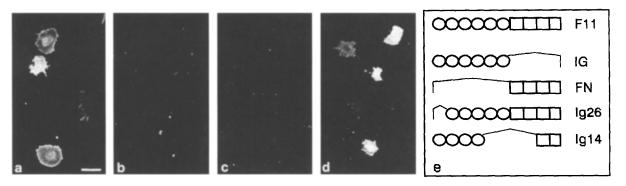
Figure 6. Binding of $^{2-3}$FNIII-like domain of TN-R to F11 deletion polypeptides expressed on the surface of transfected COS cells. COS cells transfected with different F11 deletion constructs were grown to confluence. Monolayers comprising 5–25% transfected cells were incubated with bacterially expressed $^{2-3}$FNIII domains of TN-R fused to glutathione-S-transferase. After washing and fixation, bound fusion protein was detected by immunofluorescence analysis using polyclonal antibodies directed to glutathione-S-transferase. (*a*) Construct IG comprising the Ig-related part of F11; (*b*) construct FN containing the FNIII-related domains of F11; (*c*) clone Ig26 lacking the aminoterminal domain of F11; (*d*) construct Ig14 lacking Ig-like domains 5 and 6 and the 1st and 2nd FNIII-related domains; (*e*) Schematic representation of mutant polypeptides of F11 used to narrow down the TN-R binding region on the F11 polypeptide. Ellipses symbolize Ig-like domains, and boxes represent FNIII-related repeats. NH$_2$ termini are on the left. Bar, 100 μm.

with Ig-like domains of F11 and confirms our previous finding that the first four Ig-like domains of F11 are sufficient to bind TN-R (Brümmendorf et al., 1993). Taken together, FNIII-like domains 2–3 of TN-R bind to the Ig-like domains 1–4 of F11 if F11 is presented immobilized on

beads or on the surface of living cells.

TN-R Enhances F11-Mediated Neurite Extension

To study further the influence of the interaction between F11 and TN-R on neurite extension, tectal cells were
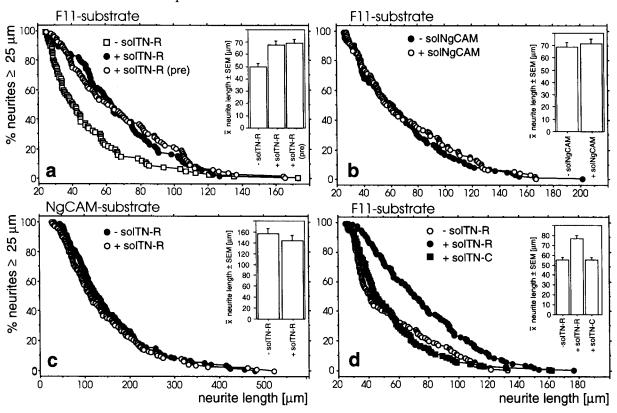

