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# Functional Mapping of Cytotactin: Proteolytic Fragments Active in Cell-Substrate Adhesion

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*Abstract.* Cytotactin is an extracellular matrix glycoprotein with a restricted distribution during development. In electron microscopic images, it appears as a hexabrachion with six arms extending from a central core. Cytotactin binds to other extracellular matrix proteins including a chondroitin sulfate proteoglycan (CTB proteoglycan) and fibronectin. Although cytotactin binds to a variety of cells including fibroblasts and neurons, in some cases it causes cells in culture to round up and it inhibits their migration.

To relate these various effects of cytotactin on cell behavior to its binding regions, we have examined its ability to support cell-substrate adhesion and have mapped its cell-binding function onto its structure. In a cell-substrate adhesion assay, fibroblasts bound to cytotactin but remained round. In contrast, they both attached and spread on fibronectin. Neither neurons nor glia bound to cytotactin in this assay. In an assay in which cell-substrate contact was initiated by centrifugation, however, neurons and glia bound well to cytotactin; this binding was blocked by specific anti-cytotactin antibodies. The results suggest that neurons and glia can bind to cytotactin-coated substrates and that these cells, like fibroblasts, possess cell surface ligands for cytotactin.

After applying methods of limited proteolysis and fractionation, these assays were used to map the binding functions of cytotactin onto its structure. Fragments produced by limited proteolysis were fractionated into two major pools: one (fraction I) contained disulfide-linked oligomers of a 100-kD fragment and two minor related fragments, and the second (fraction II) contained monomeric 90- and 65-kD fragments. The 90- and 65-kD fragments in fraction II were closely related to each other and were structurally and immunologically distinct from the fragments in fraction I. Only components in fraction I were recognized by mAb M1, which binds to an epitope located in the proximal portion of the arms of the hexabrachion and by a polyclonal antibody prepared against a 75-kD CNBr fragment of intact cytotactin. A mAb (1D8) and a polyclonal antibody prepared against a 35-kD CNBr fragment of cytotactin only recognized components present in fraction II. In cell-binding experiments, fibroblasts, neurons, and glia each adhered to substrates coated with fraction II, but did not adhere to substrates coated with fraction I. Fab' fragments of the antibody to the 35-kD CNBr fragment strongly inhibited the binding of cells to cytotactin, supporting the conclusion that fraction II contains a cell-binding region. In addition, Fab' fragments of this antibody inhibited the binding of cytotactin to CTB proteoglycan and to fibronectin. The binding of fibroblasts to components in fraction II and to intact cytotactin was also inhibited by peptides containing the sequence RGD. These combined results suggest that a cell-binding site containing the sequence RGD is present in the distal portion of the arms of the cytotactin hexabrachion near binding sites for CTB proteoglycan and fibronectin and that the disulfide-bonded portion of the hexabrachion does not contain these sites.



CELL adhesion plays a major role among the developmental processes leading to pattern formation. Molecules involved in cell adhesion include cell-cell adhesion molecules (12) and cell-substrate adhesion molecules (SAMs; 44).[1] The protein cytotactin is a SAM with a characteristic and striking distribution during development (9, 22). In the chick embryo, it appears first during gastrulation, is later expressed in basement membranes of the developing neural tube (9), and is seen in neural crest cell pathways, including the rostral half of sclerotomal mesenchyme of each somite (40) where neural crest cells accumulate. At later times of development, cytotactin is present at high levels in the central nervous system and in a number of nonneuronal tissues, especially around smooth muscle and along basement membranes of lung and kidney (9).

1. *Abbreviations used in this paper*: CTB proteoglycan, cytotactin-binding proteoglycan; SAM, cell-substrate adhesion molecule.

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Electron microscopic analyses of the cytotactin molecule (24) have revealed a six-armed structure with a central core, designated a hexabrachion (16). Biochemical analyses suggest that cytotactin is a disulfide-linked oligomer (22). Although the polypeptide subunits of cytotactin that are expressed during development in different organs vary in molecular mass, all of these polypeptides appear to be the products of a single gene (26). In cytotactin preparations from 14-d embryonic chicken brains similar to those used in the present studies, the predominant subunit had a molecular mass of 220 kD (22, 24). Molecules known as brachionectin (16, 17), and tenascin (or myotendinous antigen [4, 5, 42]) have subunit sizes and electron microscopic images that are similar to those of cytotactin, and they are probably closely related or identical proteins.

