Functional Interactions of the Immunoglobulin Superfamily Member F11 Are Differentially Regulated by the Extracellular Matrix Proteins Tenascin-R and Tenascin-C*

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The axon-associated protein F11 is a GPI-anchored member of the immunoglobulin superfamily that promotes axon outgrowth and that shows a complex binding pattern toward multiple cell surface and extracellular matrix proteins including tenascin-R and tenascin-C. In this study, we demonstrate that tenascin-R and tenascin-C differentially modulate cell adhesion and neurite outgrowth of tectal cells on F11. While soluble tenascin-R increases the number of attached cells and the percentage of cells with neurites on immobilized F11, tenascin-C stimulates cell attachment to a similar extent but decreases neurite outgrowth. The cellular receptor interacting with F11 has been previously identified as NrCAM; however, in the presence of tenascin-R or tenascin-C cell attachment and neurite extension are independent of NrCAM. Antibody perturbation experiments indicate that $\beta_1$ integrins instead of NrCAM function as receptor for neurite outgrowth of tectal cells on an F11-TN-R complex. Cellular binding assays support the possibility that the interaction of F11 to NrCAM is blocked in the presence of tenascin-R and tenascin-C. Furthermore, a sandwich binding assay demonstrates that tenascin-R and tenascin-C are able to form larger molecular complexes and to link F11 polypeptides by forming a molecular bridge.

These results suggest that the molecular interactions of F11 might be regulated by the presence of tenascin-R and tenascin-C.

Cell adhesion molecules (CAMs) of the immunoglobulin superfamily (IgSF) act in concert with other cell surface molecules and extracellular matrix (ECM) proteins to regulate cell migration, axonal growth, and guidance during development of the nervous system. IgSF members coexist on many extending axons and show a transient expression pattern during early stages of development. The multidomain nature of glycoproteins of the IgSF suggest that they regulate axonal pathfinding by multiple complex interactions with other axonal and ECM molecules (1).

The axon-associated F11 glycoprotein is composed of six N-terminal Ig domains followed by four fibronectin type III (FNIII) domains and a glycosylphosphatidylinositol anchor and has been implicated in axonal growth and fasciculation (2–6). As found for other axonal members of the IgSF, the F11 polypeptide shows a broad binding activity. Interactions with the cell surface proteins NgCAM, NrCAM, neurofascin, Caspr, and RPTP$\beta$/$\zeta$ and the ECM glycoproteins tenascin-R (TN-R) and tenascin-C (TN-C) have been revealed by in vitro assays (7–16). The N-terminal Ig domains 1–4 of the F11 polypeptide are sufficient for interactions with NgCAM, NrCAM, TN-R, and TN-C, although binding assays with domain-specific anti-F11 monoclonal antibodies and with F11 domain deletion mutants suggest that individual domains of the four N-terminal domains might be more important for specific bindings (8–10, 15). The interaction between immobilized F11 and neuronal NrCAM induces neurite outgrowth of tectal cells (10).

TN-R and TN-C are two major members of the tenascin family of ECM glycoproteins. These multidomain proteins are composed of a cysteine-rich segment, epidermal growth factor-like repeats, FNIII-like domains, and a segment similar to the $\beta$- and $\gamma$-chains of fibrinogen (for a review, see Ref. 17). TN-R and TN-C show striking functional analogies, but within the nervous system TN-R has a more restricted localization than TN-C, has a different developmental time course, and is synthesized by oligodendrocytes and a subpopulation of neurons rather than predominantly by astroglia (18). TN-R and TN-C form oligomeric structures as revealed by rotatory shadowing electron microscopy (19, 20). Multiple ligands have been described for TN-R and TN-C including cell surface proteins such as F11, axonin-1, CALEB, RPTP$\beta$/$\zeta$, integrins, and ECM glycoproteins and proteoglycans such as neurocan, phosphacan, versican, brevican, heparin, and fibronectin (21–29). Interactions between TN-R or TN-C and cell surface molecules affect cell adhesion and neurite growth. The responses can be either stimulatory or inhibitory, depending on the specific neuronal cell type studied, the assay design (choise situation on patterned substrates or homogeneous substrate), and they are probably mediated by separate domains. Neurite outgrowth-promoting, cell-binding, antiadhesive, and nonpermissive regions have been identified in TN-R and TN-C (15, 30–36). These observations could also reflect the differential expression of receptor complexes on the responding cells or of downstream effector mechanisms that control the growth cone. The short term attachment site for retinal cells within TN-R was localized to FNIII domain 8, while the site interacting with F11 has been mapped to the FNIII domains 2 and 3. Furthermore, TN-R FNIII domain 2 has been shown to mediate homophilic interaction (15). Cell attachment sites within TN-C have been identified in FNIII domains 3 and 6–8 and in the fibronogen-like glob (25, 26, 29, 35, 37), whereas F11 binds to FNIII domains 5 and 6 (24). The multidomain and oligomeric struc-
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MATERIALS AND METHODS