Figure 7. Neurite extension of tectal cells on immobilized F11 in the presence of TN-R. Culture dishes were coated with F11 (*a, b*, and *d*) or NgCAM (*c*) immunoaffinity purified from adult chick brain (5 μl of 100 μg/ml solution) and blocked with culture medium containing 10% FCS. Tectal cells from 6-d-old chick embryos were plated at a density of 1 × 10⁶ cells/ml for 40 h in the absence (−*sol*) or presence (+*sol*) of soluble TN-R, TN-C, or NgCAM. In one set of experiments, (*a*) immobilized F11 was preincubated (pre) for 2 h with TN-R (*open circles*), followed by washing and plating of the cells. The percentage of neurons (vertical axis) with neurites longer than or equal to 25 μm (horizontal axis) is plotted as introduced by Chang et al. (1987). The inserts show the mean neurite length in micrometers, and the bars on the columns indicate SEM. The distributions of neurite lengths on F11 compared with F11 plus TN-R in solution or preincubated are significantly different ($p < 0.0001$; Mann-Whitney U-test), whereas neurite lengths are not significantly different with TN-C or NgCAM in solution.
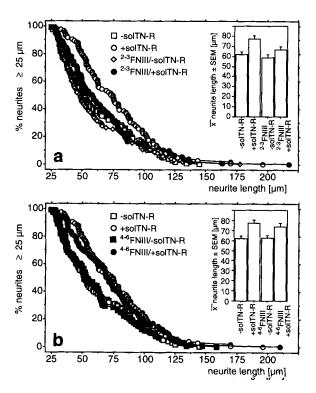
Figure 8. Neurite extension on the F11/TN-R complex and competition by recombinant proteins. (a) Chick tectal cells were cultivated on F11, as indicated in the legend of Fig. 7, in the absence (open squares) or presence (open circles) of TN-R, in the presence of ²⁻³FNIII domains (open squares), or in the presence of ²⁻³FNIII domains plus TN-R (filled circles). (b) Tectal cells grown on immobilized F11 in the absence (open squares) or presence (open circles) of TN-R in the presence of ⁴⁻⁶FNIII domains (filled squares) or in the presence of ⁴⁻⁶FNIII domains plus TN-R (filled circles). The distributions of neurite length on F11/TN-R in the presence or absence of the TN-R fragment containing domains 2–3 are significantly different ($p = 0.0022$; Mann-Whitney U-test). The inserts show the mean neurite length in micrometers, and the bars on the columns indicate SEM.
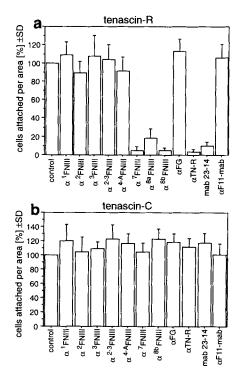
Figure 9. Inhibition of retinal cell attachment to a TN-R substrate by antibodies. (a) TN-R substrate was coated on culture dishes at 50 μg/ml, blocked with bovine serum albumin, and incubated 15 min with polyclonal antibodies generated to specific TN-R fragments. Unbound antibodies were removed, the substratum was washed with PBS, and retinal cells from 9-d-old embryos were added. Chick retinal cells were plated at a density of 1 × 10⁶ ml, incubated for 90 min, washed, and fixed; attached cells per area were counted by microscopy. The values for cells attached are normalized to control samples without antibodies. Mean values with standard deviations are shown. Each experiment was repeated five times. Antibodies (α) directed to the FNIII-like domain 7 or 8 of TN-R inhibited cell attachment nearly completely, whereas antibodies directed to other FNIII domains or to the FG domain of TN-R had no effect. Cell attachment could also be blocked by polyclonal antibodies directed to TN-R and by mAb 23-14, which binds to the FNIII domain 8. A mixture of mAbs directed to the Ig-related part of F11, which comprises the TN-R binding site, had no effect on retinal cell attachment on immobilized TN-R. (b) The same set of polyclonal and monoclonal antibodies had no effect on retinal cell attachment on immobilized TN-C.

grown on immobilized F11 in the presence of soluble TN-R or TN-R adsorbed to F11. As reported previously, tectal neurons generate long axons on an F11 substrate after 2 d of cultivation (Brümmendorf et al., 1993; Morales et al., 1993), while TN-R presented as substratum does not induce the extension of neurites (Rathjen et al., 1991). If TN-R is added to cultures of tectal neurons at the time of cell plating, TN-R was not found to neutralize the permissive properties of F11. In contrast, neurons extend neurites that are 30–40% longer than those on F11 alone (Fig. 7 a). The same result was obtained if TN-R was preincubated with immobilized F11, allowing formation of F11/TN-R complexes. No enhancement was observed when tectal cells were cultivated on substratum-bound NgCAM in the presence of soluble TN-R, indicating that TN-R specifically modulates F11-mediated outgrowth (Fig. 7 c). Similarly, if soluble NgCAM (Fig. 7 b) or soluble TN-C (Fig. 7 d) was added to neurons cultivated on an F11 substratum, no change in the length of the neurites was measured. The generation of longer neurites was reduced by mAbs against F11, which are known to block the F11–TN-R interaction (see Fig. 4), indicating that a direct interaction