A variety of experiments in vitro have suggested that cytotactin is involved in cell adhesion, regulation of cell migration, and pattern formation. Antibodies to cytotactin inhibit the binding of dissociated neurons to glial monolayers (22) and they interfere with the migration of external granule cells out of the molecular layer in explants of cerebellar cortex (6). Cytotactin both binds fibronectin and has an inhibitory effect on the migration of neural crest cells on fibronectin in vitro (40); it may have an analogous effect on these cells in vivo as they invade the rostral half of somites (40).

The ability of cytotactin to bind to various cells and to other extracellular matrix proteins has been analyzed in detail in assays in which the molecule was coupled to microscopic fluorescent beads (23, 24). Such cytotactin-coated beads bind to neurons and fibroblasts and to other beads that were coated either with fibronectin or with the so-called cytotactin-binding proteoglycan (CTB proteoglycan; 23). When soluble CTB proteoglycan is incubated with cytotactin-coated beads before mixing them with cells, the binding of the beads to the cells is strongly inhibited suggesting that an interaction between CTB proteoglycan and cytotactin blocks cytotactin binding to cells (23, 24). Soluble cytotactin itself blocks the binding of fibronectin-coated beads to cells in a similar manner (23). It has also been reported that tenascin inhibits the binding of cells to fibronectin (31). These various molecules appear to be members of a network of extracellular matrix proteins whose abilities to bind to cells may be altered by their mutual binding interactions. Other probable members of this SAM network include the various matrix proteoglycans which have been shown to inhibit cell attachment to fibronectin (36, 38). To understand this complex of interactions, it is important to study each individual protein in terms of its structure–function relationships.

In the present study, we have examined further the ability of various cell types to attach to cytotactin-coated substrates and have compared these cell-binding properties of the intact molecule with those possessed by its proteolytic fragments. Although cells attached to cytotactin-coated substrates, this interaction did not promote cell spreading, in marked contrast to interactions with fibronectin for which cell binding is accompanied by spreading. To map the binding functions of cytotactin onto its structure, we prepared and characterized fragments of cytotactin produced by limited proteolytic cleavage. Cells were found to bind to a domain in the arms of the cytotactin hexabrachion that does not include the region in which the polypeptide chains are linked by disulfide bonds. On the basis of these and other studies, we present a model in which the known functional and structural properties of cytotactin are localized in particular regions of the molecule.

## Materials and Methods

### Reagents

Cytotactin and CTB proteoglycan were purified from 14-d embryonic chicken brains (24). Other reagents included human plasma fibronectin (New York Blood Center, New York, NY), BSA (ICN Biomedical, Inc., Lisle, IL), deoxyribonuclease I, trypsin (2× crystallized), and alpha-chymotrypsin (3× crystallized) (Cooper Biomedical, Malvern, PA), *Staphylococcus aureus* V8 protease (Miles Scientific, Naperville, IL), proteinase K (Beckman Instruments, Inc., Palo Alto, CA), CNBr (Aldrich Chemical Co., Milwaukee, WI), laminin and tissue culture media (Gibco Laboratories, Grand Island, NY), synthetic peptides (Peninsula Laboratories, Belmont, CA), Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ), phenylmethylsulfonylfluoride (PMSF) (Eastman Kodak Co., Rochester, NY), guanidine–HCl ultra pure (Schwartz/Mann Biotech, Cleveland, OH), $Na^{125}I$ (New England Nuclear, Boston, MA), and Covaspheres (Duke Scientific Corp., Palo Alto, CA). Purified chondroitin 6-sulfate was provided by Drs. M. B. Mathews and J. A. Cifonelli (University of Chicago, Chicago, IL) under National Institutes of Health contract AM-5-2205.

### Cells

Fibroblasts (24), neurons (1), and glia (21) were prepared from 10-d embryonic chickens, as previously described, except that cells were dissociated with 20 μg/ml of trypsin in the presence of 1 mM $CaCl_2$.

### Gravity Cell Attachment Assay

To prepare protein-coated substrates, eight drops (2 μl each in PBS) containing different proteins or different concentrations of protein were placed in a circular array near the center of a polystyrene dish in a humid atmosphere to prevent drying (Falcon 1008, Becton Dickinson Labware, Oxnard, CA). After 30 min, the dish was washed three times with PBS containing 10 mg/ml BSA and the last wash was kept in the dish until cells were added (1 h). 1–2 × $10^5$ cells in 250 μl of medium (1 mg/ml BSA, 50 μg/ml DNase I, DME) were placed in the middle region of dishes containing the adsorbed protein dots for 60 min at 37°C in a 10% $CO_2$ incubator. The dishes were washed four times with PBS, and the bound cells were fixed with 1% glutaraldehyde, observed by phase microscopy, and counted using a 20× objective and an eyepiece reticle. Cells were counted in four predetermined fields that combined represented 10% of the dot area. To quantitate cell spreading, attached cells were examined using an inverted microscope and the number of phase-dark, polygonal, flattened cells determined visually (20). Examples of cells that have attached and spread are shown in Fig. 2 *B*, while cells that have attached but not spread are shown in Fig. 2 *A*.