Proteins and Antibodies—NgCAM, NrCAM, TN-R, and TN-C were purified from detergent (CMs) and urea (TNs) extracts, respectively; F11 and axonin-1 were purified from phosphatidylinositol-specific phospholipase C-treated extracts of plasma membrane preparations of adult chicken brains followed by immunoaffinity chromatography as described previously (4, 8, 10, 16, 47, 48). The purity of isolates was analyzed by SDS-PAGE. Isolation of monoclonal antibodies and generation of Fab fragments of polyclonal antibodies to these antigens are detailed elsewhere (2, 10, 49, 50). TN-R fragments were expressed as glutathione S-transferase fusion proteins and purified as described (15).

Monoclonal anti-β1 integrin antibody WIB10 was purchased from Sigma (Deisenhofen, Germany), and JG22 was purified from the supernatant of hybridomas that were obtained from the Developmental Studies Hybridoma Bank (John Hopkins University School of Medicine, Baltimore, MD).

Transfection of COS7 Cells and Microsphere Binding Assay—Immunoaffinity-purified F11 and NrCAM were conjugated to red fluorescent microspheres of 0.5 μm in diameter according to the manufacturer’s protocol (Biolean, Duke Scientific Corp., Palo Alto, CA), and residual binding sites were blocked by bovine serum albumin.

COS7 cells were transiently transfected with F11- or NgCAM-encoding plasmids using the DEAE-dextran method as described previously (8, 51). After 24 h, transfected cells were transferred to poly-L-lysine (100 μg/ml)-coated eight-well multiscr (ICN, Costa Mesa, CA), grown overnight, and then incubated with 30 μl of Dulbecco’s modified Eagle’s medium, 10% fetal calf serum containing 0.3 μl of microsphere solution.

For competition binding assays, NrCAM-coated microspheres were allowed to bind to F11-expressing COS7 cells in the absence or presence of F11 (10 μg/ml), TN-C (20 μg/ml), NrCAM (20 μg/ml), or NgCAM (20 μg/ml). Monoclonal antibodies to F11 (10 μg/ml), laminin (10 μg/ml), and bovine serum albumin (10 μg/ml) that was spread over 1 cm² delineated with a silicon fitting at 4°C overnight. Note that in previously published experiments, 10 μl of a F11 solution at a concentration of 100 μg/ml was used for coating (10, 15). Residual binding sites were blocked by washing and incubating with Dulbecco’s modified Eagle’s medium/10% fetal calf serum for 30 min at 37°C. Single cell suspensions were obtained by dissociation of chick tecta of embryonic day 6 in a trypsin solution (1 mg/ml, 20 min at 37°C) and subsequent trituration. After resuspension in Dulbecco’s modified Eagle’s medium/10% fetal calf serum, 15,000 cells/well were plated on immobilized F11 and grown in the absence or presence of TN-R (0.6–50 μg/ml), TN-C (1.2–20 μg/ml), NrCAM (20 μg/ml), F1 (20 μg/ml), or axonin-1 (20 μg/ml). Monoclonal antibodies to β1 integrin WIB10 and JG22 were added at the time of plating at a final concentration of 20 μg/ml each. All other monoclonal antibodies and Fab fragments of polyclonal antibodies to different proteins were used at a final concentration of 10 and 200 μg/ml, respectively. For preincubation studies, immobilized F11 was incubated with TN-R (10 μg/ml) or TN-C (20 μg/ml) after blocking for 1 h at 37°C. Unbound TN-R and TN-C were removed by extensive washing with Dulbecco’s modified Eagle’s medium, 10% fetal calf serum prior to the addition of cells. After cultivation for 40 h, cells were fixed in 4% formaldehyde/phosphate-buffered saline and stained by indirect immunofluorescence using monoclonal antibody A2B5 and Cy3-conjugated anti-mouse polyclonal antibodies (Dianova; Hamburg, FRG). The number of attached cells and the number of extending neurites were quantified with Genias imaging software (Image Works; Teltow, Germany (52)) and calculated as the percentage of control cultures.