between F11 and TN-R is required to induce the enhancement. Interestingly, the fragment of TN-R containing the F11 binding site, FNIII domains 2–3, was unable to accelerate F11-mediated neurite extension; however, it could reduce the enhancement on F11 induced by intact TN-R (Fig. 8 a). Furthermore, the TN-R fragment containing domains 4–6, which does not participate in binding of TN-R to F11, did not neutralize the effect (Fig. 8 b). Antibodies to Nr-CAM, an axon-associated glycoprotein, which has been identified as an axonal receptor of tectal neurons mediating neurite extension on immobilized F11 (Morales et al., 1993), completely blocked the extension on the complex of F11/TN-R (data not shown), indicating that the primary signal to extend neurites is provided by F11 via NrCAM.
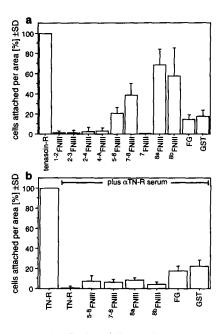
Figure 10. Retina cell attachment to recombinant TN-R fragments. TN-R fragments expressed as GST-fusion proteins were immobilized on culture dishes at 50 μg/ml. Retinal cells from 9-d-old chick embryos were added at a density of 1 × 10⁶ per ml, incubated for 90 min, and fixed; attached cells per area were counted by microscopy. (*a*) TN-R fragments containing FNIII-like domain 8 showed a cell attachment activity of 60–70% of the level of intact TN-R. The cell attachment to the FNIII domain 8 can be inhibited by polyclonal antibodies generated against TN-R. (*b*) The cell attachment on glutathione-S-transferase (20%) or FG domain (18%) was not inhibited by antibodies directed to TN-R. The values are normalized to cell attachment on intact TN-R. Mean values with standard deviations are shown. Each experiment was repeated five times.

Localization of the Cell Attachment Site within TN-R

Retinal cells adhered efficiently to immobilized TN-R but did not spread as detailed previously (Rathjen et al., 1991). Antibody inhibition experiments revealed that the attachment site for retinal cells mapped to the distal region of a TN-R arm (Nörenberg et al., 1992). To localize the domain containing the cell attachment site, antibodies to recombinant FNIII-like and FG-like domains of TN-R were generated in mice or rabbits. Antibodies to FNIII-like domains 7 or 8 completely inhibited the attachment to TN-R, while other domain-specific antibodies were found not to affect the adherence of cells (Fig. 9 *a*). Similarly, mAb 23-14, which binds to FNIII-like domain 8 (data not shown), strongly reduces the attachment to intact TN-R (Fig. 9 *a*), suggesting that FNIII-like domains 7 and 8 are important for the attachment of retinal cells. These antibodies did not block attachment of retinal cells to TN-C, which is structurally related to TN-R (Fig. 9 *b*). Furthermore, a mixture of mAbs directed to the Ig-related part of F11, which composes the TN-R binding site, had no effect on retinal cell attachment to immobilized TN-R, indicating that the observed cell attachment is distinct of the F11 binding (Fig. 9 *a*).

To localize further the cell attachment site, the various fusion proteins were immobilized on culture dishes. Only

the fragment containing the FNIII-like domain 8 showed cell attachment activity and reached 60–70% of the level of the intact protein (Fig. 10). This result is seen by two independently generated plasmid constructs of FNIII domain 8 ([8a]FNIII and [8b]FNIII). Combination of the type III domain 8 with other type III units in one fusion protein, as exemplified in the TN-R fragment containing FNIII-like domains 5–8 or a combination of two single domains, did not result in improving cell attachment activity (Fig. 10 *a*, and data not shown). The attachment to the FNIII-like fragments containing the FNIII-like domain 8 was specifically inhibited by antibodies directed to intact TN-R (Fig. 10 *b*) or by mAb 23-14 (not shown). Cell attachment to domain 8 was dependent on the fragment concentration used to immobilize it on the dish (data not shown). In conclusion, we demonstrated that retinal cells attach to FNIII-like domain 8 of TN-R. Because there is no cross-reactivity of antibodies directed to FNIII-like domain 7 with FNIII-like domain 8 and vice versa (data not shown), the inhibition of cell attachment by antibodies directed to the FNIII-like domain 7 could be explained by sterical effects.

Discussion

The multidomain structure of TN-R suggests the existence of multiple independent functions within the TN-R polypeptide. To analyze the structure-function relationships of the different domains of TN-R, we have used fusion proteins produced in bacterial cells. The approach of generating single domains implies that each FNIII segment, or two tandem repeats, folds correctly, independent of its neighbors, into a native structure. Such strategies have also been successfully employed for FNIII-like domains of TN-C (Leahy et al., 1992; Prieto et al., 1992; Aukhill et al., 1993) and fibronectin (Baron et al., 1992; Main et al., 1992). We have characterized two distinct regions within TN-R that interact with the surface of neural cells, including a cell attachment site in FNIII-like domain 8 and a binding site for the axon-associated protein F11 within FNIII domains 2–3.