### Centrifugation Cell Attachment Assay

96-well polystyrene (Falcon 3910) or polyvinyl chloride microtiter plates (Falcon 3911) with U-shaped wells were incubated with 40 μl/well of protein in PBS and the wells were washed and blocked with BSA as described above for the substrates used in the gravity assay. 200 μl of a cell suspension containing 1–5 × $10^4$ cells was placed in each well and the plate was centrifuged at 250 *g* for 1 min. The pattern of cells in each well was observed using dark field microscopy and interpreted in terms of a balance between centrifugal force and cell–substrate adhesion; on a nonadhesive substrate, centrifugal force predominates and cells are driven into a pellet at the bottom of the well. As the strength of cell–substrate adhesion increases, cells become more likely to bind to the substrate as they contact it. Therefore, the area covered by cells after centrifugation increases as the size of the central cell pellet decreases (see Figs. 3 and 6).

### Quantitation of Proteins on Substrates

To estimate the amount of protein that was associated with the substrate in cell-attachment assays, radioiodinated molecules were incubated on polystyrene as described above for the gravity assay and the centrifugation assay. In both assays, similar levels of adsorption were obtained. The concentrations of the various solutions used to coat substrates and the resulting levels

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of adsorbed protein were as follows: intact cytotactin 2 μg/ml, 0.84 ng/mm²; 3.7 μg/ml, 1.5 ng/mm²; 6 μg/ml, 2.5 ng/mm²; 11 μg/ml, 4.6 ng/mm²; 20 μg/ml, 8.4 ng/mm²; 33 μg/ml, 14 ng/mm²; 100 μg/ml, 34 ng/mm²; 300 μg/ml, 64 ng/mm²; fraction I 10 μg/ml, 1 ng/mm²; fraction II 5 μg/ml, 0.5 ng/mm²; fibronectin 3.7 μg/ml, 1.6 ng/mm²; 11 μg/ml, 4.8 ng/mm²; 20 μg/ml, 7.6 ng/mm²; 33 μg/ml, 13 ng/mm²; 100 μg/ml, 23 ng/mm²; 300 μg/ml, 45 ng/mm², and laminin 20 μg/ml, 4.1 ng/mm²; 100 μg/ml, 16 ng/mm². When labeled proteins were incubated with the substrate in the presence of 10 mg/ml of BSA, adsorption to the substrate was inhibited >90%. To confirm that substrate-associated counts in these experiments represented labeled protein and not free iodine, the adsorbed material was quantitatively eluted with SDS, resolved on SDS gels (28), and found to contain polypeptides with the same specific radioactivity (~$1 \times 10^8$ cpm/mg) as the starting material.

ELISAs using the various anti-cytotactin antibodies described below were also performed on these radiolabeled samples of cytotactin and fragments of cytotactin that had been adsorbed to substrates. The combined data from the quantitative adsorption experiments described above and these ELISAs allowed the construction of a standardization curve that could be used to convert the results of ELISAs into protein concentrations on the substrate. This curve was used to estimate the levels of adsorbed cytotactin and fragments of cytotactin in the experiment shown in Fig. 7.

### Preparation of Cytotactin Fragments

To prepare chymotryptic fragments, 500 μg of cytotactin purified from 5,000 14-d chicken embryo brains (24) was dissolved in 2 ml PBS/2 M urea and was incubated with 5 μg α-chymotrypsin for 3 h at 37°C. Proteolysis was stopped with 1 mM PMSF and the sample was fractionated by gel filtration using Sephacryl S-300 equilibrated with 4 M guanidine hydrochloride/0.1 M Tris (pH 7.6). The major fragments of cytotactin were found in two regions of the eluate, a breakthrough peak called fraction I and a second peak with a $K_{av}$ = 0.1 called fraction II. Samples to be analyzed in adhesion assays were dialyzed against PBS and stored at −70°C. The protein concentration in solutions containing cytotactin or other SAMs was determined using the method of Lowry (30). Because solutions containing fragments of cytotactin were very dilute, their concentrations were estimated by spectrophotometry at 230 nm, using a solution of intact cytotactin whose concentration had been determined by the Lowry method as a standard.

