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Binding of contactin/F11 to the fibronectin type III domains 5 and 6 of tenascin is inhibited by heparin

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Abstract The structural basis for the interaction between tenascin-C and the neuronal cell adhesion molecule, contactin/F11, was investigated using plasmon surface resonance technology. The binding site on tenascin-C for contactin/F11 is shown to span the two fibronectin type III homology domains 5 and 6. Either domain alone is insufficient for binding. Heparin, heparan sulfate and dermatan sulfate inhibit this interaction through binding to a conserved heparin-binding site on domain 5. In contrast, chondroitin sulfates A and C have no such effect.

Key words: Contactin/F11; Tenascin; Glycosaminoglycan; Heparin

1. Introduction

Tenascin-C gives its name to a family of extracellular matrix (ecm) molecules implicated in the regulation of cell-ecm interactions. Structurally, they are composed of a series of colinear domains whose homologues are found in a wide range of proteins with adhesive and signalling functions (see Fig. 1). Functionally, tenascin-C can be adhesive or repulsive for cells and supports cell migration and neurite outgrowth. The natures of the cellular ligands mediating these interactions are particularly diverse and include integrins, proteoglycans, cell adhesion molecules (CAMs) and a receptor tyrosine phosphatase [1–3]. To understand how interactions between tenascin and these various receptors might be coordinated, it is essential to map the receptor binding sites on tenascin, to provide a structural basis for functional investigations.

Here, we examine how the interactions of one of the more intriguing receptors, the neuronal CAM contactin/F11 [4,5], can be modulated by glycosaminoglycans. Contactin/F11 is a member of the immunoglobulin superfamily (IgSF) and can bind to both tenascin-C [6] and tenascin-R [7] (see also [1,8] for recent reviews). The binding site on contactin/F11 for tenascin-C lies within the amino-terminal three Ig domains, whereas the binding site in tenascin-C is thought to span the boundary of the alternatively spliced region of tenascin, as contactin/F11 binds preferentially to the 190 kDa tenascin isoform [6].

To examine the properties of this region more closely, we prepared bi-domain fusion proteins spanning the alternatively spliced region of chicken tenascin (Fig. 1). These constructs represent the tenascin-C isoforms ten190 (TNfn56) and ten200 (TNfnD6 and TNfn5D). This was achieved by cDNA fragments of adjacent type III domains being synthesized using polymerase chain reaction technology from a cDNA clone encoding the entire tenascin-C sequence. Only fusion proteins containing the fifth type III homology region of tenascin bound to heparin-Sepharose and bound biotin-labelled heparin in solid phase assays. Using this assay system, we also localised the binding of heparan sulfate and dermatan sulfate to domain five. Molecular modelling of this domain revealed a conserved heparin-binding motif which we proposed as the putative binding site [9].

Here we present evidence that the TNfn56 binds specifically to contactin/F11, an interaction which can be inhibited by heparin, heparan sulfate and dermatan sulfate. This raises the possibility that cell surface contactin/F11 and heparan sulfate proteoglycans compete for the same site on tenascin.

2. Material and methods

2.1. Purification of proteins

Contactin/F11 was purified from chick embryo brains [6]. Briefly, embryo day 17 chick brains were extracted with 0.5 M NaCl in detergent-free buffer A (20 mM Tris-HCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM PMSF, 25 mM ε-amino caproic acid, 0.02% NaN_3; pH 7.5). The extract was cleared by centrifugation (100 000 × *g*, 90 min, 4°C), diluted with buffer A to 0.2 M NaCl and passed through a column of Sepharose 4B, to remove material binding non-specifically to Sepharose, followed by columns of T16-Sepharose (anti-tenascin) and 8D9-Sepharose (anti-NgCAM) to specifically absorb tenascin-C and NgCAM, respectively. The eluate was then applied to a column of Concanavalin A-Sepharose. After washing with buffer A containing 0.2 M NaCl (buffer B), the Concanavalin A column was eluted with buffer B containing 0.1 M methyl-α-D-mannoside and the eluted peak subsequently loaded onto a column of 4D1-Sepharose (anti-contactin/F11). After a wash with buffer B and then with buffer C (20 mM Tris-HCl, 1 mM PMSF, 50 mM NaCl, 0.02% NaN_3; pH 7.5), contactin/F11 was eluted with 1 M triethylamine buffer (pH 11.5; 50 mM NaCl, 0.02% NaN_3). The collected fractions were neutralised with 1 M Tris-HCl pH 5.8 and the purity was controlled by SDS PAGE with detection by silver staining or immunoblotting with antibodies specific for tenascin-C or tenascin-R or Ng-CAM.

