Coordinate Regulation of Cadherin and Integrin Function by the Chondroitin Sulfate Proteoglycan Neurocan

Hedong Li,* Tin-Chung Leung,* Stanley Hoffman,‡ Janne Balsamo,* and Jack Lilien*

*Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202; and ‡Division of Rheumatology and Immunology, Medical University of South Carolina, Charleston, South Carolina 29425-2229

Abstract. N-cadherin and β1-integrins play decisive roles in morphogenesis and neurite extension and are often present on the same cell. Therefore, the function of these two types of adhesion systems must be coordinated in time and space to achieve the appropriate cell and tissue organization. We now show that interaction of the chondroitin sulfate proteoglycan neurocan with its GalNAcPTase receptor coordinately inhibits both N-cadherin– and β1-integrin–mediated adhesion and neurite outgrowth. Furthermore, the inhibitory activity is localized to an NH₂-terminal fragment of neurocan containing an Ig loop and an HA-binding domain. The effect of neurocan on β1-integrin function is dependent on a signal originating from the cadherin cytoplasmic domain, possibly mediated by the nonreceptor protein tyrosine kinase Fer, indicating that cadherin and integrin engage in direct cross-talk. In the developing chick, neural retina neurocan is present in the inner plexiform layer from day 7 on, and the GalNAc cPTase receptor becomes restricted to the inner nuclear layer and the ganglion cell layer (as well as the fiber layer), the two forming a sandwich. These data suggest that the coordinate inhibition of cadherin and integrin function on interaction of neurocan with its receptor may prevent cell and neurite migration across boundaries.

Key words: cadherin • integrin • adhesion • neurite outgrowth • tyrosine kinase Fer

Introduction

The characteristic cellular patterns that arise during development are a result of complex cell and tissue rearrangements. Nowhere is this complexity more apparent than in the development of the nervous system. The number and type of cell-surface receptors that transduce signals from environmental cues to the cytoplasmic machinery that ultimately directs growth cone guidance are extensive. Environmental cues too come in many forms including the following: diffusible molecules, cell-surface molecules, and extracellular matrix molecules. Whereas the identity of the cell-surface receptors, their environmental triggers, and even the nature of many of the signaling pathways has been unfolding (for reviews see Goodman and Tessier-Lavigne, 1997; Mueller, 1999; Song and Poo, 1999), the machinery that integrates and coordinates the responses to various cues within a single growth cone remains relatively unknown.

β1-Integrins and N-cadherin have long been recognized as two transmembrane adhesion receptors critical to neurite outgrowth (Bixby et al., 1988; Neugebauer et al., 1988; Tomaselli et al., 1989). We have previously suggested that a cell-surface glycosyltransferase (GalNAcPTase)¹ may be a critical component of one regulatory circuit that coordinates the activity of these two adhesion systems (Gaya-Gonzalez et al., 1991; Lilien et al., 1997, 1999). The GalNAc cPTase is anchored to the plasma membrane by a glycosphatidylinositol linkage (Balsamo and Lilien, 1993), and specifically associates with N- and E-cadherin (Balsamo and Lilien, 1990; Bauer et al., 1992) but not β1-integrins (Lilien et al., 1999). Binding of one unique mAb to the GalNAc cPTase initiates a signal that results in coor-

¹Abbreviations used in this paper: 250kD PG, 250-kD chondroitin sulfate proteoglycan; CBP, catenin binding peptide; COP, control antennapedia peptide; GalNAc cPTase, cell-surface glycosyltransferase; HA, hyaluronic acid; IPL, inner plexiform layer; JMP, juxtamembrane peptide; OPL, outer plexiform layer.
dinate inhibition of N-cadherin and β1-integrin-mediated neurite outgrowth (Gaya-Gonzalez et al., 1991). We hypothesized that the activity of this antibody reflected the activity of an endogenous ligand. Indeed, we subsequently identified and purified a 250-kD chondroitin sulfate proteoglycan (250kD PG) that binds to the same, or an overlapping domain of the GalNAcPTase. It also initiates a signal that results in retention of the phosphate on tyrosine residues of β-catenin and uncoupling of cadherin from its association with actin, with concomitant inhibition of N-cadherin-mediated adhesion and neurite outgrowth (Balsamo et al., 1995, 1996).

To further study the molecular mechanism of the interaction between 250kD PG and its receptor, GalNAcPTase, we have isolated the complete cDNA coding for the 250kD PG and show that binding of the protein backbone to the GalNAcPTase initiates a signal cascade that results in coordinate inhibition of cadherin- and integrin-mediated adhesion and neurite outgrowth. Sequence analysis shows that the 250kD PG is the chicken homologue of neurocan. Chicken neurocan contains the conserved NH2- and COOH-terminal protein motifs characteristic of the aggrecan/versican/neurocan/brevican family of chondroitin sulfate proteoglycans (Roslashi, 1996; Schwartz et al., 1999). The conserved motifs are ~70% similar to rat neurocan, but there is little sequence similarity (~10%) in the central region, where the majority of the consensus sites for attachment of chondroitin sulfate side chains occur. The coordinate regulation of cadherin and integrin function is due to a direct interaction between the NH2-terminus of neurocan containing the Ig loop and hyaluronic acid (HA-binding) domain and the GalNAcPTase, as removal of the GalNAcPTase with PI-PLC abrogates the coordinate regulatory activity. Furthermore, coordinate regulation appears to be due to a signal originating from the cytoplasmic domain of cadherin directly affecting integrin function. Our results suggest a mechanism whereby neurite outgrowth may be directionally controlled by environmental cues defining axonal trajectories.