In vitro neurite outgrowth experiments demonstrate that the combination of F11 and TN-R, if offered to neurons, results in an enhancement of F11-mediated but not NgCAM-mediated neurite extension. This enhancement requires the direct binding of TN-R to F11 and was not observed with NgCAM or TN-C. An enhancement of length has also been observed for hippocampal neurites on a poly-ornithine substratum (Lochter et al., 1994). It is conceivable that the binding of TN-R to F11 induces a conformational shift within the F11 polypeptide, allowing axons to interact more effectively with F11. Alternatively and more likely, TN-R, which becomes attached through its binding site to F11, might provide an additional signal for neurite extension, which, together with the F11-NrCAM signal, supports axonal growth. This signal presented by TN-R alone might be insufficient to induce axonal extension because it was found that TN-R offered alone does not induce the formation of axons (Rathjen et al., 1991). The observation that the binding fragment of TN-R domains 2–3 alone did not enhance neurite extension on F11 might favor the second explanation. If this explanation is correct, it should be possible to identify and map a site

within the TN-R polypeptide that provides such an additional signal. Current investigations are aimed to localize such a region within TN-R.

The binding of TN-R to F11 might be of regulatory influence on F11-mediated neurite outgrowth in vivo. For example, it is possible that TN-R secreted by glial cells might bind to surface-associated F11 and thereby offer a more favorable substratum for NrCAM-positive axons. The local enhancement of axonal elongation might then influence pathway choices of growth cones. In this context it is interesting that the synthesis of TN-R could be modulated by PDGF (Jung et al., 1993).

The site of interaction between F11 and TN-R can be mapped to the second and third FNIII-like domain of TN-R, while the binding site of TN-R on F11 resides in the first four Ig-like domains of F11. The NH_2-terminal–localized C2 domains of F11 have also been observed to interact with NgCAM and NrCAM (Brümmendorf et al., 1993; Morales et al., 1993), two transmembrane proteins composed of multiple Ig- and FNIII-related units (Moos et al., 1988; Burgoon et al., 1991; Grumet et al., 1991). It is currently unknown whether the NH_2-terminal C2 domains of F11 also bind to the type III repeats within these proteins. However, because neurite outgrowth of tectal cells on F11 as well on the F11/TN-R complex is mediated by NrCAM, it can be concluded that TN-R does not compete with the binding of NrCAM to F11. In a previous study we demonstrated that the 2nd and 3rd domains of F11 are important for TN-R binding (Brümmendorf et al., 1993). In this previous work, mutant Ig26, lacking the NH_2-terminal domain of F11, was found to bind TN-R–coated covaspheres less well than wild-type F11 polypeptide and less well than other mutants examined (Brümmendorf et al., 1993, Table 1). In the present study, however, soluble proteins were used—TN-R fragment $^{2-3}$FNIII (Fig. 6 c) and soluble TN-R (data not shown)—and both failed to bind to mutant Ig26. Furthermore, mAb 25, which was mapped to domain 1 or 2 of F11, was found to have no effect on TN-R–F11 interaction in the former study, which used covaspheres and COS cell transfectants, whereas it inhibits the interaction in the present study, employing flow cytometric analyses of covasphere–covasphere interactions (Fig. 4, a and d). Both discrepancies are likely caused by different sensitivities of the assays employed. It is possible that covasphere binding to transfected COS cells is more sensitive than covasphere–covasphere interaction or binding of soluble protein to COS cell transfectants. The greater sensitivity is caused by "catching" of the F11 molecules between the covasphere and the cell surface, because the GPI-linked F11 molecules are likely to move freely within the membrane. No such "catching" effects can be expected in either of the other assays. Unfortunately, the covasphere-coated TN-R fusion proteins, expressed in bacteria and lacking carbohydrates, revealed high background if incubated with COS cells and therefore could only be used as soluble proteins. On the basis of the present study, the NH_2-terminal domain of F11 seems to contribute to TN-R binding. Binding between F11 and TN-R requires the two FNIII modules of TN-R in series, suggesting that both domains participate directly in binding to the C2 domains of F11, or the conformation of one of these two FNIII units is dependent on the presence of its neighbor. A conformational link of two

