Molecular Forms, Binding Functions, and Developmental Expression Patterns of Cytotactin and Cytotactin-binding Proteoglycan, An Interactive Pair of Extracellular Matrix Molecules

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Abstract. Cytotactin is an extracellular matrix protein that is found in a restricted distribution and is related to developmental patterning at a number of neural and non-neural sites. It has been shown to bind specifically to other extracellular matrix components including a chondroitin sulfate proteoglycan (cytotactin-binding [CTB] proteoglycan) and fibronectin. Cell binding experiments have revealed that cytotactin interacts with neurons and fibroblasts. When isolated from brain, both cytotactin and CTB proteoglycan contain the HNK-1 carbohydrate epitope. Here, specific antibodies prepared against highly purified cytotactin and CTB proteoglycan were used to correlate the biochemical alterations and modes of binding of these proteins with their differential tissue expression as a function of time and place during chicken embryo development. It was found that, during neural development, both the levels of expression of cytotactin and CTB proteoglycan and of the molecular forms of each molecule varied, following different time courses. In addition, a novel M_r 250,000 form of cytotactin was detected that contained chondroitin sulfate. The intermolecular binding of cytotactin and CTB proteoglycan and the binding of cytotactin to fibroblasts were characterized further and found to be inhibited by EDTA, consistent with a dependence on divalent cations. Unlike the molecules from neural tissue, cytotactin and CTB proteoglycan isolated from non-neural tissues such as fibroblasts lacked the HNK-1 epitope. Nevertheless, the intermolecular and cellular binding activities of cytotactin isolated from fibroblast culture medium were comparable to those of the molecule isolated from brain, suggesting that the HNK-1 epitope is not directly involved in binding. Binding experiments involving enzymatically altered molecules that lack chondroitin sulfate suggested that this glycosaminoglycan is also not directly involved in binding.

Although they clearly formed a binding couple, the spatial distributions of cytotactin and CTB proteoglycan in the embryo were not always coincident. They were similar in tissue sections from the cerebellum, gizzard, and vascular smooth muscle. In contrast, CTB proteoglycan was present in cardiac muscle where no cytotactin is present, and it was seen in cartilage throughout development unlike cytotactin, which was present only in immature chondrocytes. Cell culture experiments were consistent with the previous conclusion that cytotactin was specifically synthesized by glia, whereas CTB proteoglycan was specifically synthesized by neurons. Both molecules were found either associated with the cell surface or in an intracellular, perinuclear pattern. These observations are in accord with the idea that binding of central glia to neurons occurs in part through the cytotactin–CTB proteoglycan couple. The combined results of this study support the hypothesis that structural modulations and dynamic interactions among extracellular matrix components contribute to the control of cell behavior during development.

MORPHOGENESIS results from the coordinate expression of various cellular primary processes, among which cell adhesion and migration play pivotal roles at certain stages of development. Specific adhesion molecules are important to an understanding of the regulation of cell motion and adhesion that leads to pattern formation (11). Such molecules include cell–cell adhesion molecules, or CAMs[1] (12), as well as extracellular matrix proteins (36, 39) including various cell–substrate adhesion molecules.

We have previously identified an extracellular matrix protein, cytotactin, that has an unusual site-restricted distribution during embryogenesis (8), and is involved in neuron–glia interaction (17). When isolated from embryonic brain, cytotactin appears as multiple polypeptides of M_r 220,000, 200,000, and 190,000, all of which express the HNK-1 carbohydrate antigenic determinant (17). Polyclonal antibodies recognizing these components in extracts of em-

1. *Abbreviations used in this paper*: CAMs, cell–cell adhesion molecules; CTB proteoglycan, cytotactin-binding proteoglycan; RGD, Arg-Gly-Asp.

bryonic brain were found to recognize two HNK-1 antigen-bearing polypeptides of M_r 160,000 and 180,000 in extracts of adult brain; as shown by one-dimensional peptide maps, these components were related to each other but not to the three components of cytotactin (17). Further analysis led to the finding that a chondroitin sulfate proteoglycan that co-purifies with cytotactin binds to it (20). The M_r 280,000 core protein of this proteoglycan isolated from embryonic brain tissue carried the HNK-1 determinant. This proteoglycan binds specifically to cytotactin and we therefore designate it CTB (cytotactin-binding) proteoglycan. CTB proteoglycan inhibits the binding of cytotactin to cells (20); in contrast, fibronectin, which also binds to cytotactin, does not inhibit its cell binding.

The potential importance of cytotactin and CTB proteoglycan in affecting cell movement and morphogenesis was suggested by observations (8) that cytotactin appears in the developing embryo in a succession of cephalocaudal waves. This molecule and the CTB proteoglycan exhibit a striking asymmetric distribution during somite formation and neural crest cell migration that is correlated with the position of crest cells in the sclerotome (34). Functional tests in vitro showed that cytotactin and CTB proteoglycan caused cell rounding and halted the migration of neural crest cells on fibronectin substrates (34), suggesting that these molecules may differentially affect the cell migration known to occur on fibronectin (32). Consistent with this idea, perturbation experiments with specific antibodies have shown that cytotactin is also involved in the control of cell migration in the developing cerebellum (7).

The current study was undertaken to relate the expression of cytotactin and CTB proteoglycan at various sites and times of development, to determine at which sites they might interact during histogenesis, and to correlate these findings with their biochemical and binding properties. Using highly specific antibodies to the purified proteins, we found various biochemical changes in the forms and amounts of authentic cytotactin and CTB proteoglycan at various sites during development, differentially glycosylated forms of these molecules, and complex interactions among cytotactin, CTB proteoglycan, and fibronectin that affected their interactions with cells. The results further support the idea that an interactive network of matrix components that are synthesized at different times and places and are modulated by binding interactions may control cell behavior during morphogenesis.

Materials and Methods

Purification of Cytotactin and CTB Proteoglycan

Both molecules were purified from 95,000 *g* supernatants of 14-d-old embryonic brain tissues homogenized in NaCl (8 g/liter)/KCl (0.3 g/liter)/NaH_2PO_4 (0.025 g/liter)/KH_2PO_4 (0.025 g/liter)/$NaHCO_3$ (1 g/liter)/glucose (2 g/liter)/1 mM phenylmethylsulfonyl fluoride (PMSF; Eastman Kodak Co., Rochester, NY)/2% (vol/vol) trasylol (Mobay Chemical Corp., Pittsburgh, PA). The extract from 1,000 brains in 400 ml was incubated for 3 h at 4°C with 50 ml of DE52 (Whatman Inc., Clifton, NJ). The DE52-bound fraction contained ~90% of the CTB proteoglycan and ~25% of the cytotactin and was, therefore, used as the source of CTB proteoglycan. After washing with PBS (NaCl [8 g/liter]/KCl [0.2 g/liter]/KH_2PO_4 [0.2 g/liter]/Na_2HPO_4 [1.15 g/liter]), the DE52 was eluted with 100 ml of 2.7 M NaCl in PBS. When the DE52 eluate from 5,000 brains had been collected, it was dialyzed versus H_2O, lyophilized, and dissolved in 4 M guanidine-HCl/0.1 M sodium phosphate (pH 7.4) plus 0.6 g/ml CsCl. This solution was centrifuged in a VTi50 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 170,000 *g* for 24 h at 20°C. The resulting density gradient was fractionated, and those fractions with a density >1.42 g/ml that contained no polypeptides that entered a 6% gel before chondroitinase treatment were pooled, dialyzed versus H_2O, and lyophilized. For the preparation of chondroitinase-treated CTB proteoglycan, the sample was then resuspended in PBS and treated with 0.5 U of chondroitin ABC lyase (Miles Laboratories Inc., Naperville, IL) for 4 h at 37°C. This treatment removed ~92% of the uronic acid from CTB proteoglycan as quantitated by standard methods (1). CTB proteoglycan or chondroitinase-treated CTB proteoglycan was then dissolved in 4 M guanidine-HCl/0.1 M sodium phosphate (pH 7.4) and further fractionated either by centrifugation (24 h, 20°C, 150,000 *g*) on sucrose gradients (5–20% sucrose in the same buffer) or by gel filtration on Sephacryl S-500 in the same buffer. In either case, after fractionation, fractions containing purified CTB proteoglycan were identified by gel electrophoresis and Stains-all (Eastman Kodak Co.) staining (15), pooled, dialyzed versus H_2O, and lyophilized.

The DE52-unbound fraction described above was the source of cytotactin. This material was incubated for 3 h at 4°C with 50 ml of Sepharose CL-2B beads to which 150 mg of 1D8 (a monoclonal antibody specific for a polypeptide epitope in cytotactin) had been covalently coupled using CNBr (9). The beads were washed with PBS and eluted with 100 ml of 50 mM diethylamine; the eluate was then neutralized with 10 ml of 1 M sodium phosphate (pH 6.0). When the 1D8 eluate from 5,000 brains had been collected, it was dialyzed versus H_2O, lyophilized, and run on a CsCl density gradient as described above for CTB proteoglycan. In this case, fractions with a density <1.38 g/ml were pooled, dialyzed versus H_2O, and lyophilized. The sample was then fractionated by gel filtration on Sephacryl S-500 in 4 M guanidine-HCl/0.1 M sodium phosphate (pH 7.4). Both of these steps removed residual CTB proteoglycan, which was found in dense fractions in the CsCl gradient fractionation and in lower molecular weight fractions than cytotactin during gel filtration. Fractions containing purified cytotactin (k_{av} 0.37–0.54) were identified by SDS gel electrophoresis, pooled, dialyzed versus H_2O, and lyophilized.