RESULTS

The ECM Glycoproteins TN-R and TN-C Compete with NrCAM and NgCAM to Bind to F11—An interesting feature of axonal IgSF members and ECM glycoproteins is their complex binding pattern. The binding sites for NrCAM, NgCAM, TN-R, and TN-C have been mapped to a similar region of the F11 polypeptide comprising Ig domains 1–4. This makes it important to evaluate whether these proteins can bind simulta-
The presence of TN-C (FNIII 4 to A (E)) coated microspheres in the absence of TN-R or TN-C, respectively, has been detected so far in different overlapping regions within the F11 polypeptide. To address this question, we established a competitive cellular binding assay and analyzed the interaction between F11 and NrCAM and between F11 and NgCAM in the presence or absence of soluble TN-R, TN-C, NgCAM, or NrCAM (Fig. 1). Binding of microspheres was quantified and related to the expression of F11 as described (52); see "Materials and Methods". Each value is the mean ± S.E. of three independent experiments. The binding of F11-coated beads to individual NrCAM-expressing COS7 cells in the presence or absence of competitor (see bottom row: TN-C, TN-R, TN-R-FNII, or TN-R-A-F) was quantified and related to the expression of NrCAM as described in G. H and J illustrate schematically the binding assays performed in the presence of TN-R or TN-C. Circles indicate fluorescent beads conjugated with NrCAM (Nr) or F11. TN-R forms three-armed oligomers (19), and TN-C is a hexamer (20).

The addition of soluble TN-R or TN-C competes for binding of NrCAM or NgCAM to the F11 polypeptide. A–F, F11-expressing COS7 cells were incubated with NrCAM-coated microspheres in the absence (A) or presence of TN-C (B) (20 µg/ml), NgCAM (C) (20 µg/ml), TN-R (D) (10 µg/ml), TN-R FNIII 2 and 3 (E) (50 µg/ml), or TN-R FNIII 4 to A (F) (50 µg/ml) in solution. Double fluorescence images obtained using a confocal microscope are shown. The left half of each micrograph reveals F11 expression on the COS7 cells by indirect immunofluorescence in the fluorescein isothiocyanate channel, while the right half depicts the binding of NrCAM-conjugated microspheres in the same microscopic field as detected in the Texas Red channel. Each microscopic field also contains unstained cells that do not express F11 and concomitantly do not bind beads. The bar in the left corner of A indicates 100 µm. Both TN-R and TN-C block the binding of NrCAM-conjugated microspheres to F11 expressed on the surface of COS7 cells. G, the binding of NrCAM-coated beads to individual F11-expressing COS7 cells in the presence or absence of competitor (see bottom row: TN-C, NgCAM, TN-R, TN-R-FNIII, or TN-R-A-F) was quantified and related to the expression of A–F) was quantified and related to the expression of NrCAM as described in G. H and J illustrate schematically the binding assays performed in the presence of TN-R or TN-C. Circles indicate fluorescent beads conjugated with NrCAM (Nr) or F11. TN-R forms three-armed oligomers (19), and TN-C is a hexamer (20).

The interaction between F11 and NgCAM was analyzed by quantifying the binding of F11-coated microspheres to NgCAM expressing COS7 cells (Fig. 1). Similar to the NrCAM-F11 interaction described above, the addition of soluble TN-R and TN-C resulted in a significant reduction of F11 coated beads to bind to NgCAM expressing COS7 cells (Fig. 1). However, in this assay system the recombinant TN-R fragment FNII 2–3 was able to mimic the inhibitory effect of intact TN-R on NrCAM binding is probably caused by steric hindrance. Taken together, these results suggest that the ECM glycoprotein TN-R or TN-C can block binding of the IgSF members NrCAM or NgCAM to F11 probably by competing for overlapping binding sites and/or by steric hindrance as illustrated in
TN-R Increases Cell Attachment and Neurite Outgrowth of Tectal Cells on Immobilized F11—Previous studies by us have shown that tectal cells extend long neurites on immobilized F11, and the cellular receptor mediating neurite outgrowth has been identified as NrCAM (10). To study the cell biological significance of the competition between TN-R and NrCAM (for binding to F11 as observed) in the binding assays described above, we analyzed in vitro long term cell attachment and neurite outgrowth assays. For this purpose, we slightly modified our previously used in vitro neurite outgrowth and cell attachment assays (10, 15) in a way that would allow us to add TN-R and TN-C in an excess large enough to compete with the F11-NrCAM interaction (for details, see “Materials and Methods”).