## Materials and Methods

### Antibodies

Preparation of anti-PG-250 antibody (Balsamo et al., 1995) and the antineurocan antibody (Zanin et al., 1999) were described previously. A nitrocellulose antibody NCD-2 is a monoclonal rat IgG (the cell line was provided by M. Takeichi, Kyoto University, Kyoto, Japan; Hatta and Takeichi, 1986). The anti-β1-integrin antibody, JG G2, is a mouse monoclonal IgG (Greve and Gottlieb, 1982; Tomasselli et al., 1986) and the anti-FAK antibody is a monoclonal IgG (Transduction Laboratories). A nitrocellulose antibody is a rabbit polyclonal IgG directed against a synthetic 15-amino acid sequence (Balsamo et al., 1995) and a mouse mAb (Transduction Laboratories). The antiactin antibody is a monoclonal mouse IgG (Chemicon International, Inc.). The antiphosphotyrosine antibody, PY 20, was purchased from Transduction Laboratories. Two anti-GalNAc CPTase antibodies, 1B11 and 7A2, are mouse IgMs prepared as described previously (Scott et al., 1990; Balsamo et al., 1995). A nitrosyntain antibody BPC-1 is a mouse monoclonal IgG purchased from Sigma Chemical Co. The following three mAbs were provided by Dr. V. Lance Lemmon (Case Western Reserve University, Cleveland, OH): 8D9 (anti-chicken L1), 3G3, and 3A7. Secondary antibodies, Cy3-conjugated goat anti-mouse IgG, FITC-conjugated donkey anti-rat IgG, FITC-conjugated goat anti-rabbit IgG, and rhodamine-conjugated goat anti-mouse IgM were purchased from Jackson ImmunoResearch Laboratories, Inc.

### Isolation of the cDNA Encoding Neurocan

Total RNA was purified from day 10 embryonic chicken brain (E10) and retina using an RNeasy kit (Qiagen). RNA was reverse transcribed into first-strand cDNA using a degenerate primer based on the amino acid sequence of the most highly conserved region of the HA-binding domain present in all members of the hyaluronic acid binding family of proteins, CD44, CD44v10, TAC1, and L1 (Balsamo et al., 1995, 1996). PCR was performed using two sets of nonoverlapping primers derived from microsequencing of the NH2-terminus and a cyanoan bromide fragment of the 250 kD PG core protein. Inosine (I) was used to reduce degeneracy.

The NH2-terminal amino acid sequence with the two nested primers underlined is as follows: QDGDEKVIHSRVNOA4VRVGLGEVYP-A(LP)5-GGIA(AAGAGTIAATICTATGTKGCTC1-3′, and 5′-GGTGAGCAGCCGITGICTCITICTIC3′. The internal sequence with two nested primers underlined are as follows: DNSAVIA3PHQLQ... and QAAEDFYDNDN, 5′-TTATG(TC)CAG-TAICTCCAGCTCTGTACGTTGTG3′, and 5′-TGIA-(AG)AGCT(GTC)CIGGIGC(A)TAG(C1T1C1G1C3′.

A 450-bp product was obtained, subcloned into the TA vector (Invitrogen Corp.) and sequenced. This sequence showed 71% similarity to rat and mouse neurocan. The PCR product was radiolabeled and used as a probe to screen a chick brain λ-Zap cDNA library. Eight positive clones were isolated and cloned into pBluescript SK (Stratagene). Restriction pattern analysis revealed two distinct clones with inserts of 1.6 and 2.0 kb, with 1.5 kb of overlap yielding 2.3 kb of unique sequence. To obtain the full-length cDNA sequence, reverse transcriptase-inverse-PCR was used (Zeiner and Gehring, 1994). In brief, first-strand cDNA was made from E10 chick brain total RNA using an oligo-dT primer, followed by second-strand cDNA synthesis and blunt-end circularization of the double-stranded cDNA. The circularized cDNA was used as a template for a set of nested primers based on the 5′ and 3′ ends of the 2.3 kb of known sequence pointing away from the known sequence, allowing amplification of regions flanking the 2.3-kb fragment. A complete clone was constructed by recombinant PCR using the high fidelity polymerase, elongase (GIBCO BRL) and sequenced (sequence data available from GenBank/EMBL/DDJB) under accession number A116856).

### Northern Blot Analysis

Total RNA was isolated from E10 chick brains using the RNeasy kit (Qiagen). 5 μg of total RNA was separated on 1% denaturing agarose gel, transferred to Hybond-N+ membrane (Amersham), and hybridized with a probe of 1.1 kb from the central region of the cDNA sequence. This probe was used as it is the least conserved region of the full-length clone. A fer high stringency washes, (0.1× SSPE, 0.1% SDS) the membrane was exposed to Biomax X-film (Eastman Kodak Co.).

### Preparation of Recombinant His-tagged Bacterial Fusion Protein

The complete open reading frame lacking the signal peptide (nucleotides 128–4467), with adaptor sequences, was obtained by PCR, digested with Ndel and BglII, and ligated into pET-15b (Novagen, Inc.), which was pre-digested with the same enzymes. Fusion protein fragments representing the NH2- and COOH-terminal domains were generated as follows (see Fig. 2). A NH2-terminal fragment was generated by cutting the full-length construct with Ndel and partially with XhoI, ligating the released DNA fragment containing nucleotides 128–1400 into the pET-15b vector that was pre-digested with the same enzymes. A COOH-terminal fragment was generated by cutting the full-length construct with BamHI and the released DNA fragment, containing nucleotides 2668–4467, was ligated into BamHI-linearized pET-15b vector. The NH2-terminal construct was further cut with XhoI to generate an Ig (nucleotides 128–518) and an HA (nucleotides 518–1400) fragment. A second Ig fragment containing a piece of HA-binding region was generated by PCR using the 5′ primer used to clone the complete open reading frame and a 3′ primer corresponding to nucleotides 599–622. All fragments were also ligated into pET-15b at appropriate restriction sites and confirmed by sequencing. The constructs were transformed into BL21(DE) (Novagen, Inc.) and induced bacteria were pelleted and stored at ~80°C until needed. Fusion proteins were extracted from the cell pellet in buffer containing 6 M urea, purified on Ni2+ columns following the manufacturer's recommendations (Novagen, Inc.), and refactored by a series of dialyses in PBS with 0.1 M DTT. The final products were aliquoted and frozen at ~80°C.
Single Cell Preparation and Adhesion Assays