Purification of CTB Proteoglycan and Cytotactin from Fibroblast Culture Medium

Fibroblasts were isolated from the body walls of 9- to 11-d-old chicken embryos, were cultured by standard methods for 2–4 d, and the conditioned medium was collected. For the purification of CTB proteoglycan, dry guanidine-HCl was added to a final concentration of 4 M, and CsCl was added to 0.6 g/ml. The pH was adjusted to 7.4 with NaOH, and density fractionation was performed as described above. For the purification of cytotactin, the fibroblast-conditioned medium was incubated with monoclonal antibody 1D8 covalently linked to Sepharose CL-2B, and the eluate was further purified on sucrose gradients. Both of these steps were performed as described above.

Purification of M_r 180,000 Protein

A mixture of proteins bearing the carbohydrate epitope recognized by monoclonal antibody HNK-1 was purified from adult chicken brain extracts as previously described (17). This material, which contained the M_r 180,000 and 160,000 polypeptides, was injected into mice. Hybridomas were prepared, and their culture supernatants were screened by their ability to immunoprecipitate the M_r 180,000 polypeptide. The monoclonal antibodies synthesized by two clones identified in this manner were found to immunoprecipitate specifically the M_r 180,000 polypeptide but were not useful in immunoblotting and in immunohistochemistry. To purify quantities of the M_r 180,000 polypeptide sufficient to prepare polyclonal antibodies, the same extracts used as a source of cytotactin and CTB proteoglycan were incubated with these monoclonal antibodies coupled to Sepharose CL-2B, and were washed, eluted, and concentrated as described above in the case of cytotactin and monoclonal antibody 1D8.

Gel Electrophoresis and Immunoblotting

SDS gels (25) were run and immunoblotted (37) or stained with Coomassie Blue or silver (28) by standard methods. Immunoblots were quantitated by cutting out appropriate regions of the nitrocellulose and determining the amount of bound ^{125}I-protein A as previously described (21).

Preparation of Antibodies

Cytotactin, chondroitinase-treated CTB proteoglycan, or the M_r 180,000 polypeptide was purified as described above, and 50-μg aliquots were in-

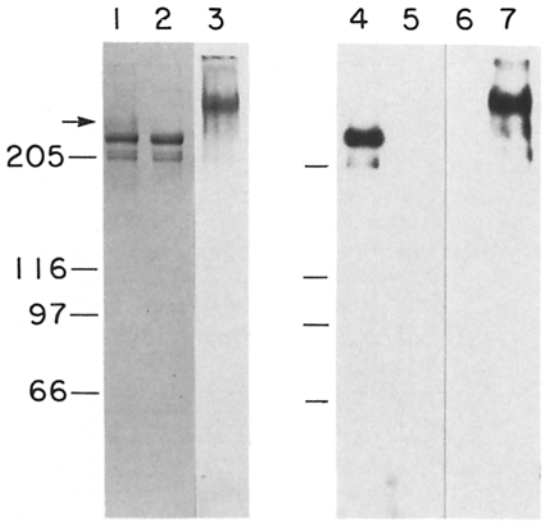

Figure 1. Electrophoretic and immunological comparison of purified cytotactin and CTB proteoglycan. Cytotactin and chondroitinase-treated CTB proteoglycan were prepared as described in the Materials and Methods and resolved on SDS gels containing 6% acrylamide. Lane *1*, 4 μg cytotactin; lane *2*, 4 μg cytotactin treated with 0.005 U of chrondroitin ABC lyase for 30 min at 37°C; lane *3*, 4 μg chondroitinase-treated CTB proteoglycan; lanes *4* and *6*, 2 μg cytotactin; and lanes *5* and *7*, 2 μg chondroitinase-treated CTB proteoglycan. The detection methods used were Coomassie Blue staining (lanes *1* and *2*), silver staining (lane *3*), and immunoblotting with anti-cytotactin antibodies (lanes *4* and *5*) or anti-CTB proteoglycan antibodies (lanes *6* and *7*). The migration and $M_r \times 10^{-3}$ of standard proteins are indicated. The arrow indicates the position of the chondroitinase-sensitive M_r 250,000 component of cytotactin.

jected into rabbits. Aliquots of the cytotactin and CTB proteoglycan used for injection are shown in Fig. 1, lanes *1* and *3*. IgG and Fab' fragments (3) were prepared from the immune sera. Monoclonal anti-Ng-CAM antibodies were prepared as previously described (16).

Preparation of Extracts

The indicated cells or tissues were collected, suspended in 5 vol of PBS/2% (vol/vol) trasylol/1 mM PMSF, and disrupted using a Polytron homogenizer (Brinkmann Instruments Co., Westbury, NY). The homogenates were centrifuged for 10 min at 10,000 *g*. The supernatants were saved, and the pellets were resuspended in 0.5% NP-40/PBS and recentrifuged. These supernatants were also saved, and the pellets were resuspended in 8 M guanidine-HCl/0.2 M sodium phosphate (pH 7.4) and recentrifuged. Again, the supernatants were saved, and all three supernatants were dialyzed versus H_2O before resolution on SDS gels. The first supernatant is referred to as the "PBS extract," the second as the "NP-40 extract," and the third as the "guanidine extract." When extracts were to be chondroitinase-treated, 25-μl aliquots of the dialyzed extracts were incubated with 0.01 U of chondroitin ABC lyase for 30 min at 37°C in the presence of 5 mM EDTA, 5 mM *N*-ethyl maleimide, and 3 mM PMSF.

Immunohistochemistry

Paraffin sections were prepared for staining (26). Briefly, whole embryos or dissected organs were frozen in isopentane cooled in liquid nitrogen and immediately immersed in methanol at −70°C. After 40–70 h, the embryos were serially transferred to methanol equilibrated at −20°, 4°, and 20°C, incubating them for at least 3 h at each step. The samples were then immersed twice in xylene for 15 min and then transferred to a solution of 50% Paraplast (Monoject Scientific, St. Louis, MO) in xylene at 45°C for 15 min, infiltrated twice with Paraplast in a vacuum oven at 45°C for 10 min, and embedded in Paraplast. Sections 10-μm thick were cut using a model 3010 microtome (Reichert Scientific Instruments, Buffalo, NY). For immunofluorescent staining, the deparaffinized sections were incubated sequentially with the primary antibody (1:100 dilution of serum in PBS/5% normal goat serum) and fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA), mounted in 90% gycerol/PBS, and observed in a Zeiss microscope. Sections to be incubated with anti-CTB proteoglycan antibodies were first treated with 0.025 U/ml in PBS of chondroitin ABC lyase for 30 min at 25°C. Little or no labeling was obtained without this treatment.

To determine whether CTB proteoglycan is synthesized by neurons or glia, 9-d-old embryonic chicken brain cells were cultured on glass coverslips and fixed as previously described (18). Cells were permeabilized by including 0.5% NP-40 in all buffers and were chondroitinase-treated by incubation with 0.1 U/ml of chondroitin ABC lyase for 1 h at 25°C. For immunofluorescent staining with polyclonal rabbit antibodies, samples were treated as described above. In double-labeled experiments, samples also were incubated sequentially with 100 μg/ml of a monoclonal antibody in PBS/5% normal goat serum and rhodamine-conjugated goat anti-mouse IgG (Cappel Laboratories).

Preparation and Use of Covaspheres

To prepare Covaspheres (Duke Scientific Corp., Palo Alto, CA) for binding studies, 50-μl aliquots of Covaspheres were incubated with 10 μg of cytotactin, CTB proteoglycan, or human fibronectin (New York Blood Center) and processed and stored as previously described. Under these conditions, 100% of the cytotactin or fibronectin became covalently attached to the Covaspheres, but only 20% of the CTB proteoglycan bound to the beads.

In Covasphere to Covasphere binding assays, Covaspheres were sonicated for 20 s in a bath sonicator, and 10-μl aliquots of Covaspheres were mixed in the presence of 0.5 mM $CaCl_2$. After 30-min incubation at 25°C, the samples were diluted to 20 ml in PBS that had been filtered with a 0.45-μm filter, and 0.5 ml-aliquots were analyzed in a Coulter Counter model ZBI (Coulter Electronics, Hialeah, FL) with a 100-μm aperture set at amplification 1/8, aperture current 0.177, and threshold 10–100 (these settings should count aggregates of ~40 Covaspheres). In the reported experiments where strong aggregation was observed, particle counts were >10,000. At this level of aggregation, <20% of monomer Covaspheres remain outside of aggregates.

In Covasphere to fibroblast binding assays, cells were prepared by the light trypsin method (2), but in the presence of 1 mM $CaCl_2$ instead of EDTA. Cells (1×10^6 in 100 μl suspension culture medium [No. 330-1650; Gibco, Grand Island, NY]/1 mM $CaCl_2$) plus 10-μl aliquots of sonicated Covaspheres bearing radioiodinated proteins (50,000 cpm) plus the indicated other molecules were incubated for 20 min at 25°C, and cells with bound Covaspheres were separated from unbound Covaspheres as previously described (20). In a typical experiment, ~5% of cytotactin-coated Covaspheres or fibronectin-coated Covaspheres bound to cells. To confirm that the remaining unbound Covaspheres were equally capable of binding to cells, in a control experiment, unbound cytotactin-coated Covaspheres and fibronectin-coated Covaspheres were each reincubated with a second aliquot of cells and found to bind at the 5% level, indicating no depletion of binding activity during the initial incubation.