Under these conditions, the percentage of tectal cells with neurites and the percentage of attached cells increased significantly on an F11 substratum in the presence of increasing TN-R concentrations, reaching a saturation at 5 μg/ml TN-R (Fig. 2, A and B). TN-R induced a maximally 2-fold increase in the percentage of cells with neurites as well as in the total number of attached cells. This stimulation of neurite formation and cell adherence could be specifically inhibited by Fab fragments of polyclonal antibodies to TN-R (Fig. 3, A and B). Neurite extension could be completely blocked and cell attachment could be reduced by 85% by Fab fragments of polyclonal antibodies to F11 in the presence of TN-R (Fig. 2, C and D). In the absence of TN-R, the same antibody preparation both blocked adhesion and neurite extension completely (8, 15). Stimulation of neurite outgrowth and neural cell attachment was also observed if the F11 substratum was preincubated with soluble TN-R, followed by washing away unbound TN-R before adding tectal cells (Fig. 2, C and D), suggesting that binding of TN-R to the F11 substratum is required. TN-R did not enhance cell attachment and neurite extension on other immobilized proteins like NgCAM, axonin-1, and bovine serum albumin (data not shown). Furthermore, immobilized TN-R did not allow long term cell attachment and neurite elongation of E6 tectal cells (16). To provide further specificity, we cultivated tectal cells on immobilized F11 in the presence or absence of other soluble proteins known to interact with F11 or NrCAM (Fig. 2, C and D). Soluble TN-R almost completely inhibited neurite extension and cell attachment, probably by competing with the immobilized F11 substrate. Soluble NrCAM strongly reduced neurite outgrowth on F11 possibly due to binding to immobilized F11. Soluble axonin-1, however, had no effect.

In summary, these data indicate that the modulation of cell attachment and neurite outgrowth by TN-R was due to a complex formation between soluble TN-R and immobilized F11. TN-R Induces NrCAM-independent Tectal Cell Attachment and Neurite Outgrowth on Immobilized F11—On a pure F11 substratum and at low TN-R concentrations (0.6 and 1.2 μg/ml) neurite extension and cell attachment could be almost completely inhibited by Fab fragments of polyclonal antibodies to NrCAM (Figs. 3, A and B, and 4, A and D). This is in agreement with our previous results and confirms that NrCAM functions as axonal receptor to extend neurites on a pure F11 substratum (10, 15). However, in the presence of increasing amounts of TN-R, starting at 2.5 μg/ml, neurite outgrowth and cell attachment on immobilized F11 showed a reduced sensitivity toward Fab fragments of polyclonal antibodies to NrCAM (Fig. 3, A and B). By using TN-R at a concentration of 5 μg/ml or higher, neurite extension was only slightly inhibited, and cell attachment remained unaffected by Fab fragments of polyclonal antibodies to NrCAM as depicted in Fig. 4, B and E, and quantified in Fig. 3, A and B. Soluble NrCAM and anti-NrCAM monoclonal antibody (mAb) 3 were used to support the observations with polyclonal antibodies to NrCAM. Both blocked neurite extension on an F11 substrate in the absence of TN-R (Fig. 3C). Similar to polyclonal antibodies to NrCAM, in the presence of TN-R soluble NrCAM only partially reduced neurite extension, and the anti-NrCAM mAb 3 had no inhibitory effect on neurite outgrowth (Fig. 3C). In contrast to Fab fragments of polyclonal antibodies to NrCAM, soluble NrCAM and
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FIG. 3. TN-R induces a receptor switch during neurite outgrowth and cell attachment on F11. A and B, culture dishes were coated with F11 (100 µl of a 2 µg/ml solution) overnight and blocked, and tectal cells were cultivated for 40 h in the presence of increasing amounts of TN-R (0.6–10 µg/ml) with or without Fab fragments of anti-NrCAM polyclonal antibodies (200 µg/ml). The number of neurites (A) and the number of attached cells (B) were measured using the Genias imaging software, and the percentage of inhibition by anti-NrCAM antibodies was calculated. C and D, tectal cells were cultivated for 40 h on immobilized F11 in the absence or presence of TN-R (10 µg/ml), Fab fragments of polyclonal antibodies to NrCAM (200 µg/ml), anti-NrCAM mAb 3 (10 µg/ml), and NrCAM (20 µg/ml). The protein in solution and the applied antibody are given at the bottom. The number of neurites (C) and the number of attached cells (D) were calculated as the percentage of control cultures. Each value is the mean ± S.E. of three independent experiments.