For N-cadherin-mediated adhesion, E9 chick neural retina single cells were prepared by trypsinization of tissues in the presence of 1 mM Ca\(^{2+}\) as previously described (Balsamo et al., 1995) with minor modifications. Single cells were washed 2× in HBSSG (20 mM Heps, pH 7.4, 150 mM NaCl, 2 mM glucose, 3 mM KCl, and 1 mM CaCl\(_2\)) and resuspended in HBSSG Ca at 2× 10\(^5\) cells/ml. For integrin-mediated adhesion, E9 chick neural retina cells were prepared as above; however, calcium was omitted and trypsinization was performed for 10 min in contrast to the usual 30 min. The cells were preincubated on ice for 15 min with or without additives and 100-μl aliquots containing 10\(^5\) cells were added to each well of a 96-well plate previously coated with N-cadherin or laminin. Glass coverslips were coated with 250 μg/ml poly-L-lysine (Sigma Chemical Co.). At 37°C for 2 h, washed, with water, and postcoated with NCD-2 or laminin as described previously (Aregui et al., 1999). The coverslips were subsequently blocked with 1% BSA in HBSSG for a minimum of 2 h at room temperature. E7 chick neural retina cells (10\(^5\) cells/ml), prepared by trypsinization in the absence of Ca\(^{2+}\), were plated on each coverslip in F12 medium (GIBCO BRL) including 1% ITS (insulin/transferin/selenium; GIBCO BRL), 2% glucose, and 0.5% gentamycin (Sigma Chemical Co.) as previously described (Gaya-Gonzalez et al., 1991). A 2 h in culture, and thereafter every 3 h, neurocan peptides were added to the culture medium. A 12 h, cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and images captured with a SenSys CCD camera (Photometrics) and analyzed with a MetaMorph image analysis system. Cells bearing neurites longer than one cell diameter were considered positive for neurite extension.

Interaction of Neurocan with the Cell-surface GalNAcPTase

E9 retina cells, prepared by trypsinization in the presence of Ca\(^{2+}\) as described above, were incubated with neurocan fusion peptides (~20 × 10\(^5\) cells/100 μl of HBSSG Ca containing ~0.2 mM fusion peptide or BSA) for 1 h at 4°C. Cells were pelleted, resuspended in 0.25 mM DTTSSP cross-linker (Pierce Chemical Co.) in PBS, and incubated at 4°C for 30 min. The cells were again pelleted, washed in HBSSG Ca, and lysed in homogenization buffer (1% Triton X-100, 20 mM Tris, pH 7.5, 150 mM NaCl, 1:1,000 AESSF [Sigma Chemical Co.], 100 μg/ml D Nase, 2 mM 0-α-vanadate, 1 mM NaF) at 4°C for 30 min. The lysates were centrifuged at 14,000 g for 5 min, and the supernatant was mixed with an equal volume of Immunomix (1% Triton X-100, 0.5% DOC, 1% SDS, 0.1% BSA in 25 mM Tris, pH 7.5, 150 mM NaCl, 1 mM PM SF). The solution was cleared by centrifugation and incubated with monoclonal anti-GalNAcPTase antibody for 2 h at 4°C. The precipitates were collected using goat anti-mouse IgG attached to magnetic beads, washed in Immunomix, and fractionated by SDS-PAGE. Western transfers were probed with polyclonal neurocan antibody or HRP nickel (KPL) as previously described (Balsamo et al., 1995). To control, peptides were added to cells immediately after treatment with the anti-β-catenin antibody (Transduction Laboratories) and developed with alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson Immunor Research Laboratories, Inc.).

Peptides

Peptides containing the cell permeable sequence derived from the antenapedia homeodomain (Perez et al., 1992; Derosil et al., 1994; Prochiantz, 1996) and sequences from the N-cadherin cytoplasmic domain (Aregui et al., 2000) were synthesized and purified to >90% by HPLC (Genemed Biotechnologies, Inc.). All peptides were dissolved in sterile deionized water, stored in small aliquots at −70°C, and used at 2 μM, a concentration that gives maximal inhibition without toxicity (Aregui et al., 2000). The three peptides used are as follows: COP, RQIKIWFQNRRMKWKK (antennapedia sequence alone); CBP, ROIKIFQONRMRKWKSLLDVFRDYEGSGSTAAGSL (antennapedia plus the β-catenin binding region); and JMP, RQIKIWFQNRRMKWKKRQQAQLLIDPEDDVRD-NILK (antennapedia plus the juxtamembrane region).

Immunohistochemistry

Chicken eyes from embryos at different ages were carefully dissected in cold PBS, and fixed in freshly made 4% paraformaldehyde at 4°C overnight. Fixed eyes were washed three times in PBS (10-15 min each), and the neural retina and pigmented retina layers were removed and maintained in 30% sucrose in PBS overnight at 4°C and frozen in Histoprep medium (Fisher Scientific) at −80°C. 20-μm sections were cut at −25°C, picked up on gelatin-coated coverslips, air-dried on a 37°C hot plate, and frozen at −20°C. Before staining, the sections were heated to 60°C on a hot plate, rehydrated, and washed three times with PBS. A filter blocking with 3% goat serum in PBS for 2 h at room temperature, the sections were incubated with the appropriate primary antibody in 3% goat serum in PBS at room temperature for 1 h. A filter washing three times with PBS, the sections were incubated with secondary antibody in 3% goat serum in PBS at room temperature for 1 h, followed by another three washes with PBS. Finally, the sections were mounted on slides with VectaShield (Vector Laboratories, Inc.) anti-fading mounting medium, sealed with nail polish, and analyzed as for neurite outgrowth.