Results

Purification of Cytotactin and CTB Proteoglycan from Embryonic Chicken Brain and Characterization of Specific Antibodies

In a previous study (20), purified cytotactin and CTB proteoglycan were found to bind to each other. To overcome the low levels of cross-contamination that were observed in cytotactin and CTB proteoglycan preparations and that may result from their mutual affinity, more stringent schemes were devised to yield extremely pure molecules for generating polyclonal antibodies. As described in detail in the Materials and Methods, the fractionation of the proteoglycan took advantage of its high negative charge and high density. The fractionation of cytotactin took advantage of the availability of a specific monoclonal anti-cytotactin antibody as well as of the lower density of the cytotactin molecule. For both CTB proteoglycan and cytotactin, density fractionation

and size fractionations were performed in the presence of 4 M guanidine-HCl to minimize intermolecular interactions. In accord with previous studies (17, 20), purified cytotactin preparations contained three major components of M_r 220,000, 200,000, and 190,000 (Fig. 1, lane *1*). In addition, a minor component of M_r 250,000 was detected that was sensitive to chondroitinase treatment (compare lane *2* with lane *1*). This component was also recognized on immunoblots by a monoclonal antibody specific for a polypeptide epitope in cytotactin, indicating that it is an authentic form of the molecule (data not shown). As previously described (20), chondroitinase-treated CTB proteoglycan migrated with an apparent M_r of 280,000 (lane *3*). Upon storage, components of M_r 250,000, 220,000, 150,000, and 110,000 routinely appeared in chondroitinase-treated CTB proteoglycan preparations, all of which were recognized by specific antibodies to the proteoglycan.

To evaluate the purity of these cytotactin and proteoglycan preparations, polyclonal rabbit antibodies were prepared against the isolated molecules. In immunoblotting experiments, anti-cytotactin antibodies detected purified cytotactin (Fig. 1, lane *4*) but not chondroitinase-treated CTB proteoglycan (lane *5*). Although the M_r 220,000 component of cytotactin was predominant in lane *4*, all components of cytotactin were recognized by this antibody. In contrast, the anti-CTB proteoglycan antibody (prepared against the chondroitinase-treated CTB proteoglycan) detected chondroitinase-treated CTB proteoglycan (lane *7*) but not cytotactin (lane *6*). Immunoblotting could not be used to analyze intact CTB proteoglycan because the molecule transfers poorly to nitrocellulose. However, when intact CTB proteoglycan was spotted directly onto nitrocellulose, it was recognized by anti-CTB proteoglycan antibodies and not by anti-cytotactin antibodies.

Molecules with very similar properties to cytotactin have been isolated from fibroblasts (13) and cartilage (38) and were shown to appear in electron micrographs as six-armed structures that Erickson and Inglesias (13) called hexabrachions. To determine whether cytotactin isolated from the brain extracts has a similar morphology, the purified molecule was rotary shadowed and observed by Dr. Erickson. Hexabrachions that were indistinguishable from those previously described (13, 38) were found in the preparations (Fig. 2). In addition, many four- or five-armed but otherwise similar structures were observed. These results indicate that all of these molecules have similar three-dimensional structures and suggest that they are closely related or identical.

Molecular and Cellular Binding Properties of Cytotactin and Proteoglycan

In a previous study (20), cytotactin-coated beads were found to bind to CTB proteoglycan-coated beads, to fibronectin-coated beads, and to cells including neurons and fibroblasts. In the present study, these molecular and cellular binding mechanisms were investigated further. Fibroblasts were found to be particularly useful to study the binding of cytotactin to cells because they bind substantially more cytotactin-coated beads on a per cell basis than do neurons.

Binding of cytotactin-coated beads to proteoglycan-coated beads or to fibronectin-coated beads was found to be inhibited by EDTA (Table I). Furthermore, the inhibition of both intermolecular binding modes was reversed by added $CaCl_2$ and not by $MgCl_2$, and the binding was also inhibited by EGTA, which is a more specific chelator of Ca^{++} ions (data not shown). These results suggest that these binding mechanisms are Ca^{++}-dependent. Both binding mechanisms were also inhibited by soluble cytotactin or CTB proteoglycan, and by anti-cytotactin Fab′ fragments. As expected, only the cytotactin–CTB proteoglycan binding mechanism was inhibited by anti-CTB proteoglycan Fab′ fragments. Soluble fibronectin inhibited cytotactin–fibronectin binding only weakly and had no effect on cytotactin–CTB proteoglycan binding as previously described (20). Neither binding mechanism was inhibited by a synthetic Arg-Gly-Asp (RGD)-containing peptide, an amino acid sequence that forms part of

Table I. Effects of Various Molecules on Covasphere–Covasphere Aggregation

	Cytotactin-coated Covaspheres + CTB proteoglycan-coated Covaspheres		Cytotactin-coated Covaspheres + fibronectin-coated Covaspheres	
Soluble molecule	Superthreshold particles*	Inhibition	Superthreshold particles*	Inhibition
		%		%
–	20,200	–	15,100	–
EDTA	10,600	48	5,700	62
Anti-cytotactin Fab′	900	96	1,700	89
Anti-proteoglycan Fab′	11,900	41	13,800	9
GRGDS	19,900	1	18,300	(21)‡
Cytotactin	2,500	88	2,500	83
CTB proteoglycan	2,300	89	2,800	81
Fibronectin	20,100	0	12,200	19

Covasphere–Covasphere binding assays were performed as described in the Materials and Methods. Amounts or concentrations of soluble molecules were: EDTA, 2 mM; anti-cytotactin or anti-proteoglycan Fab′, 20 μg; GRGDS (Peninsula Laboratories, Inc., Belmont, CA; Code 9137), 0.5 mg/ml; cytotactin, CTB proteoglycan, or fibronectin, 1 μg.

* Average of duplicate points. In all cases, SD was <1,500 superthreshold particles. Each species of Covasphere, when incubated alone, resulted in <1,000 superthreshold particles.

‡ Promotion.

Table II. Effects of Various Molecules on Covasphere to Cell Binding

Soluble molecule	Cytotactin-coated Covaspheres		Fibronectin-coated Covaspheres	
	Bound cpm*	Inhibition	Bound cpm*	Inhibition
		%		%
–	1,990	–	2,510	–
EDTA	510	74	610	76
Anti-cytotactin Fab'	230	88	2,460	2
Anti-proteoglycan Fab'	1,210	39	2,430	3
GRGDS	1,830	8	1,490	41
GRGESP	1,830	8	2,250	10
Cytotactin	1,350	32	1,240	51
CTB Proteoglycan	570	71	1,020	59
Fibronectin	1,910	4	1,640	35

Covasphere to fibroblast binding assays were performed as described in the Materials and Methods. Amounts or concentrations of soluble molecules were: EDTA, 2 mM; anti-cytotactin or anti-proteoglycan Fab' fragments, 150 μg; GRGDS or GRGESP (Peninsula Laboratories, Inc.; Codes 9137 and 9135, respectively), 0.5 mg/ml; cytotactin, CTB proteoglycan, or fibronectin, 10 μg.
* Average of duplicate points. In all cases, SD was <200 cpm. Nonspecific binding, as estimated by incubation of fibroblasts with BSA-coated Covaspheres, was 200 cpm.

the active site involved in fibronectin binding to cells (22, 33, 40).

To evaluate whether there were similarities in the molecular mechanisms of binding of these various extracellular proteins to cells, the binding of cytotactin-coated Covaspheres or of fibronectin-coated Covaspheres to fibroblasts was compared (Table II). Binding of both kinds of beads was inhibited by EDTA, consistent with the interpretation that the binding of cytotactin to cells, like the binding of fibronectin to cells (29), is divalent cation-dependent. Soluble CTB proteoglycan strongly inhibited cytotactin binding (20) and fibronectin binding. Soluble cytotactin also inhibited the binding of either cytotactin-coated Covaspheres or fibronectin-coated Covaspheres to fibroblasts. In contrast, differential effects on binding were obtained with other molecules tested. Anti-cytotactin Fab' fragments and anti-CTB proteoglycan Fab' fragments inhibited cytotactin binding but had no effect on fibronectin binding. Soluble fibronectin and soluble RGD-containing peptide inhibited fibronectin binding but had little effect on cytotactin binding; a control peptide containing Arg-Gly-Glu had little effect on either cytotactin or fibronectin binding.

The observations that both cytotactin and CTB proteoglycan contain chondroitin sulfate chains and carbohydrate epitopes recognized by the HNK-1 antibody led us to evaluate the role of these carbohydrates in the various binding mechanisms. Chondroitin sulfate was enzymatically removed using chondroitin ABC lyase. Cytotactin lacking the HNK-1 epitope (see below) was isolated from fibroblast culture supernatants. When the binding of cytotactin-coated Covaspheres to CTB proteoglycan-coated Covaspheres or to fibronectin-coated Covaspheres was examined (Table III, *A*), neither epitope was found to be directly involved in binding. Similarly, the absence of chondroitin sulfate or HNK-1 epitopes from cytotactin did not affect its ability to bind to fibroblasts (Table III, *B*). To confirm that the adhesive activities of Covaspheres coated with chondroitinase-treated cytotactin or fibroblast cytotactin were due to the cytotactin and not to contaminating molecules, these experiments were repeated in the presence of anti-cytotactin Fab' fragments. In all cases, binding was inhibited >90% (data not shown), indicating that cytotactin was the active ligand on these beads.