The expression and native purification of fusion proteins have been previously described [9]. Briefly, M15 bacteria were transformed with expression plasmids containing the sequence coding for six histidines, the factor Xa digestion site (Ile-Glu-Gly-Arg) and the appropriate chicken tenascin-C type III domains (Fig. 1). The expression of the fusion proteins was induced by adding IPTG to a final concentration of 2 mM. After 5 h, the cells were harvested and resuspended in 1/30th detergent-free buffer (25 mM Tris-HCl, 150 mM NaCl, 0.2 mM PMSF, 2 mM iodacetamide; pH 8.0) and lysed with a sonifier (Cell disrupter, SKAN; setting 4, 6 min, 75%) cooled with ice water. After clearing by centrifugation (12 000 × *g*, 15 min, 4°C; Sorvall SS34), the supernatant was diluted with 1.5 vols. loading buffer (67 mM sodium phosphate, 300 mM NaCl, 0.02% NaN_3; pH 8.0) and loaded onto a Ni-NTA column. The column was washed with 67 mM phosphate buffer (300 mM NaCl, 0.02% NaN_3; pH 6.0) and the fusion protein eluted with 100 mM acetate buffer (300 mM NaCl, 0.02% NaN_3;

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Abbreviations: HBS, HEPES-buffered saline; TNfnxx, fibronectin type III domain xx of tenascin-C; RU, response units; LMWH, low molecular weight heparin; CAM, cell adhesion molecule; GPI, glycosylphosphatidylinositol; CN, contactin/F11

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pH 3.8). The collected fractions were neutralised with 1 M Tris-HCl pH 9. The purity was assayed on 10–15% SDS-PAGE and Coomassie blue staining. The molecular weights of the three fusion proteins TNfn5D, TNfn56 and TNfnD6 are 22638, 22089 and 22153, respectively, which are sufficiently close to give a similar signal for each fusion protein in surface plasmon resonance analysis.

The monoclonal antibody 1C10 against the leading polyhistidine sequence of the fusion proteins was produced by injecting TNfn56 into mice with subsequent hybridisation of the spleen cells with myeloma cells. Antibody clones which additionally recognised the fusion proteins TNfn5D and TNfnD6 were screened for their affinity to the polyhistidines. The factor Xa processed fusion protein missing the polyhistidine sequence (TNfn56w/oHis) was used as a negative control. One clone selected, designated 1C10, was grown in bulk culture roller bottles. The pH of the harvested medium was adjusted to 8.0 and the medium subsequently loaded onto a TNfnD6 column. After washing the column with 67 mM phosphate buffer (300 mM NaCl, 0.02% NaN_3; pH 8.0), the antibody was eluted with 100 mM acetate buffer (300 mM NaCl, 0.02% NaN_3; pH 3.8) and the collected fraction was neutralised with 1 M Tris, pH 9.0. The purified antibody 1C10 was tested for specificity with ELISA on coated TNfn5D, TNfn56 and TNfnD6.