Single cells were prepared and plated on laminin-coated coverslips as described above for neurite outgrowth assays at a density of 100,000 cells/cover slip. The cells were cultured overnight, and fixed in 4% paraformaldehyde for 20 min at room temperature. For immunostaining, cells were treated similarly to tissue sections, except that 0.4% saponin was included in the incubation buffer when cell permeabilization was required. Tissue sections and cells were observed under phase and epifluorescence using a Zeiss universal microscope. Images were captured and analyzed as for neurite outgrowth.

Results

The 250kD PG cDNA Encodes the Chicken Homologue of Neurocan

The complete cDNA clone has an open reading frame of 3,861 bp, encoding 1,287 amino acids with a calculated molecular mass of 141.6 kD. This is flanked by 65 bp of 5'-untranslated region, 705 bp of 3'-untranslated region including a polyadenylation signal, AATAAA, and the poly-A tail. Database searches at both the nucleic acid and deduced amino acid levels indicate that the cDNA encodes the chicken homologue of neurocan (Fig. 1). All four neurocins thus far sequenced (rat [Rauh et al., 1992], mouse [Rauh et al., 1995], human [Prange et al., 1998], and chick) are organized similarly: the NH\(_2\) terminus contains an Ig-like domain, followed by the hyaluronic acid binding domain, a central region rich in proline, and the COOH terminus with two EGF-like repeats, a lectin-like domain, and a complement regulatory-like domain.
The NH₂- and COOH-terminal regions have ~70% sequence identity between chicken and rat, whereas the central region shows little similarity, <10%.

Analysis of the deduced amino acid sequence indicates the presence of four NXS/T consensus sequences for potential N-glycosylation (Bause, 1983) and six potential GAG attachment sites based on the presence of an SG dipeptide, closely preceded by an acidic amino acid, or followed by XG, where X is any amino acid (Bourdon et al., 1987; Kreuger et al., 1990). Two RGD sequences are also present in the central region of chick neurocan (Fig. 2), whereas the rat has only one (Rauch et al., 1992, 1995), which is not at the same position as either in the chicken, but none appear in the mouse or human homologues. In addition, the COOH terminus contains a stretch of histidine residues (1,251–1,266; Fig. 1). The entire neurocan molecule appears as two globular domains separated by an extended linear region, composed of the unique central domain that bears all of the chondroitin sulfate side chains (Retzler et al., 1996b). Except for the HA-binding domain, little is known about the physiological or developmental functions of these domains.

Northern analysis with total RNA from E10 chicken brain reveals a single band of ~5.4 kb (not shown). This is significantly smaller than the rat or human mRNA (Rauch et al., 1992; Prange et al., 1998). Given the conserved size of the coding region, this discrepancy in message size is most likely because of the differences in the 3' and/or 5' noncoding regions.

To determine whether the cloned cDNA represented the 250kD PG, recombinant neurocan produced in bacteria was reacted with anti-250kD PG antibody (Fig. 3). Neurocan is recognized by affinity-purified anti-250kD PG antibody. Additional bands recognized by the anti-250kD antibody are most likely degradation products. The recombinant polypeptide has a calculated molecular mass...
Neurite Outgrowth Fragment Inhibit N–Cadherin-mediated Adhesion and the stiff, rodlike structure of the central region of the molecule (Retzler et al., 1996b).

The neurocan polypeptide backbone was expressed as a His-tagged fusion protein, purified on a Ni²⁺ column, and assayed for its effect on cadherin-mediated adhesion and neurite outgrowth. Full-length neurocan fusion protein (R P) at 20 μg/ml inhibits N–cadherin-mediated cell adhesion by 60% (Fig. 4 A). This indicates that the polypeptide backbone of neurocan is sufficient for inhibition, and that the sugar chains on tissue-derived neurocan core protein are unlikely to be contributing to the inhibitory effect. That the assay reflects N–cadherin-mediated adhesion is seen by the ability of the anti-N–cadherin antibody NCD-2 to completely block adhesion (Fig. 4 A). The anti-GalNAcPTase antibody 1B11 also functionally blocks N–cadherin–mediated adhesion by 60% (Fig. 4 A, RN versus RC). Inhibition by the NH₂-terminal fragment is dose-dependent exhibiting half maximal inhibition at ~5 μg/ml (Fig. 4 B). The fact that the NH₂-terminal fragment is more active than the full-length protein in a soluble state, possibly be-

Figure 2. (A) Diagram of chick neurocan showing its multiple domains and (B) the regions expressed as fusion peptides. N indicates the position of potential N-linked oligosaccharides, and cs indicates potential chondroitin sulfate attachment sites. RGD indicates the presence of the consensus integrin-binding sequence (A rg-Gly-Asp). SP refers to the signal peptide and CRL to complement regulatory-like domain. Small numbers refer to residue number.

of 141.6 kD, however, the apparent molecular mass on SDS-PAGE is >200 kD. This discrepancy in migration rate was also seen when recombinant rat neurocan was fractionated by SDS-PAGE. This has been attributed to the stiff, rodlike structure of the central region of the molecule (R etzler et al., 1996b).

Recombinant Neurocan and Its NH2-terminal Fragment Inhibit N–Cadherin-mediated Adhesion and Neurite Outgrowth

The neurocan polypeptide backbone is recognized by anti-250kD PG antibody. Lysates of bacteria transformed with plasmids containing the complete chick neurocan cDNA were fractionated by SDS-PAGE and stained with Coomassie brilliant blue (CBB), or transferred to polyvinylidene fluoride membranes and immunoblotted with anti-250kD PG antibody. U, lysate from noninduced bacteria; and I, lysate from bacteria induced with IPTG. Numbers to the left refer to the migration of molecular mass markers (10³).
anti-GalNAcPTase mAb 1B1 indicate that neurite outgrowth is mediated by N-cadherin. Importantly, neurite outgrowth on poly-L-lysine is unaffected by neurocan (not shown). Thus, it is specifically the function of cadherin (or integrin, see below) that is affected, not other aspects of the machinery required for neurite outgrowth.