Expression of Cytotactin and CTB Proteoglycan Polypeptides during Development

Previous studies have indicated that the contributions of CAMs and substrate adhesion molecules to cell adhesion are dependent both on their absolute local concentrations (19,

Table III. Binding Properties of Cytotactin and CTB Proteoglycan Lacking Certain Oligosaccharides

A. Covasphere-Covasphere aggregation

Cytotactin-coated Covaspheres*	Ligand on Covaspheres*	Superthreshold particles‡
Control cytotactin	Control proteoglycan	18,600
Cytotactin w/o CS	Control proteoglycan	12,900
Cytotactin w/o HNK-1	Control proteoglycan	17,100
Control cytotactin	Proteoglycan w/o CS	26,400
Control cytotactin	Fibronectin	15,300
Cytotactin w/o CS	Fibronectin	11,500
Cytotactin w/o HNK-1	Fibronectin	13,400

B. Covasphere to fibroblast binding

Protein on Covaspheres*	Bound cpm§
Control cytotactin	2,420
Cytotactin w/o CS	2,300
Cytotactin w/o HNK-1	2,280

Binding assays were performed as described in the Materials and Methods and legends of Tables I and II.
* Control cytotactin is cytotactin purified from brain extracts, cytotactin w/o CS is cytotactin purified from brain extracts with chondroitin sulfate removed by chondroitinase treatment after purification, cytotactin w/o HNK-1 is cytotactin purified from fibroblast culture medium and therefore lacking the HNK-1 carbohydrate epitope, control proteoglycan is CTB proteoglycan, and proteoglycan w/o CS is CTB proteoglycan with chondroitin sulfate removed by chondroitinase treatment.
‡ Average of duplicate samples. SD in all cases is <1,500 superthreshold particles.
§ Average of duplicate samples. SD in all cases is <200 cpm.

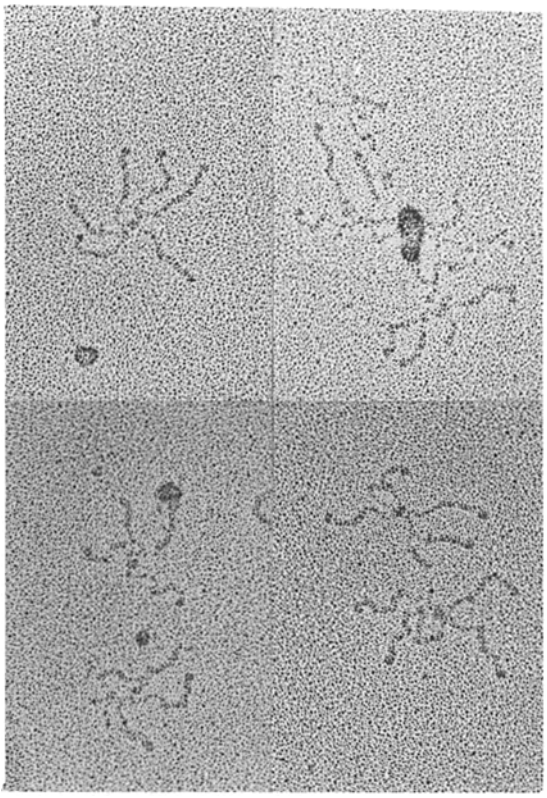

Figure 2. Electron micrograph of selected hexabrachions from purified cytotactin. Samples were sprayed onto mica, vacuum-dried, and rotary-shadowed (14). This photograph was taken by Dr. Harold P. Erickson, Duke University. Bar, 100 nm.

20) and on their concentrations relative to other adhesive molecules present (21). Because CTB proteoglycan binds to cytotactin and thereby inhibits its ability to bind to cells (20), it was particularly important to determine the relative amounts and sites of expression of these molecules during development.

Cytotactin and CTB proteoglycan were routinely prepared in these studies from the soluble supernatant obtained by centrifugation of 14-d-old embryonic chicken brains homogenized in an isotonic detergent-free buffer. To confirm that most of the cytotactin and CTB proteoglycan was present in this fraction and not in the complementary membrane fraction, aliquots representing an equal percentage of each fraction were immunoblotted with anti-cytotactin and anti-CTB proteoglycan antibodies and the binding quantitated. Most of the cytotactin (74%) and almost all the CTB proteoglycan (94%) were found in the supernatant fraction; the rest of the cytotactin and CTB proteoglycan was recovered in the membrane fraction, and their molecular forms were indistinguishable from those found in the supernatant.

To evaluate the levels and molecular forms of cytotactin expressed in the developing brain, PBS extracts from several growth stages ranging from 6-d-old embryo to adult were prepared and immunoblotted with anti-cytotactin antibodies (Fig. 3). Sample loadings were normalized on the basis of tissue volume, and each sample was analyzed both with and without chondroitinase treatment. Cytotactin was present at all stages examined; the highest levels were observed in 6- to 12-d-old embryos and the lowest in hatchlings and adults. Quantitation of these immunoblots indicated that the levels of cytotactin in extracts from 18-d-old brains and adult brains were respectively only 25 and 15% of the level found in extracts from 6-d-old brains. The M_r 220,000 and 200,000 components of cytotactin were present at similar levels in 6- and 9-d-old embryos, but by 18 d, the M_r 220,000 component was predominant. The chondroitinase-sensitive M_r 250,000 component was particularly prominent in extracts from 9-, 12-, and 15-d-old brains.

In previous studies on cytotactin (17) and the J1 antigen (24), polyclonal antibodies that recognized M_r 220,000 polypeptides in embryonic brain extracts were also reported to recognize M_r 180,000 and 160,000 polypeptides in adult brain extracts. A one-dimensional peptide analysis (17) of these M_r 180,000 and 160,000 polypeptides suggested, however, that they were structurally related to each other but not to the M_r 220,000 protein (cytotactin). The highly specific anti-cytotactin antibodies and anti-CTB proteoglycan antibodies used in the present study did not recognize these M_r 180,000 and 160,000 polypeptides even though they were readily detectible in the adult extract using a previously described anti-cytotactin antibody (Fig. 3, lane *15*). Moreover, a polyclonal antibody was prepared against the purified M_r 180,000 polypeptide (Fig. 4, lane *1*), and this antibody did not recognize purified cytotactin (lane *2*) even though it detected the M_r 180,000 and 160,000 polypeptides in embryonic extracts (lane *3*) and at significantly higher levels in hatchling extracts (lane *4*). These results strongly suggest that the adult M_r 180,000 and 160,000 polypeptides and the embryonic M_r 220,000 and 200,000 polypeptides are not structural variants of the same molecule as has been suggested in studies of the so-called J1 antigen (24). On the other hand, the myotendinous antigen (4), or tenascin (5), appears

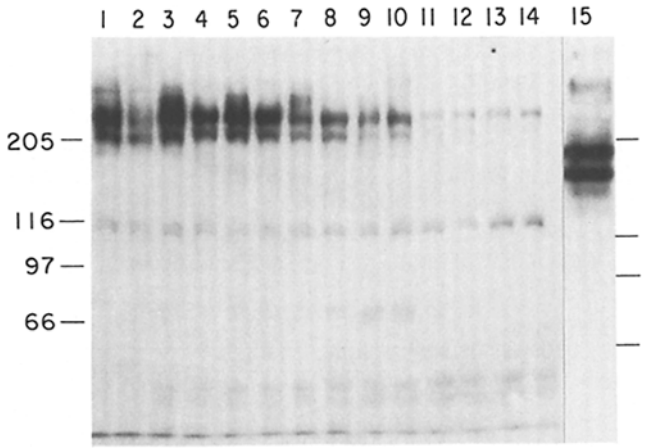

Figure 3. Developmental expression of cytotactin in brain extracts. PBS extracts of 6-, 9-, 12-, 15-, 18-d-old embryonic, hatchling, and adult brain tissue were prepared and chondroitinase treated as described in the Materials and Methods. 25-μl aliquots were resolved on SDS gels containing 6% acrylamide. Lanes *1, 3, 5, 7, 9, 11*, and *13*, untreated extracts from the stages described above; lanes *2, 4, 6, 8, 10, 12*, and *14*, chondroitinase-treated extracts from the stages described above; and lane *15*, adult extract. The detection methods used were immunoblotting with anti-cytotactin antibodies prepared against the highly purified molecule (lanes *1–14*) and immunoblotting with the previously described (17) anti-cytotactin antibodies (lane *15*). The migration and $M_r \times 10^{-3}$ of standard proteins are indicated.

to contain the same polypeptide chain(s) as cytotactin, as evidenced by our observations that both monoclonal and polyclonal antibodies to the myotendinous antigen recognize purified cytotactin (data not shown).

In contrast to the ready detectability of cytotactin, the reactivity with the anti-CTB proteoglycan antibody required prior treatment of extracts with chondroitinase (Fig. 5). Levels of CTB proteoglycan expression in the brain increased ninefold from 6-d-old embryos to 18-d-old embryos and decreased only ~25% between 18-d-old and adult tissue. Although the anti-CTB proteoglycan antibody was prepared against M_r 280,000 material (see Fig. 1, lane *3*), it also reacted with molecules of lower molecular weight in chondroitinase-treated extracts. From 6-d-old embryos to 15-d-old embryos, the M_r 280,000 component was predominant; however, components of M_r 220,000 and 150,000 were also present and became an increasing fraction of the total as development progressed. At later times, as many as eight components ranging from M_r 110,000 to 280,000 were apparent, and the components were similar to those observed upon storage of purified intact CTB proteoglycan. Whether this differential degradation or processing has functional significance remains to be determined.