2.2. Plasmon surface resonance analysis

Plasmon surface resonance analyses were performed with the BIAcore biosensor (Pharamacia) using the sensor chip CM5 (Pharmacia). All experiments were carried out in HBS (10 mM HEPES, 150 mM NaCl, 0.02% NaN_3; 0.005% surfactant P20) at a flow rate of 5 μl/min at 25°C. Immobilisation of contactin/F11 or 1C10 antibody to the chip surface was carried out according to the supplier's instructions. Briefly, prior to immobilisation, contactin/F11 was dialysed against 2.5 mM HEPES (pH 7.4). Immediately before the immobilisation procedure, 84 μl of contactin (48 μg/ml) was mixed with 24 μl of 20 mM acetate buffer (pH 3.5). The chip surface was activated for 10 min with a freshly prepared mixture of 0.2 M *N*-ethyl-*N'*-(dimethylaminopropyl)carbodiimide (EDC) and 0.05 M *N*-hydroxysuccinimide (NHS). The contactin/F11 was allowed to immobilise for 9 min, then the remaining activated groups were blocked with 1 M ethanolamine for 7 min. The surface was regenerated during 1 min with 10 mM HCl. The coupling of antibody 1C10 was carried out similarly. From a stock solution of 1C10 (375 μg/ml in 2.5 mM HEPES) 13.5 μl were mixed with 86.5 μl 10 mM acetate buffer (pH 5.0) immediately before immobilisation. Inhibition studies were performed with chondroitin sulfates A and C, dermatan sulfate, heparan sulfate, heparin and low molecular weight heparin (average M_r 3000), all purchased from Sigma.

3. Results

3.1. Immobilisation of contactin/F11 and 1C10

To investigate the binding of fusion proteins to contactin/F11 by means of the BIAcore facility, the contactin/F11 needed to be covalently immobilised to the chip surface. The efficiency of coupling can be directly estimated from the increase in signal arising after washing away unbound material. In the BIAcore system, the signal is measured in response units (RU), where 1 RU is equal to about 1 pg/mm^2 protein [10,11]. From the increase in RU of 4529 resulting from the immobilisation of contactin/F11 (135 kDa), its surface concentration can be estimated to be 33.6 fmol/mm^2. As a positive control, we immobilised the monoclonal antibody 1C10. This antibody binds specifically to the polyhistidine sequence, which is common to all fusion proteins used in this work (Fig. 1) [9,12]. Similarly to contactin/F11, the immobilisation of 1C10 gives a difference of 5000 RU, equivalent to a surface concentration of 33.3 fmol/mm^2 antibody.

3.2. Binding of fusion protein TNfn56 on contactin/F11

Contactin/F11 binds preferentially to the TN190 isoform where the fibronectin type III domains 5 and 6 are adjacent

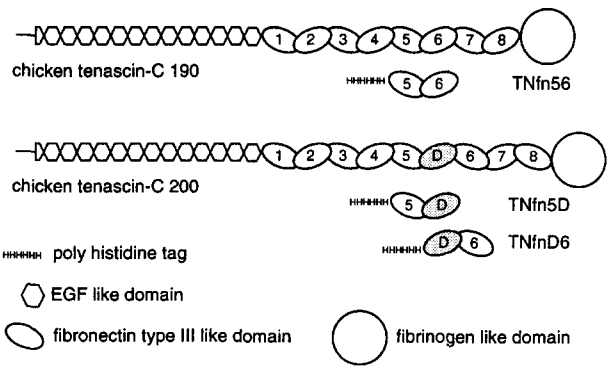

Fig. 1. Schematic illustration of fusion proteins and the tenascin-C isoforms ten190 and ten200. Tenascin isoforms differ in the number of fibronectin type III homology domains, additional domains being spliced inbetween TNfn5 and 6. The fusion proteins used in this study, TNfn56, TNfn5D and TNfnD6 are representative for this region of ten190 and ten200, respectively.

(Fig. 1) [6]. This raised the possibility that both domains are required for binding and that either domain alone would be insufficient. To test this, the fusion proteins TNfn56, TNfn5D and TNfnD6 were passed over the contactin/F11 surface at different concentrations (Fig. 2). Binding to the immobilised ligand can most easily be judged from the off-rate phase, when the chip surface is being rinsed with HBS buffer alone. If binding occurs, the protein is retained on the chip surface and elutes in a long drawn out curve. In the case of non-binding, there is a fast drop in the signal, as exhibited by the negative control, BSA on the 1C10 surface. Neither TNfn5D nor TNfnD6 appeared to bind, both eluting without any retention to immobilised contactin/F11. The binding curves for TNfn56 on contactin/F11 clearly differ and show drawn out dissociation phases.