The NH₂-terminal Fragment of Neurocan Inhibits β1-Integrin–mediated Adhesion and Neurite Outgrowth

We have previously demonstrated that binding of the anti-GalNAcPTase antibody 1B11 to chick retina cells results in inhibition of both cadherin- and integrin-mediated adhesion (Gaya-Gonzalez et al., 1991), and our working hypothesis is that neurocan is an endogenous ligand for the cell-surface GalNAcPTase (Balsamo et al., 1995). This led us to determine if the NH₂-terminal fragment of neurocan is also active in inhibiting β1-integrin–mediated adhesion and neurite outgrowth. E8 chick retina cells were assayed for adhesion to laminin-coated wells in the presence and absence of the NH₂-terminal neurocan fragment. The anti-chick β1 antibody JG22 (Greve and Gottlieb, 1982; Tomaselli et al., 1986; Gaya-Gonzalez et al., 1991), and the anti-N-cadherin antibody NCD-2 serve as controls for the extent and specificity of adhesion. NCD-2 has no effect on adhesion to laminin, whereas JG22 maximally inhibits adhesion by ~60% (Fig. 6). This residual adhesion may be a result of nonspecific interactions or the function of non-integrin laminin receptors (Powell and Kleinman, 1997). Like JG22, the NH₂-terminal fragment inhibits adhesion to laminin by ~60% at 10 μg/ml whereas the COOH-terminal fragment has little or no effect (Fig. 6). This suggests that the NH₂-terminal fragment inhibits β1-integrin–mediated adhesion by close to 100%. β1-Integrin–mediated neurite outgrowth is similarly affected; the NH₂-terminal, but not the COOH-terminal fragment, inhibits neurite extension (Fig. 7 A) with maximal inhibition by the NH₂-terminal fragment of ~60% at 10 μg/ml (Fig. 7 B). In agreement with our previous observations (Gaya-Gonzalez et al., 1991), the anti-GalNAcPTase antibody 1B11 inhibits neurite extension on laminin. Thus, interaction of the NH₂-terminal fragment of neurocan with cells results in coordinate inhibition of cadherin- and integrin-mediated adhesion and neurite outgrowth.

Neurocan-mediated Inhibition of Cadherin and Integrin Function Is via the GalNAcPTase

Native neurocan core protein binds to the glycosphatidylinositol-linked cell-surface GalNAcPTase (Balsamo et al., 1995). To determine if recombinant neurocan and its NH₂-terminal fragment also bind directly to the GalNAcPTase, cells were incubated with the full-length neurocan, the NH₂- and COOH-terminal fragments, followed by the reducible cross-linking reagent DTSSP. After lysis and precipitation with the anti-GalNAcPTase antibody, Western transfers of SDS-PAGE were probed with anti-neurocan antibody that recognizes both fragments. Full-length neurocan and the NH₂-terminal fragment, but not the COOH-terminal fragment, are present (Fig. 8), indicating that neurocan interacts with the GalNAcPTase through the NH₂ terminus.

The GalNAcPTase is associated with the cell surface through a glycosphatidylinositol linkage (Balsamo and Lilien, 1993). Removal of the GalNAcPTase by treating cells with PI-PLC eliminates the effect of the 250kD PG/neurocan on cadherin-mediated adhesion and neurite outgrowth.

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Similarly, treatment of E9 chick retina cells with PI-PLC removes the GalNAcPTase (Fig. 9, box at top) and eliminates the effect of neurocan on β1-integrin–mediated neurite outgrowth (Fig. 9).

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**The Effect of Neurocan on Integrin-mediated Adhesion Is Mediated by N-Cadherin**

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We previously suggested that the inhibitory effect of the 250kD PG/neurocan on cadherin function was due to a signal initiated on binding to the GalNAcPTase that results in retention of phosphate on tyrosine residues of β-catenin with concomitant uncoupling of N-cadherin from the actin-containing cytoskeleton (Balsamo et al., 1995). Similarly, treatment of E9 chick retina cells with recombinant neurocan or its NH2-terminal fragment, but not the COOH-terminal fragment, reduces the amount of actin associated with N-cadherin and increases the phosphotyrosine content of β-catenin. Disruption of the cadherin/actin connection and enhancement of the phosphotyrosine content of β-catenin is very rapid, occurring within 5 min after incubation of cells with neurocan and remaining relatively constant through 15 min (Fig. 10). In contrast to the pattern of β-catenin phosphorylation, disassembly of the cadherin–cytoskeletal connection, as measured by coimmunoprecipitation of actin, increases during the first 15 min of incubation with neurocan (Fig. 10, top).
N-cadherin- and integrin-mediated adhesion. The signal initiated on binding of neurocan to the GalNAcPase results in disassembly of the complex of proteins associated with the cytoplasmic domain of N-cadherin (see also Balsamo et al., 1991, 1995). Further, the GalNAcPase is intimately associated with N-cadherin (Balsamo and Lilien, 1990), but no association with integrin has been detected (Lilien et al., 1999). Based on these data, we speculated that one or more of the protein effectors, originally associated with the cytoplasmic domain of N-cadherin, may be translocated to the integrin complex where it has an inhibitory effect on integrin-mediated adhesion and neurite outgrowth.