Cytotactin and proteoglycan are readily extracted from neural tissues in nondissociative buffers (see above). In previous studies (8), however, it was found that harsher treatments were required to extract cytotactin from non-neural tissues. To evaluate the conditions under which CTB proteoglycan is extracted from non-neural tissues, anti-proteoglycan antibodies were used to immunoblot sequential extracts of embryonic chicken gizzards, hearts, and fibroblasts prepared using PBS, an NP-40-containing buffer, and 8 M guanidine-HCl. Unlike cytotactin, in all cases proteoglycan was found primarily in the PBS extract and required prior chondroitinase treatment for detection (data not shown).

Histological Distribution of Cytotactin and CTB Proteoglycan in Neural Tissue

The microscopic distribution of cytotactin and CTB proteoglycan in the brain was examined by immunohistochemistry using the cerebellum as an example (Fig. 6). In 15-d-old embryos, cytotactin was seen in the intermediate layer and on developing Bergmann glial fibers as previously described (7, 8, 17). At this stage, staining with anti-CTB proteoglycan antibodies was very weak in the absence of chondroitinase treatment. After chondroitinase treatment, however, the distribution of CTB proteoglycan was quite similar to that of cytotactin, consistent with the possibility of a role for this neuronal molecule in the interactions of neurons migrating on Bergmann glia (7). Both molecules remained in the molecular layer of the adult cerebellum.

Previous immunofluorescent staining experiments using neurons and glia in culture indicated that, in the central nervous system, cytotactin is specifically synthesized by glial cells and is found both in an extracellular fibrillar pattern and a punctate, perinuclear pattern (17). To evaluate the site of synthesis of CTB proteoglycan, cells from 9-d-old embryonic chicken brains were cultured and immunofluorescently labeled with anti-CTB proteoglycan antibodies. In double-label experiments (Fig. 7), cells with neuronal morphology were found to be labeled by both polyclonal anti-CTB proteoglycan antibodies and monoclonal anti-Ng-CAM antibodies, which are specific for neurons in the central nervous system (10, 18, 35). Proteoglycan staining occurred in two distinct patterns, either in a punctate, perinuclear pattern (*B*) or associated with the cell surface of cell bodies and neurites (*E*). Chondroitinase pretreatment was required for optimal levels of anti-CTB proteoglycan staining, and permeabilization was required to observe the perinuclear staining. No staining of flat cells (presumably glia) was detected with the

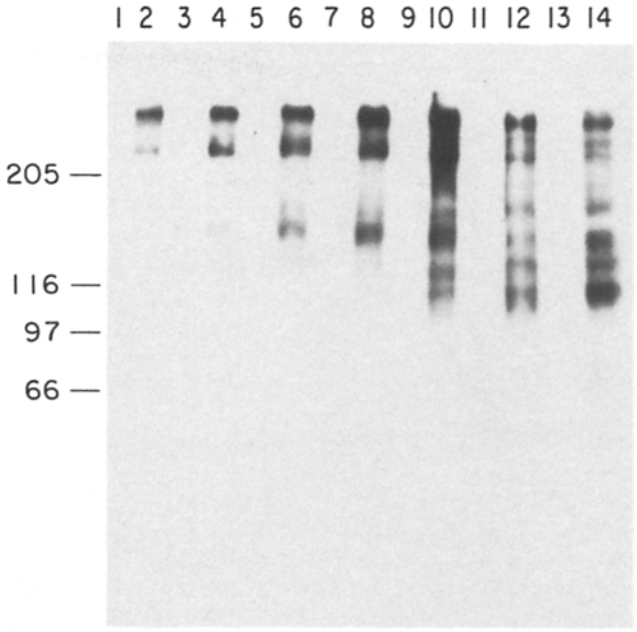

Figure 5. Developmental expression of CTB proteoglycan in brain extracts. PBS extracts of 6-, 9-, 12-, 15-, 18-d-old embryonic, hatchling and adult brain tissue were prepared and chondroitinase-treated as described in the Materials and Methods. 25-μl aliquots were resolved on SDS gels containing 6% acrylamide. Lanes *1*, *3*, *5*, *7*, *9*, *11*, and *13*, untreated extracts from the stages described above; and lanes *2*, *4*, *6*, *8*, *10*, *12*, and *14*, chondroitinase-treated extracts from the stages described above. The detection method used was immunoblotting with anti-CTB proteoglycan antibodies. The migration and $M_r \times 10^{-3}$ of standard proteins are indicated.

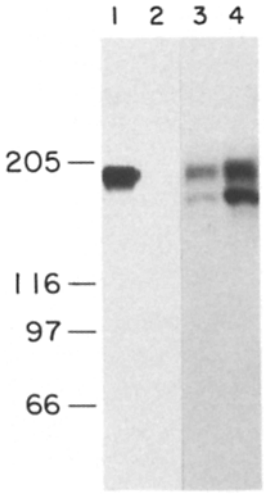

Figure 4. Characterization of anti-M_r 180,000 polypeptide antibodies. The M_r 180,000 polypeptide, cytotactin, and PBS extracts of brain tissue were prepared as described in the Materials and Methods and resolved on SDS gels containing 6% acrylamide. Lane *1*, 2 μg M_r 180,000 polypeptide; lane *2*, 2 μg cytotactin; lane *3*, 25 μl 18-d-old embryonic brain extract; and lane *4*, 25 μl hatchling brain extract. The detection method used was immunoblotting with anti-M_r 180,000 polypeptide antibodies. The migration and $M_r \times 10^{-3}$ of standard proteins are indicated.

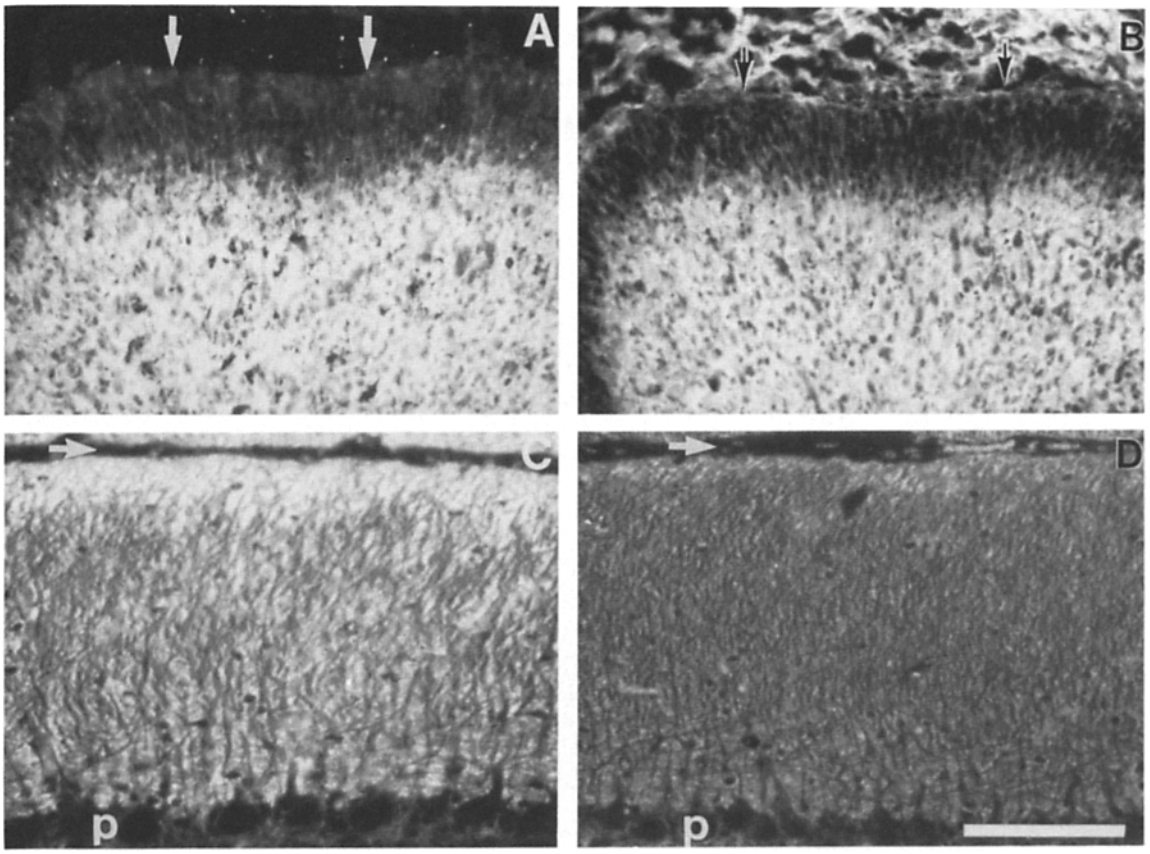

Figure 6. Distribution of cytotactin and CTB-proteoglycan in embryonic and adult cerebellum. In 15-d-old embryos, cytotactin staining is prominent in the intermediate layer and along the Bergmann glia (*A*). CTB-proteoglycan shows a similar distribution in chondroitinase-treated sections and, in addition, is found in the meninges (*B*). In adult cerebellum, both cytotactin (*C*) and CTB-proteoglycan (*D*) are strongly expressed along the fibers of the molecular layer. Arrows in *A–D* point to the pial surface; *p* denotes Purkinje cell layer. Bar, 100 μm.

anti-CTB proteoglycan or anti-Ng-CAM antibodies. Thus, although cytotactin and CTB proteoglycan were co-localized in tissue sections (Fig. 6), the data are consistent with the previous conclusion that they are specifically synthesized by different cell types: CTB proteoglycan by neurons (20) and cytotactin by glia (17).