In contrast to contactin/F11, all fusion proteins bound to the positive control, the immobilised 1C10 monoclonal antibody specific for the polyhistidine sequence (Fig. 2). Applied at a concentration of 15 μM, they bound more rapidly than TNfn56 to contactin/F11 and were retained more strongly by the antibody, indicating both a higher on-rate and slower off-rate. The form of the curves closely resembled that of TNfn56 binding to contactin/F11, confirming the specificity of this interaction.

3.3. Inhibition of binding of TNfn56 on contactin/F11 with glycosaminoglycans

The potential overlap of the contactin/F11 and glycosaminoglycan binding sites on TNfn5 raised the possibility that these two ligands may interfere in their binding to tenascin. To examine this, TNfn56 (44 μM) was incubated together with LMWH over a range of concentrations and the binding to the contactin/F11 surface followed. Relatively low concentrations (4 μM) of LMWH are sufficient to effectively inhibit the interaction of TNfn56 with the contactin/F11 (Fig. 3).

Heparin, LMWH, heparan sulfate and dermatan sulfate were previously shown to bind to TNfn5, whereas chondroitin sulfates A and C did not [9]. To examine whether the same glycosaminoglycans might inhibit the interaction of TNfn56 and contactin/F11, mixtures of 15 μM TNfn56 and 50 μg/ml glycosaminoglycans were injected over the contactin/F11 surface (50 μg/ml LMWH corresponds to 16.7 μM). The glycosaminoglycans heparin, LMWH, heparan sulfate and dermatan