In the companion paper (Arregui et al., 2000), we have used cell permeable peptides coupled to sequences mimicking specific regions of the cytoplasmic domain of N-cadherin to cause the release of effectors from the cadherin complex, and have assayed their effect on N-cadherin and β1-integrin-mediated adhesion. Consistent with the above hypothesis, a cell permeable peptide bearing a sequence mimicking the juxtamembrane region of N-cadherin (JM P) causes the specific release of the nonreceptor tyrosine kinase Fer, normally associated with the cadherin complex of cytoplasmic proteins (Kim and Wong, 1995; Rosato et al., 1998), and its translocation to the cytoplasmic complex of proteins associated with β1-integrin with concomitant loss of β1-integrin-mediated adhesion (Arregui et al., 2000). In contrast, treatment of cells with a cell permeable peptide containing a sequence that mimics the β-catenin binding region of N-cadherin (CBP) also causes the release of Fer, but in a complex with β-catenin and p120Catenin. Under these circumstances Fer is not translocated to the integrin complex and there is no effect on integrin function (Arregui et al., 2000).

To determine if treatment of cells with recombinant neurocan NH2-terminal fragment also results in translocation of Fer from cadherin to the β1-integrin complex, retina cells were treated with recombinant NH2-terminal neurocan, cell lysates immunoprecipitated with anti-N-cadherin antibody or anti-FAK antibody, and the immunoprecipitates were analyzed for the presence of Fer. Indeed, Fer is lost from the cadherin complex and appears associated with the integrin complex (Fig. 11). Like neurocan, treatment of cells with the anti-GalNAcPase antibody 1B11, but not 7A2, also results in the loss of N-cadherin and β1-integrin functions and translocation of Fer to the integrin complex (Fig. 11). If translocation of Fer from cadherin to integrin, on treatment of cells with neurocan, is causally related to the loss of integrin function, we speculated that the release of Fer complexed with p120Catenin and β-catenin, as occurs when cells are treated with CBP, might eliminate this signal, abolishing the effect of neurocan on integrin-mediated adhesion. To test this idea, we pretreated retina cells for 2 h with CBP, before addition of neurocan. Pretreatment with CBP does in fact abolish the effect of neurocan on β1-integrin-mediated adhesion (Fig. 12). Treatment with the CBP alone or control antennapedia peptide (COP) alone has no effect on integrin-mediated adhesion (Fig. 12). Thus, a specific configuration of proteins associated with the cytoplasmic domain of N-cadherin is essential for neurocan-mediated inhibition of integrin function. Furthermore, translocation of the non-receptor protein tyrosine kinase Fer from cadherin to integrin appears to be at least one component of a mechanism required for signaling from cadherin to integrin (Arregui et al., 2000).

Figure 11. Treatment of cells with the neurocan NH2-terminal fragment results in release of the protein tyrosine kinase Fer from the cadherin-associated complex of proteins and its association with the β1-integrin complex. E9 retina cells were treated with 10 μg/ml of recombinant neurocan NH2-terminal fragment (RN) or with anti-GalNAcPase antibodies 1B11 or 7A2. The cells were lysed in buffer containing nonionic detergent and the lysates were immunoprecipitated with anti-FAK or anti-N-cadherin antibodies. The immunocomplexes were subjected to SDS-PAGE, followed by immunoblotting with the indicated antibodies.

**Neurocan and Its Receptor GalNAcPase Have a Complementary Distribution in the Retina**

By affecting both cadherin- and integrin-mediated adhesion and neurite outgrowth, interaction of neurocan with its receptor GalNAcPase may have profound effects on morphogenesis. To locate regions of the developing neural retina where such interactions might take place, we immunostained retina sections from E7 to E18 with affinity-purified polyclonal antineurocan antibody and anti-GalNAcPase antibody. By E7, neurocan is expressed in the developing inner plexiform layer (IPL) and continues to be associated with this layer through E18 (Fig. 13) until hatching, when little or no staining is seen (data not shown). Neurocan expression also appears transiently in the nerve fiber layer and the outer plexiform layer between E15 and E18 (Fig. 13). The GalNAcPase is initially present throughout the retina at E7 (not shown), but becomes progressively concentrated in the ganglion cell layer, the inner nuclear layer, and the nerve fiber layer from about E15 (Fig. 13). This is consistent with previous observations made using other anti-GalNAcPase antibodies (Balsamo et al., 1990).
Thus, during development, neurocan staining in IPL is sandwiched by the GalNAcPTase expression in the ganglion cell and inner nuclear layers. This is most clearly seen in the overlay images (Fig. 13, bottom). To further define the cell type on which neurocan appears, a series of antibodies recognizing proteins associated with specific cell types were used in combination with antineurocan antibody. Consistent with the presence of neurocan in the IPL, a marker for amacrine cells, antisyntxin antibody (HPC-1; Barnstable et al., 1985), labels the same population of cells as antineurocan (Fig. 14 B). Cells that bind antineurocan antibody also express neurocan mRNA, which is concentrated in the cell bodies (not shown). Neither a bipolar cell-specific antibody 3G3 (Burdens-Gulley and Brady-Kalnay, 1999; Lemmon, V., personal communication), or a glial cell marker, 3A7 (Lemmon, 1985) recognize this population (not shown). Consistent with the morphological characteristics of the neurocan-positive cells, cells with one, unidirectional, long neurite, which are reactive with the ganglion cell-specific antibody, 8D9 (Lemmon and McLoon, 1986), do not react with antineurocan antibody (Fig. 15). However, there is a population of cells with short, multiple neurites that are reactive with both 8D9 and antineurocan (Fig. 15).