Localization of Cytotactin and CTB Proteoglycan in Particular Non-neural Tissues

To evaluate the extent of colocalization of cytotactin and CTB proteoglycan in non-neural tissues, sections of embryonic chicken gizzard (Fig. 8), heart (Fig. 9), and cartilage (Fig. 10) were immunofluorescently labeled with anti-cytotactin and anti-CTB proteoglycan antibodies. In the gizzard (Fig. 8), cytotactin was present at high levels in the smooth muscle (8) and at low levels in the myenteric ganglia. Similarly, anti-CTB proteoglycan reactivity was observed after chondroitinase treatment in both the smooth muscle and the ganglia. No staining for CTB proteoglycan was observed in these areas in the absence of chondroitinase treatment (not shown). Both anti-cytotactin and anti-CTB proteoglycan also stained vascular smooth muscle (Fig. 9, *A* and *B*). In contrast, anti-proteoglycan staining was strong in cardiac muscle (Fig. 9 *D*) in which cytotactin staining was weak or absent (Fig. 9 *C*).

An interesting pattern of expression of cytotactin and CTB proteoglycan was observed in cartilage. We previously found strong cytotactin staining in immature chondrocytes (8) and in the perichondrium of mature cartilage. As chondrocytes differentiated, the most mature central cells lost cytotactin while the most peripheral newly formed chondroblasts continued to express the molecule, predominantly in a pericellular pattern (Fig. 10 *A*). In chondroitinase-treated sections, however, while cytotactin staining was still limited to the area of immature cells, the staining appeared to be more extensive, surrounding cells and completely filling the extracellular spaces (Fig. 10 *B*). When anti-CTB proteoglycan staining was examined, a few relatively peripheral cells were found to be stained in the absence of chondroitinase treatment (Fig. 10 *C*). After such treatment, however, all cells, mature and immature, stained for proteoglycan and the extracellular space appeared to be filled with positive-staining material (Fig. 10 *D*). Clearly, the presence of chondroitin sulfate chains can inhibit antibody interactions with these molecules as present in tissue sections.

Differential Glycosylation of Molecules Isolated from Neural Versus Non-neural Sources

Cytotactin and CTB proteoglycan isolated from embryonic chicken brain were previously shown to contain carbohydrate epitopes recognized by the HNK-1 monoclonal antibody (17, 20). Other molecules bearing this epitope include

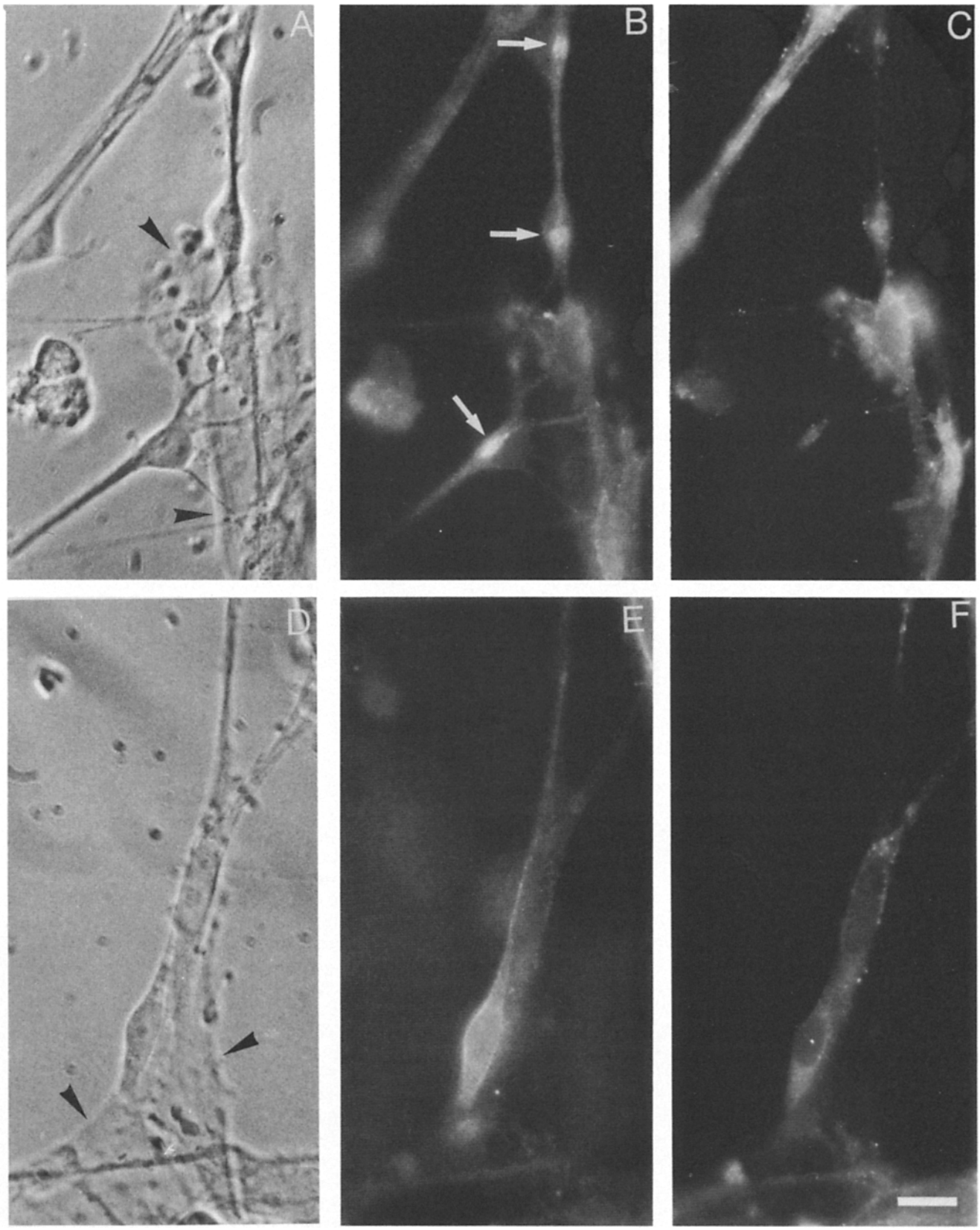

Figure 7. Immunofluorescent localization of CTB proteoglycan and Ng-CAM. Brain cells from 9-d-old embryonic chickens were cultured and double-labeled using polyclonal anti-CTB proteoglycan antibodies and monoclonal anti-Ng-CAM antibodies and the appropriate second antibodies as described in the Materials and Methods. *A–C* and *D–F* each represent a single field. Note that CTB proteoglycan is present either in an intracellular perinuclear pattern (*B, arrows*) or associated with the cell surface of cell bodies and neurites (*E*) in the same cells that express Ng-CAM (*C* and *F*), a neuronal marker. By comparison with phase micrographs of the same fields (*A* and *D*), it is clear that large, flat glial cells (*arrowheads*) do not express either CTB proteoglycan or Ng-CAM. Bar, 10 μm.

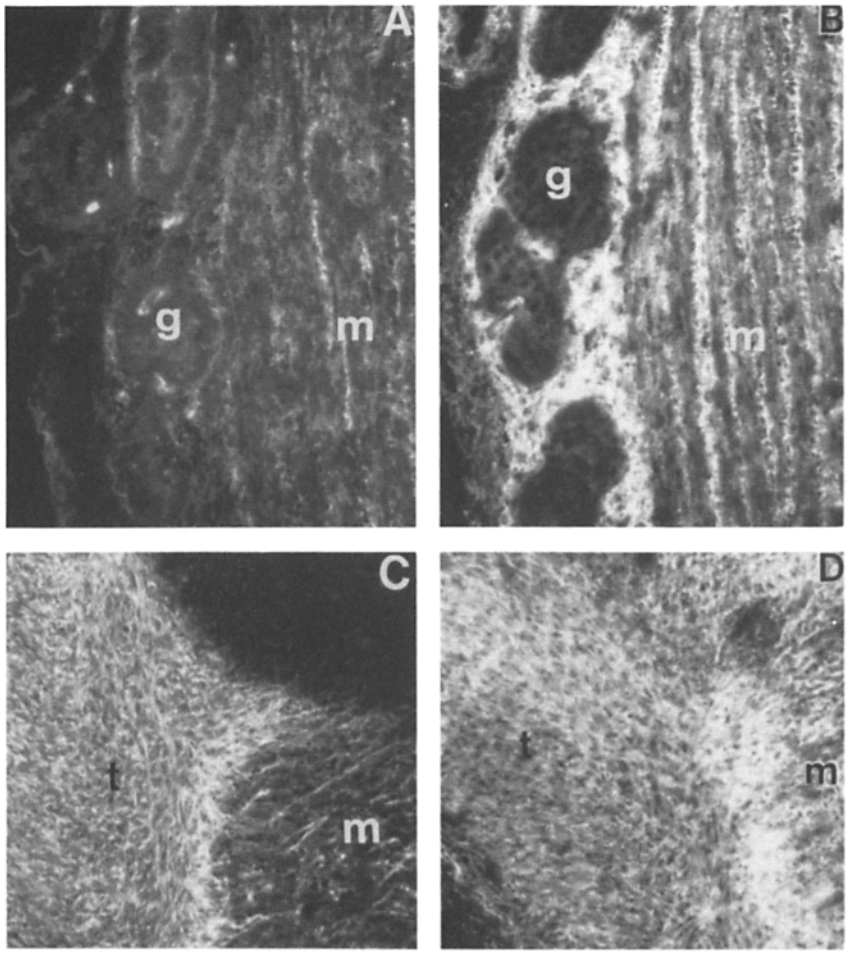

Figure 8. Localization of cytotactin and CTB proteoglycan in 11-d-old embryonic gizzard. Both cytotactin (*A*, *C*) and proteoglycan (*B*, *D*) are found in the enteric ganglia (*g*) and at high levels in the smooth muscle (*m*) (*A*, *B*). In addition, both molecules are prominent components of the large tendinous structures (*t*) bounding the gizzard muscle (*C*, *D*). Bar, 100 μm.