FIG. 4. Neurite outgrowth on immobilized F11 is modulated differentially by TN-R and TN-C. Culture dishes were coated with F11 (100 µl of a 2 µg/ml solution) overnight and blocked, and tectal cells were cultivated in the absence (A and D) or presence of TN-R (B and E) (10 µg/ml) and TN-C (C and F) (20 µg/ml) for 40 h, and representative photomicrographs are shown. Bar, 100 µm. The percentage of cells with neurites increased in the presence of TN-R and decreased in the presence of TN-C. Fab fragments of polyclonal anti-NrCAM antibodies (200 µg/ml) block neurite outgrowth and cell attachment on immobilized F11 in the absence (D) but not in the presence of TN-R (E). In the presence of TN-C, Fab fragments of anti-NrCAM antibodies (200 µg/ml) reduce neurite outgrowth but do not affect cell attachment (F).

anti-NrCAM mAb 3 had no effect on cell attachment in the absence of TN-R (Fig. 3D). The observation that neither soluble NrCAM nor anti-NrCAM mAb 3 inhibits cell attachment in contrast to neurite extension might be explained by a higher sensitivity of neurite outgrowth compared with cell attachment.

These results support the idea that NrCAM no longer functions as a cellular receptor for cell attachment and neurite outgrowth on an F11-TN-R complex in contrast to a pure F11 substrate. One explanation could be that TN-R inhibits the binding of cellular NrCAM to substrate F11 as it has been described above by competition binding assays and that the F11-TN-R complex might provide additional signal(s) for cell attachment and neurite extension, which is recognized by an alternate receptor on the surface of tectal cells.

TN-C Increases Cell Attachment and Inhibits Neurite Outgrowth of Tectal Cells on Immobilized F11—Similar to TN-R, the presence of increasing amounts of TN-C in tectal cell cultures on immobilized F11 resulted in a significant (maximal 2-fold at 20 µg/ml TN-C) increase in the number of attached cells (Fig. 5B). As observed for TN-R, preincubation of immobilized F11 with soluble TN-C followed by washing resulted in a similar increase in cell attachment (Fig. 5D), suggesting that this stimulation required the binding of TN-C to immobilized F11. On the F11-TN-C complex, Fab fragments of polyclonal antibodies to F11 strongly inhibited cell attachment (by 85%), while antibodies to TN-C reduced attachment to the same levels as when immobilized F11 alone was used as substratum (Fig. 5D). Similar to the experiments with TN-R, Fab fragments of polyclonal antibodies to NrCAM had no longer any inhibitory effect on cell attachment to immobilized F11 in the presence of TN-C (Fig. 5D). These results indicate that for tectal cell adhesion on the F11-TN-C complex NrCAM is replaced as cellular receptor as it has been described above for the F11-TN-R complex.

In contrast to TN-R, however, the presence of high concentrations of TN-C, starting at 10 µg/ml, reduced the percentage of cells with neurites on an F11 substratum to about 50% of control values (Fig. 5A). This residual neurite outgrowth on the F11-TN-C complex was almost completely blocked after the addition of Fab fragments of polyclonal antibodies to NrCAM, the anti-NrCAM mAb 3 or soluble NrCAM (Figs. 4, C and F, and 5C). Residual neurite extension observed in the presence of TN-C is therefore likely to be due to remaining uncomplexed F11.