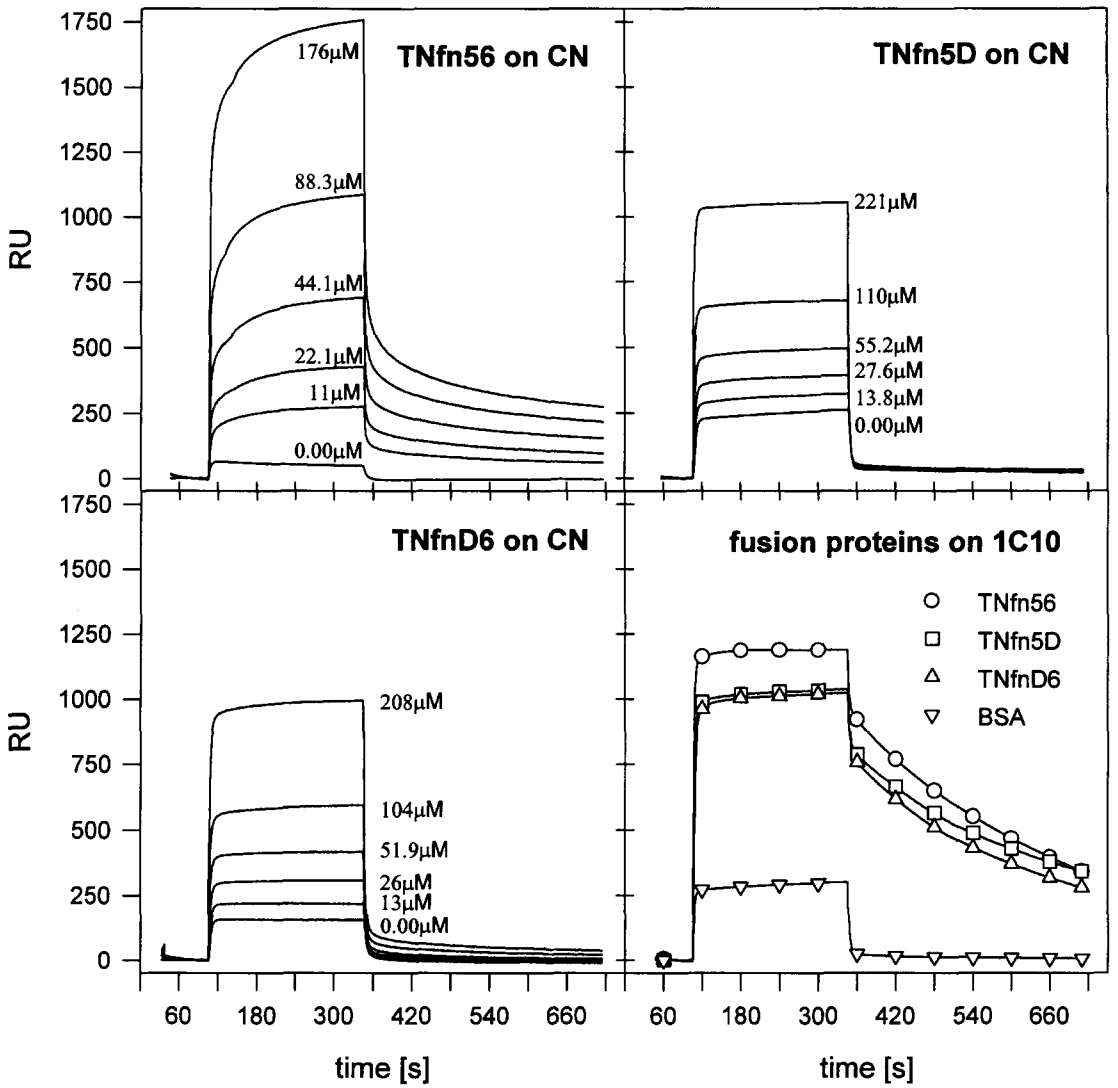

Fig. 2. Plasmon surface resonance analysis of fusion protein interaction with immobilised contactin/F11 (CN) or monoclonal antibody 1C10. Fusion proteins TNfn56, TNfn5D and TNfnD6 were injected over the immobilised contactin/F11 in a series of increasing concentrations. The passage of the buffer containing the fusion proteins over the chip surface covers the time interval from 100 to 350 s and can be seen from the square profile for the buffer alone (0.0 μM) or from the negative control of BSA in the fourth panel. In the latter, the fusion proteins at a concentration of 15 μM were passed over immobilised 1C10 antibody, specific for the polyhistidine sequence.

sulfate, also inhibited binding of TNfn5 to contactin/F11, with dermatan sulfate having the weakest effect (Fig. 4). In contrast, chondroitin sulfates A and C give a signal similar to the control without glycosaminoglycan. As expected from the previous work, only the L-iduronic acid containing glycosaminoglycans are able to inhibit the binding between TNfn56 and contactin/F11.

4. Discussion

We present evidence that the binding site on tenascin-C for contactin/F11 spans the TNfn domains 5 and 6. Both domains are necessary for binding to contactin/F11 to occur; fusion proteins containing either domain in combination with TNfnD showed no detectable affinity for immobilised contactin/F11. The double-domain requirement for binding could be independently confirmed through inhibition studies with glycosaminoglycans. Heparin, heparan sulfate and dermatan sulfate bind specifically to TNfn5, but not to TNfn6 [9]. All three glycosaminoglycans also inhibited the interaction between TNfn56 and contactin/F11 in a concentration-dependent manner. No binding between glycosaminoglycans and contactin/F11 was detected, nor has it been reported by other groups, confirming that the heparin-binding site on TNfn5 is targeted by this approach. There are no potential oligosaccharide attachment sites within the domains TNfn5 or 6, making it unlikely that carbohydrates on tenascin itself are involved in this interaction.