Discussion

Coordinate Regulation of Cadherin and Integrin

We have isolated the complete cDNA for chicken neurocan, and demonstrate that recombinant fusion protein, like the purified native proteoglycan, is active in inhibiting both N-cadherin- and β1-integrin-mediated cell adhesion and neurite outgrowth. Coordinate regulation appears to be mediated through the interaction of neurocan with its receptor GalNAcPTase. Native neurocan (Balsamo et al., 1995) and recombinant protein, as well as the smallest active fragment, the NH2 terminus containing the Ig loop and the HA-binding domain, all bind to the cell-surface GalNAcPTase. Furthermore, removal of the GalNAcPTase with PI-PLC abolishes the inhibitory effect on both adhesion systems. Further dissection of the NH2 terminus into the Ig loop alone, the HA domain alone, or the Ig loop with a fragment of the HA binding results in a loss of activity. The requirement for the HA binding domain is consistent with our previous observation that aggregates containing the 250kD PG with HA are inactive (Balsamo et al., 1995); both sets of data suggest that the exposed HA-binding domain is essential for functional interaction with the GalNAcPTase.

In Fig. 16, we have attempted to pictorially represent the interactions taking place on binding of neurocan to its GalNAcPTase receptor. Loss of cadherin function is correlated with uncoupling from the cytoskeleton after hyperphosphorylation and release of β-catenin from its association with cadherin (Balsamo et al., 1995). Hyperphosphorylation of β-catenin has consistently been correlated with the loss of adhesive function (Daniel and Reynolds, 1997; Lilien et al., 1997). Our laboratory has demonstrated that the nonreceptor protein tyrosine phosphatase PTP1B regulates β-catenin phosphorylation (Balsamo et al., 1995, 1996).
PTP1B binds to the cytoplasmic domain of N-cadherin and is essential for the continued removal of phosphate from tyrosine residues of β-catenin (Balsamo et al., 1998). Furthermore, phosphorylation of PTP1B is essential for binding to cadherin (Balsamo et al., 1996, 1998). Neurocan-GalNAcPTase interaction results in inactivation or loss of protein tyrosine kinase activity from the cadherin complex. This appears to initiate a cascade resulting in the loss of PTP1B, retention of phosphate groups on tyrosine residues of β-catenin and uncoupling of cadherin from the actin cytoskeleton (Balsamo et al., 1995, 1996, 1998). Based on the data presented in this paper and Arregui et al. (2000), the protein tyrosine kinase may be the nonreceptor tyrosine kinase Fer. Disassembly of the cadherin complex of proteins and the cytoskeletal connection on interaction of neurocan with its receptor may also be accompanied by loss of cadherin dimers, which is the functional unit required for strong adhesion (Yap et al., 1997; Takeda et al., 1999).

Our data are consistent with inhibition of integrin function by neurocan being dependent on a signal generated through cadherin. We base this on three lines of evidence. First, we demonstrate that the interaction of neurocan with the GalNAcPTase results in translocation of Fer from its association with the cytoplasmic domain of cadherin (Kim and Wong, 1995; Rosato et al., 1998) to the integrin complex of proteins. This is consistent with the fact that overexpression of Fer inhibits integrin-mediated adhesion (Rosato et al., 1998). Additionally, we have shown (Arregui et al., 2000) that inducing the release of Fer from the

Figure 14. A macrine cells express neurocan. E9 retina cells were cultured overnight before staining with antibodies. A shows the morphology of typical cells expressing neurocan. (B) Cells were double stained with antisyntaxin and antineurocan. Bar, 20 μm.

Figure 15. Typical ganglion cells are not reactive with antineurocan antibody. E9 cells cultured overnight were stained with antineurocan and 8D9, a ganglion cell marker, followed by FITC-conjugated secondary antibody. The arrowheads point to cells showing typical ganglion cell morphology, reactive with 8D9, but not antineurocan. The arrows point to cells reactive with both antibodies, but with atypical ganglion cell morphology. Bar, 20 μm.
cadherin complex using a cell permeable peptide containing a sequence mimicking the juxtamembrane region of cadherin results in its translocation to the integrin complex and loss of integrin function. Second, release of a complex of proteins containing β-catenin, p120CA T, and Fer from the cytoplasmic domain of cadherin before treatment of cells with neurocan abolishes the effect of neurocan on integrin function (this article and Arregui et al., 2000). This suggests that a specific array of effectors must be associated with the cadherin complex for transduction of the neurocan-induced signal regulating integrin function to occur. Third, cadherins, N and E (Balsamo and Lilien, 1990; Bauer et al., 1992), but not p1-integrins (Lilien et al., 1999) are intimately associated with the GPI-anchored, neurocan receptor Ga lN A cPTase, suggesting that the neurocan-induced signal is mediated by cadherin. There are a number of GPI-linked proteins that act as coreceptors in the sense that the signal is transduced through a transmembrane partner. Perhaps the most interesting, for our purposes, is the association of the receptor tyrosine kinase Ret with GPI-linked coreceptors. Ret has two extracellular cadherin-like repeats (Iwamoto et al., 1993) and associates with two GPI-linked coreceptors. Binding of the ligand to its GPI-linked coreceptors activates Ret phosphorylation setting the appropriate signaling pathway in motion (Jing et al., 1996; Treanor et al., 1996; Buj-Bello et al., 1997; Klein et al., 1997).

Loss of integrin function, either through overexpression of Fer (Osato et al., 1998) or through perturbation with a cell permeable peptide mimicking the juxtamembrane region of cadherin (A rregui et al., 2000) is correlated with hypophosphorylation of p130cas as is the inability to assemble focal adhesions (Nojima et al., 1995; Petch et al., 1995; U ori and Ruoslahti, 1995). p130cas interacts directly with the tyrosine phosphatases PTP-PEST (Garton et al., 1997) and PTP1B (Liu et al., 1996), as well as the tyrosine kinase FAK (Polte and Hanks, 1995). It has been suggested that these components compete with each other to regulate the tyrosine phosphorylation of p130cas and, therefore, assembly of focal adhesions (Garton et al., 1997). Tyrosine-phosphorylated p130cas also interacts with Crk (Matsuda and Kurata, 1996), and this interaction has the potential to protect p130cas from dephosphorylation (Birge et al., 1992). The most conservative explanation for the hypophosphorylation of p130cas on interaction of Fer with the integrin complex is that Fer affects one or more of p130cas-binding partners, PTP-PEST, PTP1B, FAK or even Crk, altering their activity, or their interaction with p130cas, changing the balance of phosphorylated tyrosine residues and, thus, integrin function.