These results also indicate that the F11-TN-C complex is not able to induce neurite outgrowth of tectal cells most likely because the F11-TN-C complex in contrast to the F11-TN-R complex does not activate alternate receptor protein(s) on the surface of tectal cells required for neurite extension. TN-C complexed to F11, however, provides additional signal(s) for long term NrCAM-independent cell attachment.
FIG. 5. Modulation of neurite outgrowth and cell attachment on immobilized F11 in the presence of TN-C. Culture dishes were coated with F11 (100 μl of a 2 μg/ml solution) overnight and blocked, and tectal cells were cultivated for 40 h in the presence of increasing amounts of TN-C (1.2–20 μg/ml) (A and B) or in the absence or presence of TN-C (20 μg/ml; Fab fragments of polyclonal antibodies to F11, TN-C, and NrCAM (200 μg/ml); anti-NrCAM mAb 3 (10 μg/ml); and NrCAM (20 μg/ml) (C and D). The protein in solution and the applied antibody are given at the bottom. In some experiments, TN-C (20 μg/ml) was preincubated for 1 h with immobilized F11 and washed out before adding tectal cells (TN-C pre). The number of neurites (A and C) and the number of attached cells (B and D) were measured using the Genias imaging software and calculated as the percentage of control cultures. Data were compiled from four different experiments. Error bars represent S.E.

β1 Integrins Are Involved in Neurite Outgrowth on an F11-TN-R Complex—To characterize the alternate cellular receptor on tectal neurons responsible for neurite extension and cell adhesion on the F11-TN-R and F11-TN-C complexes, blocking antibodies specific for various cell surface proteins were applied in these in vitro assays. Antibodies to several axonal IgSF members were found not to block neurite outgrowth on the F11-TN-R complex (data not shown). Since integrins have been implicated in cell adhesion and neurite outgrowth on TN-C, they also represent potential cellular interaction partners for the F11-TN-R or the F11-TN-C complex (25, 26, 29, 32, 36). Most interestingly, different integrins including β1-containing heterodimers are expressed in the developing chick optic tectum (53–55). To test this possibility, antibody perturbation assays with mAbs to β1 integrin (a combination of mAb JG22 and mAb W1B10) have been performed. On a pure F11 substrate and at low TN-R concentrations (0.6–1.2 μg/ml), neurite extension is not influenced by mAbs to β1 integrins, which is in accordance with NrCAM functioning as cellular receptor under these conditions (Fig. 6, A and B). However, in the presence of increasing amounts of TN-R, when neurite outgrowth becomes independent of NrCAM as axonal receptor (see above, Fig. 3A), neurite extension showed an increased sensitivity toward mAbs to β1 integrins (Fig. 6A). By using TN-R at a concentration of 10 μg/ml, neurite extension was significantly inhibited by about 40% by mAbs to β1 integrins. This incomplete inhibition might suggest that additional receptor proteins are implicated in neurite outgrowth on the F11-TN-R complex or that the applied antibodies do not completely inactivate β1 integrins on tectal cells. For comparison, these mAbs were found to inhibit neurite outgrowth of tectal cells on a laminin-1 substrate by 70% (data not shown). These antibodies had no effect on the TN-C mediated reduction in neurite outgrowth (Fig. 6C), and furthermore neither basal cell attachment nor cell adhesion stimulated by TN-R and TN-C is influenced (Fig. 6, B and D). These observations suggest that attachment and neurite extension on the F11-TN-R or F11-TN-C complexes are mediated by distinct receptor systems and that other receptor(s) distinct from β1 integrins are implicated in cell attachment to the F11-TN-R or F11-TN-C complexes.

The inhibitory effect of integrin specific mAbs indicates that β1 subunit containing integrin heterodimer(s) mediate at least in part the neurite outgrowth promoting interactions of tectal neurons with an F11-TN-R complex. The identification of the α subunit(s) interacting with the β1 subunit on tectal cells mediating neurite extension on the F11-TN-R complex awaits further investigation, since the antibodies to α subunits available to us did not block neurite outgrowth.