N-CAM (18), Ng-CAM (18), myelin-associated glycoprotein (27), and a glycolipid (6). It is striking that N-CAM from non-neural tissues does not express the HNK-1 epitope (30). Moreover, a number of tissues that express N-CAM, cytotactin, or CTB proteoglycan, for example, heart, smooth muscle, cartilage, and cultured fibroblasts, do not express the HNK-1 epitope as detected histologically (34, 35; unpublished observations). To evaluate biochemically whether cytotactin and CTB proteoglycan from one of these sources express the HNK-1 epitope, cytotactin and CTB proteoglycan purified from brain or from the culture supernatant of chick embryo fibroblasts were immunoblotted with HNK-1, and these immunoblots were compared to immunoblots of the same samples performed with rabbit antibodies to cytotactin and CTB proteoglycan. For both molecules, material from the brain was strongly recognized by both the specific rabbit antibody and HNK-1 (Fig. 11, compare lane *1* with lane *5* and lane *3* with lane *7*). In contrast, material from fibroblasts was not recognized by HNK-1 although it was readily detected with the rabbit antibodies (compare lane *6* with lane *2* and lane *8* with lane *4*). The results indicate that, in certain tissues, cytotactin and proteoglycan are expressed without the HNK-1 carbohydrate epitope. Nevertheless, the absence of the HNK-1 epitope from cytotactin does not alter its three-dimensional structure (compare Fig. 2 with references 13 and 38) or its binding functions (Table III).

Discussion

Here, we have analyzed various aspects of the structure, binding functions, expression, and distribution of cytotactin and CTB proteoglycan. Several of the results obtained appear directly relevant to understanding how these molecules may be involved in the differential control of cell behavior as parts of a network of interactive extracellular matrix components whose abilities to bind to cells are modulated by their intermolecular interactions. For example, both cytotactin and CTB proteoglycan inhibited the binding of either cytotactin or fibronectin to cells. The observed alterations in the relative expression of cytotactin and CTB proteoglycan during brain development may, through these means, have functional effects on neuron–glia adhesion (17) and cerebellar cell migration on Bergmann glia, a process shown to be perturbed by antibodies to cytotactin (7).

These observations make clear the need for careful characterization of these molecules and their properties. In previous studies, antibodies to cytotactin (18) and antibodies to J1 antigen (24) were reported to recognize M_r 220,000 and 200,000 polypeptides in embryonic brain extracts and M_r 180,000 and 160,000 polypeptides in adult brain extracts, all of which contained the carbohydrate epitope recognized by monoclonal antibody HNK-1. Although these "embryonic" and "adult" polypeptides have distinct one-dimensional pep-

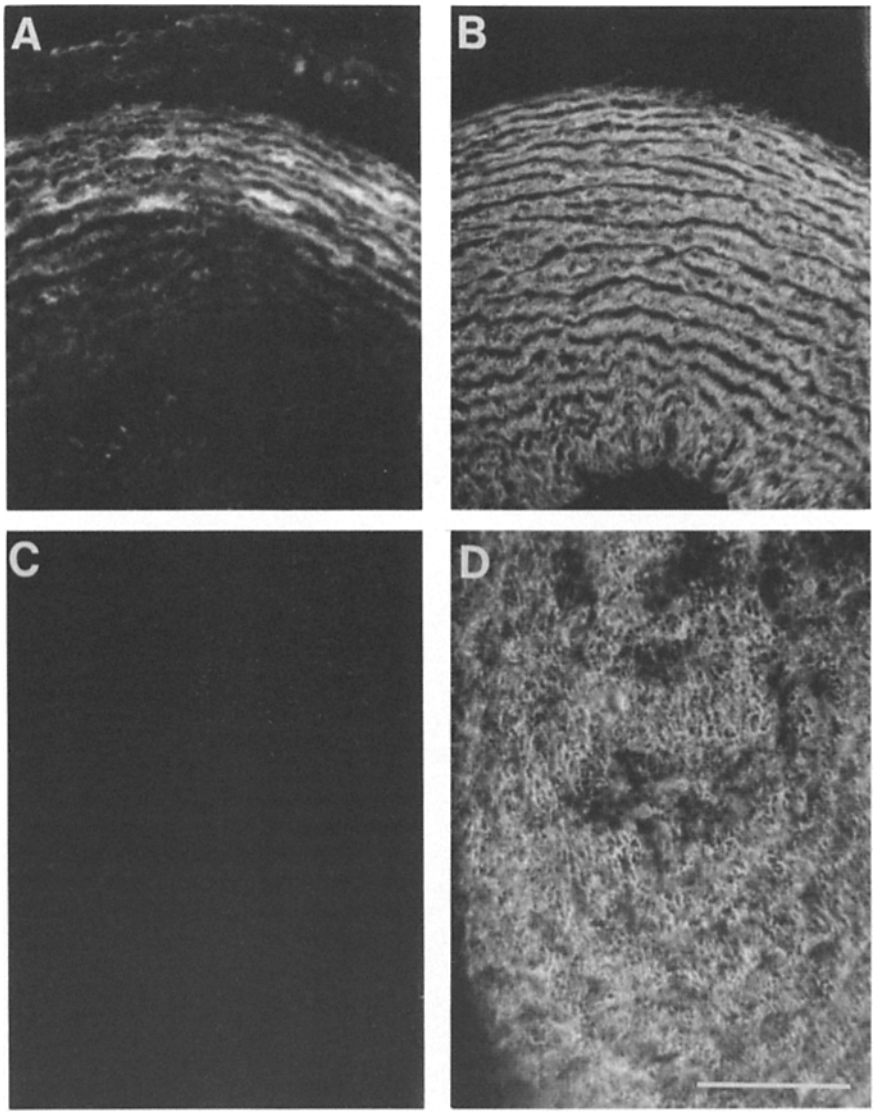

Figure 9. Cytotactin and CTB proteoglycan expression in 14-d-old vascular smooth muscle and cardiac muscle. Cytotactin staining (*A*) is found in moderate amounts in vascular smooth muscle of the great vessels of the heart and is most intense between the outermost layers of smooth muscle fibers. CTB-proteoglycan (*B*) is present throughout the layers of muscle, although in a concentric, apparently cell-associated, pattern. In contrast, no cytotactin can be seen in cardiac muscle (*C*), whereas this tissue expresses large amounts of CTB proteoglycan (*D*). Bar, 100 μm.

tide maps (18), it was suggested that, similar to N-CAM (31), the J1 antigen had structurally related embryonic and adult forms (24). The present results indicate, however, that the M_r 180,000 and 160,000 polypeptides and the M_r 220,000 and 200,000 polypeptides are unrelated. Antibodies to highly purified cytotactin do not recognize the M_r 180,000 and 160,000 polypeptides, and antibodies to the M_r 180,000 polypeptide do not recognize cytotactin. Furthermore, the M_r 180,000 and 160,000 polypeptides are present in embryonic brain extracts and are not strictly adult proteins although they do continue to increase in concentration during later development. It remains to be determined whether the M_r 180,000 and 160,000 molecules are involved in neuron–glia adhesion or cerebellar cell migration. It is clear, however, that cytotactin is involved in both processes because monoclonal antibodies specific for cytotactin inhibit neuron–glia adhesion (17) and cerebellar cell migration (7). It will also be of interest to examine whether these molecules interact with cytotactin, providing a basis for their copurification in earlier studies.

Two molecules described by other investigators, hexabrachion (13) and myotendinous antigen (4), also known as tenascin (5), appear to be closely related or identical to cytotactin. All three molecules migrate as M_r 220,000 and 200,000 polypeptides on SDS gels, and we found that monoclonal and polyclonal anti–myotendinous antigen antibodies recognize purified cytotactin. Hexabrachion and tenascin as currently purified probably represent subsets of cytotactin because they are obtained from non-neural sources and therefore lack the HNK-1 carbohydrate epitope. Nevertheless, hexabrachion (13), myotendinous antigen (38), and brain cytotactin all appear as similar six-armed figures (see Fig. 2) when analyzed by electron microscopy, suggesting that the presence of the HNK-1 carbohydrate epitope on cytotactin does not alter its three-dimensional structure.