The requirement of a binding site on tenascin-C spanning two domains implies that the binding site in the Ig-like region of contactin/F11 similarly spans two Ig-like domains. This can be inferred from the similar size and structures of fn domains and Ig-like domains [8], which raises the possibility that we are observing a side-by-side interaction between the two mo-

lecules in a manner closely analogous to that between paired immunoglobulin domains. Further evidence in support of this model is forthcoming from recent findings on the interaction between contactin/F11 and another tenascin family member, tenascin-R. The binding site on contactin/F11 for tenascin-R had previously been localised to Ig domains 23 [7], suggesting that the binding site on contactin/F11 for tenascin-C and tenascin-R is either overlapping or the same. Most recently, evidence for a contactin/F11 binding site in chick TN-Rfn23 was presented [13]. Of particular interest was the requirement of both tenascin-R domains 2 and 3 for binding, either domain alone being insufficient. This opens the possibility that TN-Cfn56 and TN-Rfn23 may compete for the same site on contactin/F11. However, no evidence has yet been presented that TN-Rfn23 carries a heparin binding site, nor is such a site evident by inspection. This implies that only the interaction between tenascin-C and contactin/F11 would be inhibited by heparan sulfate proteoglycans. Tenascin-C binds two different heparan sulfate proteoglycans, syndecan [14] and glypican [15], although whether the heparin binding site on TNfn5 or a second site in the C-terminal of tenascin [16,17] is responsible for the binding of the heparan sulfate proteoglycans is as yet unknown.

Glypican, like contactin/F11, is anchored to the membrane via glycosylphosphatidylinositol (GPI), leaving both proteins freely mobile within the plane of the membrane. Because they have no transmembrane domains, signal transduction by such GPI-anchored receptors is likely to be particularly dependent on receptor (re)organisation induced by binding to extracellular ligands. Indeed, we have recently shown that antibody-induced aggregation of contactin/F11 in neuronal cells leads to a co-aggregation of the non-receptor protein tyrosine kinase Fyn and its transient activation [18]. The competition between heparan sulfate and contactin/F11 for the same site on tenascin-C, points towards a further level of complexity in the organisation of cellular signalling complexes by tenascin and provides a fertile field for further investigations.

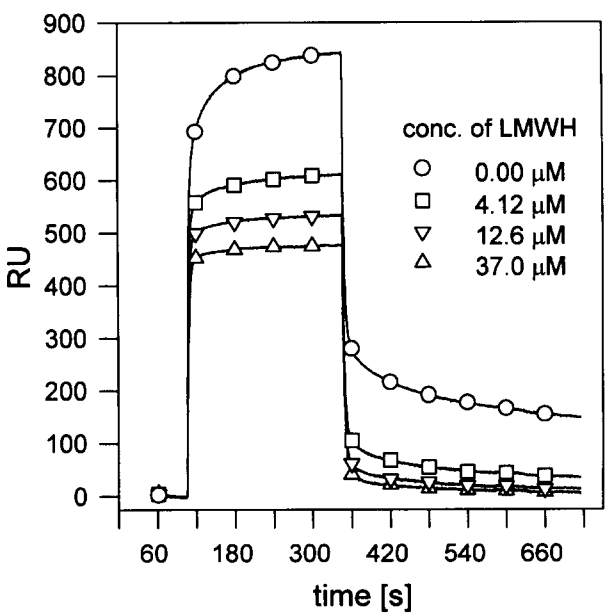

Fig. 3. Binding of TNfn56 to contactin/F11 in the presence of increasing concentrations of low molecular weight heparin (LMWH). TNfn56 (44 µM) was preincubated with LMWH at the concentrations indicated and the interaction with contactin/F11 immobilised to the chip surface measured by plasmon resonance.