TN-R and TN-C Form a Bridge between F11 Molecules—One possibility to explain the shift in receptor usage by TN-R and TN-C is that due to their oligomeric structure TN-R and TN-C may interact simultaneously with two individual F11 polypeptides on distinct cells or with an individual F11 polypeptide on one cell and a distinct receptor protein such as integrins on another cell. This might result in the formation of molecular bridges and larger molecular complexes. To address this question, a sandwich binding assay analyzing the interactions of TN-R and TN-C with F11 was chosen as a model system as illustrated in Fig. 7G. In this assay, F11-expressing COS7 cells were incubated with F11-coated microspheres in the presence or absence of soluble TN-R or TN-C. As described previously, F11 did not show homophilic interaction (Fig. 7A) (8). However, in the presence of soluble TN-R and TN-C, respectively, F11-coated beads bound to F11-expressing COS7 cells, suggesting that TN-R and TN-C might link them by forming a bridge (Fig. 7, B and D). In contrast, soluble NgCAM did not induce the formation of a similar complex (Fig. 7C), although it is known to reveal homophilic binding.

The site within TN-R interacting with F11 has previously been allocated to FNIII domains 2 and 3, and FNIII domain 2 has been shown to interact homophilically (15). Accordingly, a recombinant TN-R fragment comprising FNIII domains 2 and 3 was able to form a bridge similar to intact TN-R (Fig. 7E). For comparison, a recombinant TN-R fragment composed of FNIII...
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FIG. 6. \( \beta_1 \) integrins mediate neurite outgrowth on the F11-TN-R complex. A and B, culture dishes were coated with F11 (100 \( \mu \)l of a 2 \( \mu \)g/ml solution) overnight and blocked, and tectal cells were cultivated for 40 h in the presence of increasing amounts of TN-R (0.6–10 \( \mu \)g/ml) with or without mAbs to \( \beta_1 \) integrins (a combination of JG22 and W1B10 at a concentration of 20 \( \mu \)g/ml each). The number of neurites (A) and the number of attached cells (B) were measured using the Genias imaging software, and the percentage of inhibition by anti-\( \beta_1 \) integrin mAbs was calculated. C and D, tectal cells were cultivated for 40 h on immobilized F11 in the absence or presence of TN-R (10 \( \mu \)g/ml), TN-C (20 \( \mu \)g/ml), and mAbs to \( \beta_1 \) integrin (20 \( \mu \)g/ml). The protein in solution and the applied antibody are given at the bottom. The number of neurites (C) and the number of attached cells (D) were calculated as the percentage of control cultures. Each value is the mean ± S.E. of four independent experiments.

In summary, these sandwich assays indicate that F11 can bind to F11-TN-R or F11-TN-C complexes, suggesting that oligomeric TN-R and TN-C link F11-coated microspheres to F11-expressing COS7 cells by generating a molecular bridge. A similar complex formation could occur for the interaction of TN-R and TN-C with \( \beta_1 \) integrins in mediating NrCAM-independent neurite outgrowth on an F11-TN-R complex or with other currently unknown ligands in mediating cell attachment to the F11-TN-R or F11-TN-C complexes.

DISCUSSION

In this report, we have analyzed complex interactions of the F11 glycoprotein, a member of the IgSF, by using sandwich and competition binding assays. Because the ligands of F11 such as NgCAM, NrCAM, TN-R, and TN-C also show a complex binding pattern, it seems likely that these proteins are components of a complex network of molecular interactions (13, 22, 23, 27, 48, 56–60). In addition to other regulatory mechanisms, the formation of higher order complexes may be one possibility to modulate cell-cell and cell-ECM interactions at different sites and periods during development of the nervous system. Some of these interactions may be confined to very restricted areas and/or relatively brief developmental stages, and the multiplicity of ligands with different properties could provide a means for the fine regulation of complex developmental processes like axon guidance.

Competition and sandwich binding assays have revealed that binding of the ECM glycoproteins TN-R and TN-C to the IgSF member F11 specifically inhibits interactions of F11 with other IgSF members like NrCAM or NgCAM. However, binding of the ECM glycoproteins TN-R and TN-C to the F11 polypeptide also offers the possibility of additional interactions by linking F11 to F11 itself or to other cell surface receptors such as \( \beta_1 \) integrins and ECM glycoproteins via a TN-R or TN-C bridge. The result of this complex behavior may be a modulation of cell-cell and cell-ECM interactions, which may lead to changes in cell shape, cell migration, and neurite outgrowth during development and might be implicated in plasticity.