**Neurocan’s Modular Organization Allows Interactions with Many Adhesion-related Molecules**

Neurocan is a member of a family of structurally similar chondroitin sulfate proteoglycans (Margolis and Margolis, 1994) including aggrecan (Do ege et al., 1987), versican (Zimmermann and Ruoslahti, 1989), and brevican (Y a-mada et al., 1994; Jaworski et al., 1995). The family has been referred to as lecticans (Ruoslahti, 1996) or hyalec-

Figure 16. Pictorial representation of the known protein interactions in the cadherin and integrin complex potentially altered on binding of neurocan to its receptor GalNAcPTase. On the right is a functional cadherin complex showing the known protein interactions. The interaction of β-catenin, p120cas, and Fer is based on Arregui et al. (2000). On the left, after binding of neurocan, are the alterations induced in the cadherin complex rendering it nonfunctional and potential Fer targets in the integrin complex.
lander et al., 1994). In contrast, the Ig superfamily adhesion molecule TAG-1/axonin-1 interacts with the neurocan core protein (Milev et al., 1996). The physiological significance of these interactions is supported by the overlapping distribution of these adhesion molecules with neurocan in embryonic and early postnatal nervous tissue (Milev et al., 1996).

The COOH terminus of the neurocan polypeptide interacts with the extracellular matrix protein tenasin-C (Rauf, 1997). The COOH-terminal lectinlike domain of brevican binds cell-surface glycolipids and promotes cell adhesion (Mura et al., 1999), suggesting a similar function for the neurocan lectinlike domain. Additionally, the COOH terminus of several family members can bind simple sugars (H. alberg et al., 1988; Saleque et al., 1993; Ujita et al., 1994), suggesting lectin-like interactions.

Our data add to the group of adhesion-related molecules that interact with neurocan and further indicate a specific role for the NH2 terminus containing the Ig loop and the HA-binding domain in regulating cadherin and integrin function. The predominant expression of neurocan in the central nervous system (Ohara et al., 1994; Meyer-Puttlitz et al., 1995; Fukuda et al., 1997; Matsui et al., 1998) and its multiple interactions through distinct domains with adhesion-related molecules suggests that neurocan has evolved as a modular extracellular regulator of cell-cell and cell-matrix interactions that guide the cellular rearrangements essential to the formation and function of the nervous system.

**Neurocan May Act as a Barrier to Neurite Extension in the Developing Retina**

In the developing retina, neurocan expression predominates in the IPL, but is also transiently associated with the outer plexiform layer (OPL), whereas the GalNAcPTase, which was originally distributed to most layers in the retina, becomes restricted to the ganglion cell layer and inner nuclear layer, sandwiching the IPL. Consistent with this distribution, we find that, in vitro, it is amacrine cells with multiple processes that express and synthesize neurocan and deposit it on the substrate around cell bodies and processes.

There are two possible exceptions to this cellular distribution. First, we occasionally observe that neurocan and the amacrine cell marker HPC-1 do not overlap. This may be explained by the number of different subtypes of amacrine cells, some expressing neurocan and others not, but all being HPC-1-positive. Alternatively, HPC-1 also weakly reacts with some horizontal cells (Gleason et al., 1993), and it is possible that, in our culture system, some HPC-1-positive cells are horizontal cells, not expressing neurocan. We do observe transient neurocan staining in the OPL at around day 14, and this may be due to transient expression by horizontal cells. Second, some neurocan-positive cells with typical stellate morphology are positive for the ganglion cell marker, 8D9. It is likely that these cells are not ganglion cells. In fact, it has been shown that 8D9 does stain the IPL faintly. This may be due to either ganglion cell dendrites or processes of amacrine cells (Lemmon and McLoon, 1986). Our finding that amacrine cells express neurocan favors the latter possibility; however, we cannot exclude the possibility that some ganglion cells may also express neurocan.

The ability of neurocan to affect the function of many distinct adhesion molecules either directly or indirectly suggests that its spatiotemporal distribution may play critical roles in morphogenesis of the retina. While much is known about the distribution of adhesion molecules in the developing retina and some functional analyses have been performed using antibodies (Buskirk et al., 1980; Hoffman et al., 1986; Matsuura et al., 1988; Svennevist and Linser, 1993; Stone and Sakaguchi, 1996) and by introducing dominant negative constructs into the developing Xenopus eye (Lilienbaum et al., 1995; Riehl et al., 1996), the role of endogenous modifiers of adhesion molecule function, such as neurocan, has not been explored experimentally. Chondroitin sulfate proteoglycans have been shown to act as a barrier to neurite extension in general (Faisstner and Stein-dler, 1995; Margolis and Margolis, 1997) and have been suggested to influence the direction of retinal ganglion cell outgrowth (Snow et al., 1991). The expression of neurocan juxtaposed by its receptor, GalNAcCPTase, does indeed suggest a role for this interacting pair in restricting cell or process movement mediated by cadherin and/or integrin at such boundaries. Furthermore, because of its interactions with other adhesion molecules in the immunoglobulin superfamily, neurocan is well suited to such a role in the retina. Thus, neurocan, and possibly other chondroitin sulfate proteoglycans, may act to prevent ganglion cell projections from extending to other layers of the retina, orienting them to form the nerve fiber layer. Indeed, retinal neurons extend processes in vitro mediated by cadherin, integrin and NCA M (Neugebauer et al., 1988), and purified neurocan acts as barrier to neurite extension by retina neurons in vitro (Balsamo et al., 1996).

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