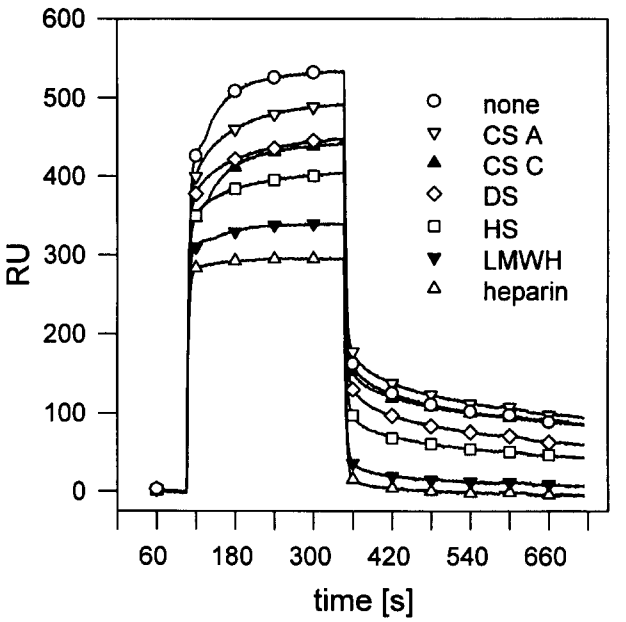

Fig. 4. Influence of different glycosaminoglycans on binding of TNfn56 to contactin/F11. TNfn56 (15 µM) was preincubated with 50 µg/ml of either chondroitin sulfates A or C (CS A or C) or dermatan sulfate (DS) or heparan sulfate (HS) or low molecular weight heparin (LMWH) or heparin and the mixture injected over contactin/F11 immobilised to the chip surface. As a positive control, TNfn56 was injected alone.

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References

[1] Vaughan, L., Weber, P., D'Alessandri, L., Zisch, A.H. and Winterhalter, K.H. (1994) Perspect. Dev. Neurobiol. 2, 43–52.
[2] Chiquet-Ehrismann, R. (1995) Experientia 51, 853–862.
[3] Faissner, A., Götz, B., Joester, A. and Scholz, A. (1995) Semin. Dev. Biol. 6, 139–148.
[4] Ranscht, B. and Dours, M.T. (1988) J. Cell Biol. 107, 1561–1573.
[5] Brümmendorff, T., Wolff, J.M., Frank, R. and Rathjen, F.G. (1989) Neuron 2, 1351–1361.
[6] Zisch, A.H., D'Alessandri, L., Ranscht, B., Falchetto, R., Winterhalter, K.H. and Vaughan, L. (1992) J. Cell Biol. 119, 203–213.
[7] Brümmendorf, T., Hubert, M., Treubert, U., Leuschner, R., Tàrnok, A. and Rathjen, F.G. (1993) Neuron 10, 711–727.
[8] Brümmendorf, T. and Rathjen, F.G. (1995) in: Protein Profile, vol. 2 (Sheterline, P. ed.) pp. 963–979, Academic Press, New York.
[9] Weber, P., Zimmermann, D.R., Winterhalter, K.H. and Vaughan, L. (1995) J. Biol. Chem. 270, 4619–4623.
[10] Karlsson, R., Michaelsson, A. and Mattsson, L. (1991) J. Immunol. Methods 145, 229–240.
[11] Granzow, R. and Reed, R. (1992) Bio/Technology 10, 390–393.
[12] Stüber, D., Matile, H. and Garotta, G. (1990) Immunol. Methods 4, 121–152.
[13] Nörenberg, U., Hubert, M., Brummendorf, T., Tarnok, A. and Rathjen, F.G. (1995) J. Cell Biol. 130, 473–484.

[14] Salmivirta, M., Elenius, K., Vainio, S., Hofer, U., Chiquet-Ehrismann, R., Thesloff, I. and Jalkanen, M. (1991) J. Biol. Chem. 266, 7733–7739.
[15] Vaughan, L., Zisch, A.H., Weber, P., D'Allesandri, L., Ferber, P., David, G., Zimmermann, D.R. and Winterhalter, K.H. (1994) Contrib. Nephrol. 107, 80–84.
[16] Aukhil, I., Joshi, P., Yan, Y. and Erickson, H.P. (1993) J. Biol. Chem. 268, 2542–2553.
[17] Fischer, D., Chiquet-Ehrismann, R., Bernasconi, C. and Chiquet, M. (1995) J. Biol. Chem. 270, 3378–3384.
[18] Zisch, A.H., Dalessandri, L., Amrein, K., Ranscht, B., Winterhalter, K.H. and Vaughan, L. (1995) Mol. Cell. Neurosci. 6, 263–279.