A wide range of experiments was performed to characterize further the binding of cytotactin to CTB proteoglycan, fibronectin, and cells, all of which were found to be divalent cation-dependent. In contrast to fibronectin binding, RGD-containing peptides did not inhibit the binding of cytotactin-coated beads to cells. Nevertheless, RGD-containing peptides do inhibit the binding of cells to cytotactin-coated

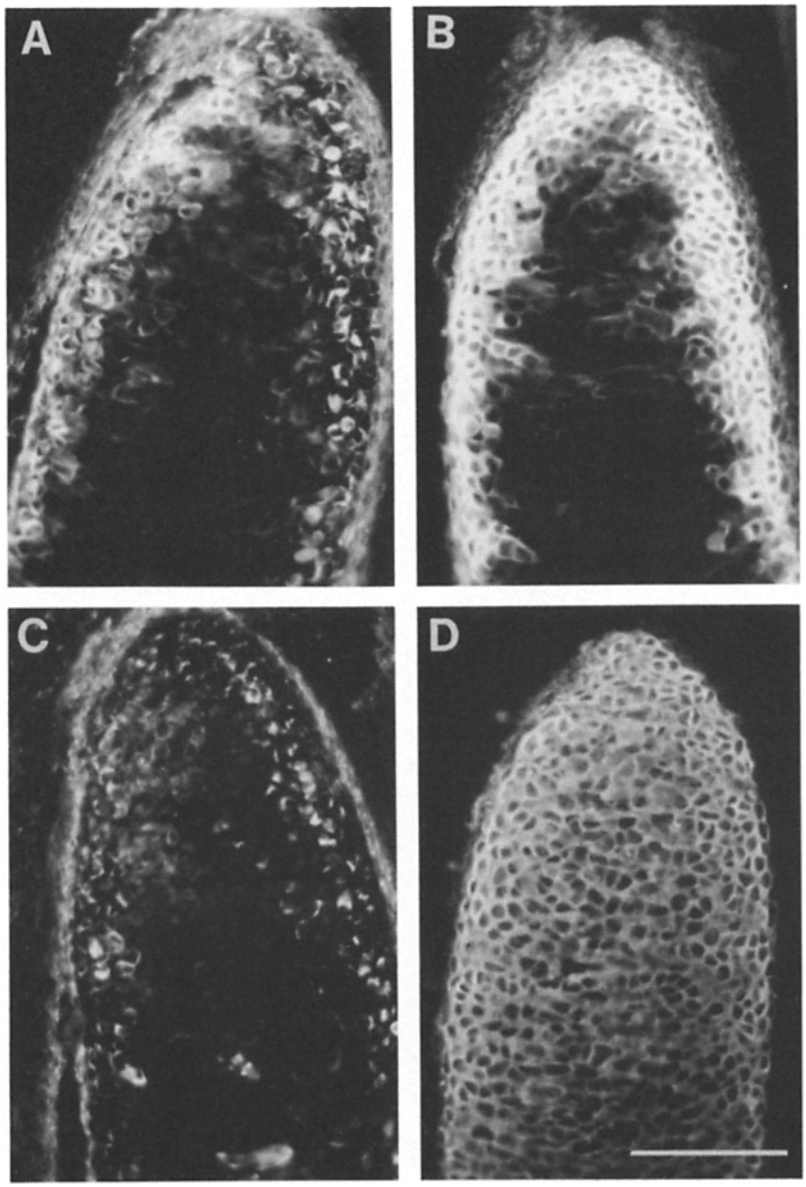

Figure 10. Distribution of cytotactin and CTB-proteoglycan in developing cartilage. Sections of 14-d-old embryo sternal cartilage were processed for immunohistology either without (*A*, *C*) or with (*B*, *D*) chondroitin ABC lyase treatment and stained with antibodies to either cytotactin (*A*, *B*) or CTB proteoglycan (*C*, *D*). Cytotactin is present only in immature chondrocytes near the periphery of the tissue (*A*), where proteoglycan can be observed even without chondroitinase treatment (*C*). Note that the staining for both molecules increases upon chondroitinase treatment; cytotactin staining is more extensive in extracellular spaces of immature chondrocytes (*B*), whereas CTB proteoglycan is found surrounding all the cells and filling their extracellular spaces (*D*). Bar, 100 μm.

culture dishes (Friedlander, D. R., S. Hoffman, and G. M. Edelman, manuscript in preparation), and analysis of cytotactin cDNA indicates the molecule contains an RGD sequence (22a). These results are consistent with the possibility that cytotactin binds to cells via both an RGD sequence and a distinct sequence that remains functional in the presence of RGD-containing peptides in the Covasphere assay.

Both cytotactin and proteoglycan appear to exist in multiple polypeptide forms. Although the particular functions of these different forms remain to be established, preliminary results suggest that multiple mRNAs exist for cytotactin and are differentially distributed in neural and non-neural tissues (22a). Thus, it is possible that the various cytotactin polypeptides within neural and non-neural tissues may include different functional domains, as have been demonstrated for other extracellular matrix proteins (36, 39).

In this study, forms of cytotactin and CTB proteoglycan were found that possessed or lacked the carbohydrate epitope recognized by monoclonal antibody HNK-1. Neither the HNK-1 carbohydrate nor the chondroitin sulfate found on forms of both molecules appears to be directly involved in their binding functions, however. Contrary to the suggestion that the HNK-1 epitope may act as a ligand in cell–cell binding (23), cytotactin lacking this carbohydrate isolated from fibroblasts bound as well to CTB proteoglycan, fibronectin, and fibroblasts, as did the cytotactin bearing this carbohydrate that was isolated from brain. Similarly, chondroitin sulfate does not appear to be a ligand in the binding interactions studied here; specific enzymatic removal of this glycosaminoglycan had little effect on the binding properties of cytotactin and proteoglycan. In fact, chondroitinase treatment of CTB proteoglycan enhanced its ability to bind to cytotactin; this may reflect an exposure of binding sites or an enhanced coupling of CTB proteoglycan to carrier beads in the absence of chondroitin sulfate chains.

Even though cytotactin and CTB proteoglycan constitute

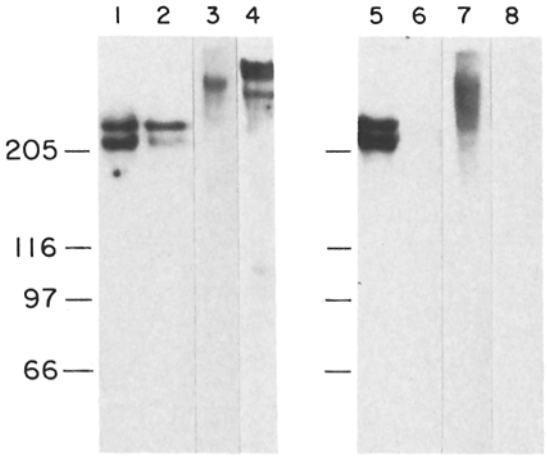

Figure 11. Immunological comparison of cytotactin and CTB proteoglycan purified from brain and fibroblasts. Cytotactin and chondroitinase-treated CTB proteoglycan were purified from brain extracts or fibroblast culture medium as described in the Materials and Methods and resolved on SDS gels containing 6% acrylamide. Lanes *1* and *5*, 2 μg cytotactin from brain; lanes *2* and *6*, cytotactin purified from 1 ml fibroblast culture medium; lanes *3* and *7*, 2 μg chondroitinase-treated CTB proteoglycan from brain; and lanes *4* and *8*, chondroitinase-treated CTB proteoglycan purified from 1 ml fibroblast culture medium. The detection methods used were immunoblotting with anti-cytotactin antibodies (lanes *1* and *2*), anti-CTB proteoglycan antibodies (lanes *3* and *4*), and monoclonal antibody HNK-1 (lanes *5–8*). The migration and $M_r \times 10^{-3}$ of standard proteins are indicated.

a binding couple, immunofluorescence localization experiments indicated that, in certain tissues, the distribution of cytotactin and its ligand, CTB proteoglycan, are similar, whereas in other tissues they are quite different. Similar distributions for the two molecules were observed in the cerebellum, although cell culture experiments indicate that cytotactin is specifically synthesized by and expressed on glia (18), whereas CTB proteoglycan is specifically synthesized by and expressed on neurons. Other cells, for example, fibroblasts, were able to synthesize both molecules. Both molecules were colocalized in gizzard and vascular smooth muscle. In contrast, whereas CTB proteoglycan was prominent in cardiac muscle and throughout developing cartilage, little or no cytotactin was found in cardiac muscle and, in cartilage, it was restricted to immature chondrocytes. These results appear to reflect differential site-related synthesis of these proteins and they raise the possibility that additional ligands for CTB proteoglycan exist in regions where the proteoglycan is present and cytotactin is absent. A more detailed study of the localization of these molecules in the histogenesis of other organs is likely to reveal correlations with patterns of morphogenesis, as already seen for neural crest development (34).

In the current study, we have identified a variety of parameters that may affect the function of the cytotactin–CTB proteoglycan binding couple including developmental alterations in their molecular forms and distributions. In addition, we have further characterized their molecular binding mechanisms. The data raise new questions about the functional role of the M_r 160,000 and 180,000 proteins and suggest experiments to explore the site-restricted regulation of cytotactin and CTB proteoglycan in development. The combined results on structure, binding function, and distribution of this binding couple, together with the results of earlier studies (20), are in accord with the hypothesis that the modulation of an interactive network of extracellular proteins may be an important factor in the control of cellular patterning in morphogenesis.

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