One example for the functional importance of the F11-TN-R interaction is illustrated by the finding that TN-R modulates cell attachment and neurite outgrowth of tectal cells on immobilized F11 and induces a shift in cellular receptor usage from NrCAM to \( \beta_1 \) integrins and most likely at least one additional protein. Such a change in receptor usage might be important within the developing retina, where in the outer and inner plexiform layers F11, NrCAM, and TN-R co-localize, in contrast to the developing spinal cord, where only F11 and NrCAM are found in the lateral and ventral axon tracts (16). It is therefore conceivable that the F11-NrCAM interaction is of importance in the latter regions, whereas in the plexiform layers of the retina the presence of TN-R reduces the F11-NrCAM interaction.

According to our sandwich binding assay, F11 represents another likely candidate for a cellular receptor mediating cell attachment and neurite outgrowth on an F11-TN-R complex. Unfortunately, it is not possible to define the role of F11 in our neurite outgrowth assay system because F11 is used here as an immobilized substrate, and this excludes antibody perturbation assays. Previous studies have shown that TN-R, if present alone as an immobilized substrate, does not allow axonal extension of tectal cells and have suggested that this protein is nonpermissive for neurite outgrowth or that additional signals might be required (16). One explanation for the functional effect of TN-R described here might be that the interaction between TN-R and F11 induces a conformation in TN-R that is suitable for the induction of neurite outgrowth. Alternatively, inhibitory sites on TN-R could be masked by interaction with F11 (14). Similar mechanisms might be discussed for the interaction between TN-C and F11. An analogous shift in cellular receptor usage in the presence of TN-R has been observed for neurite outgrowth on immobilized neurofascin. F11, axonin-1, and at least one additional protein have been implicated as...
cellular receptors mediating cell attachment and neurite outgrowth on a neurofascin-TN-R complex (7).

The interaction between F11 and TN-R or TN-C appears to be a unique example of how ECM glycoproteins regulate cell attachment and neurite outgrowth and how axonal IgSF members and ECM molecules combine to form cell recognition complexes. In parallel, the chondroitin sulfate proteoglycans neuropilin and phosphacan block the homophilic interaction of NgCAM- and NCAM-mediated neuron and glia cell adhesion and neurite outgrowth (61–63). However, phosphacan probably interferes with NgCAM-mediated neurite outgrowth by binding directly to the cell surface. The inhibitory effects may be mediated by competition for the same binding sites on the basis of affinity. Alternatively, since neurocan and phosphacan are large bulky molecules, they may also function by binding to receptors that are not in close proximity to the actual sites that mediate homophilic or heterophilic binding. Moreover, neurocan and phosphacan inhibit the binding between axonin-1 and TN-C and between axonin-1 and NCAM (56). Although these molecules show an overlapping distribution, the functional significance of this competition remains to be established.

Switching among different possible CAM and ECM interactions and the formation of larger molecular complexes is likely to underlie changes in axonal growth and fasciculation for example of commissural axons at the floor plate. The interaction between axonin-1 on commissural axons with NrCAM on floor plate cells is necessary for axonal guidance at the floor plate, but after crossing the midline axonal growth on the contralateral longitudinal tract depends on homophilic interaction of NgCAM with an NgCAM-axonin-1 complex on the growth cone (64–66). This switch is probably mediated by regulation of the expression of axonin-1 and NgCAM on the axons as described for TAG-1 and L1 (67), resulting in a competition between trans (axonin-1-NrCAM or axonin-1-axonin-1) and cis (axonin-1-NgCAM) interactions of axonin-1 (68). Furthermore, netrins act as long range chemoeactive guidance cues for commissural axons to the floor plate (69). However, after crossing the floor plate these axons do not respond to netrins any more, although the netrin receptor DCC appears to be expressed on commissural axons before and after crossing the midline (70, 71).

In summary, we were able to demonstrate that the ECM glycoproteins TN-R and TN-C regulate the interactions of the IgSF member F11 with different ligands or counterreceptors, modulate cell adhesion and neurite outgrowth of tectal cells on immobilized F11, and induce a switch in the cellular receptor usage from NrCAM to β1 integrins mediating these processes. Therefore, different complexes of CAMs with other cell surface and ECM molecules are likely to control axonal growth and guidance during development and plasticity as a consequence of their differential interaction and localization in vivo.

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