FNIII domains has also been suggested by high resolution crystallographic data on the first two FNIII domains of *Drosophila* neuroglian (Huber et al., 1994). The 13th and 14th FNIII domain within fibronectin might also form a cooperative structure, since it was found that domain 13 alone binds heparin with a lower affinity than both domains together (Ingham et al., 1993). Further evidence for intra-chain interactions of FNIII modules has been provided by the observation that the inclusion of an alternatively spliced FNIII domain results in the exposure of an antigenic site, which is explained by a shift in the conformation (Carnemolla et al., 1992).

An attachment site for retinal cells, which we mapped to FNIII domain 8 of TN-R, might be related to the adhesion site within FNIII domains 7–8 of TN-C identified in TN-C using fragments generated by limited proteolysis (Friedlander et al., 1988; Chiquet et al., 1991) or by recombinant DNA technology (Spring et al., 1989; Prieto et al., 1992). Similar to TN-R, cells attaching to this region remain rounded and do not spread. Prieto et al. (1992) mapped further adhesive regions within FNIII domains 2–6 and the fibrinogen segment of TN-C using different cell lines and a gravity cell adhesion assay. In our adhesion assays we found no further cell attachment activity in other FNIII domains of TN-R, and the F11 binding site within $^{2-3}$FNIII domains was found to be distinct of the cell attachment activity in the FNIII domain 8. Treatment of cells with heparinase or the addition of heparin in solution suggests that a cell surface proteoglycan might be important for the attachment of the cells to the fibrinogen region in TN-C (Aukhill et al., 1993). Other cellular receptors for TN-C might include the heparin sulfate proteoglycan syndecan (Salmivirta et al., 1991), integrins (Prieto et al., 1993; Wherle-Haller and Chiquet, 1993), phosphacan/RPTPζ/β (Barnea et al., 1994; Grumet et al., 1994; Maurel et al., 1994), F11 (Zisch et al., 1992), and annexin II (Chung and Erickson, 1994).

Our study represents an example of a direct interaction between Ig-like domains of the C2 subcategory (of F11) and FNIII-like domains (of TN-R). Another example of interaction of FNIII-related repeats with C2 domains might occur between the F11 protein and TN-C (Zisch et al., 1992), an observation that is controversial in the literature (Pesheva et al., 1993). The FNIII and C2 domains are common structural motifs and are found in a variety of cell surface and extracellular matrix proteins implicated in cell–cell recognition. They are frequently combined in series in glycoproteins that are primarily localized on axonal surfaces in vertebrates and invertebrates and are implicated in axonal growth and fasciculation (Brümmendorf and Rathjen, 1993, 1994). Although the primary structure of both domain types is only distantly related, high-resolution structures of FNIII domains of tenascin-C (Leahy et al., 1992), fibronectin (Main et al., 1992), and neuroglian (Huber et al., 1994), and the analysis of three C2 domains (Ryu et al., 1990; Wang et al., 1990; Jones et al., 1992; Brady et al., 1993) indicate a similar β-strand topology. Both domains are formed by two antiparallel β-pleated sheets packed face to face; the two β-sheets are composed of three to four antiparallel β-strands. Point-mutational studies are required to localize more precisely the subregions within the two FNIII-like domains of TN-R and

within the C2-type domains of F11. Such investigations might reveal whether two FNIII domains in tandem are essential to interact with the C2 domains of F11 and whether the loops connecting the β-strands are important for binding. Detailed mapping studies of IgSF molecules by site-directed mutagenesis indicate that, in principle, any exposed surface of an Ig-like domain is able to participate in binding with other proteins. Such mapping studies have not been performed extensively for FNIII domains (Brümmendorf and Rathjen, 1994).

In summary, the studies presented in this report, together with previous investigations (Brümmendorf et al., 1993; Morales et al., 1993), show that the Ig-like protein F11 regulates axonal growth in a complex interplay with TN-R and NrCAM and probably other proteins, including NgCAM and TN-C (Zisch et al., 1992). The detailed characterization of the binding regions used by the Ig- and FNIII-like domains in these proteins might help to understand how growth cones realize specific pathway choices.

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