CNBr fragments were prepared from 300 μg of cytotactin and resolved on an SDS gel containing 13.5% polyacrylamide. After staining, the prominent 75- and 35-kD fragments were cut from the gel, emulsified with Freund's adjuvant, and injected into rabbits in two equal aliquots. Injections were separated by a 2-wk interval. IgG and Fab' fragments were purified from the serum of these rabbits as previously described (2).

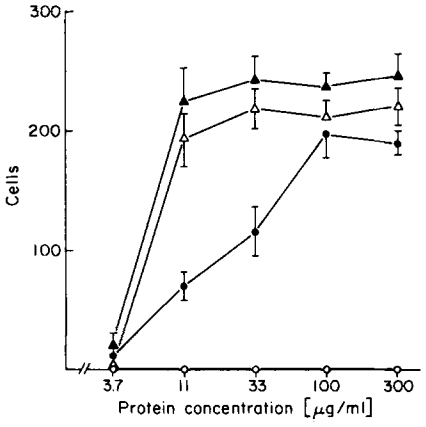


*Figure 1.* Dose dependence of fibroblast attachment and spreading on cytotactin and fibronectin in the gravity assay. Fibroblasts were incubated with dishes coated with increasing amounts of cytotactin (●, ○) or fibronectin (▲, △). Cell attachment (●, ▲) and cell spreading (○, △) were quantitated as described in Materials and Methods. Points represent averages ($n$ = 2) of cells bound or spread per 0.38 mm², and bar half-lengths are mean deviations. The concentrations of protein solutions used to coat the dishes are indicated on the abscissa.

### Radioiodination

Proteins were incubated with a final concentration of 150 μg/ml of chloramine T and 1 mCi/ml of $^{125}I$. After 5 min, 400 μg/ml of sodium metabisulfite was added to stop the reaction and free iodine was removed by dialysis against PBS in tubing with a 12–14 kD cut-off.

### Ultracentrifugation

S values for cytotactin and its chymotryptic fragments were determined by ultracentrifugation on glycerol gradients (15) using the indicated standards. The indicated molecules were reduced and alkylated by sequential incubation with dithiothreitol (5 mM, 30 min, 25°C) and iodoacetamide (15 mM, 10 min, 25°C).

### Peptide Mapping Techniques

Intact cytotactin was radioiodinated using chloramine T and fractions I and II were prepared from this material as described above. Intact cytotactin and fractions I and II were resolved on SDS gels under reducing conditions, and the indicated components were located by autoradiography, were cut from the gels, and were digested and resolved for one-dimensional peptide maps by the method of Cleveland et al. (7, 18) or for two-dimensional peptide maps by the method of Elder et al. (14).

## Results

### Fibroblasts Attach but Do Not Spread on Cytotactin-coated Substrates

When fibroblasts were incubated with cytotactin-coated substrates in the gravity assay, cell attachment increased as the concentration of protein on the substrate increased (Fig. 1, solid circles). When the abilities of fibroblasts to attach to substrates coated with cytotactin or with fibronectin were compared, the concentration dependence was found to differ. Similar numbers of cells attached to high concentrations of each protein, but attachment increased more rapidly with fibronectin concentration (solid triangles) and reached a plateau well before a plateau was reached on cytotactin.

Cytotactin- and fibronectin-coated substrates also differed in their ability to promote the spreading of attached cells. While ~80% of the attached cells spread on fibronectin during the course of the assay (see Fig. 1, open triangles, and Fig. 2 *B*), essentially no bound cells spread on cytotactin (see Fig. 1, open circles, and Fig. 2 *A*). During longer incubations, cells began to spread on cytotactin, but never to the extent observed on fibronectin. These combined results support the idea that cell–substrate adhesion and cell spreading are separable processes (19, 34).

### Cytotactin Can Inhibit or Promote the Attachment of Neurons or Glia to Substrates Depending on Assay Conditions

Two assays were used to evaluate the effects of cytotactin on the attachment of neurons and glia to polystyrene substrates, an assay in which cells settle onto the substrate (gravity assay) and an assay in which cell–substrate contact was initiated by centrifugation (centrifugation assay). Several laboratories have previously used centrifugation assays to evaluate cell–substrate adhesion. These assays fall into two classes, those in which cells are centrifuged into flat-bottomed wells (10, 19, 32) and those in which cells are centrifuged into U-shaped or V-shaped wells (13, 27, 39). In assays using flat-bottomed wells, the initial centrifugation deposits all cells in a uniform distribution on any substrate; specific attachment

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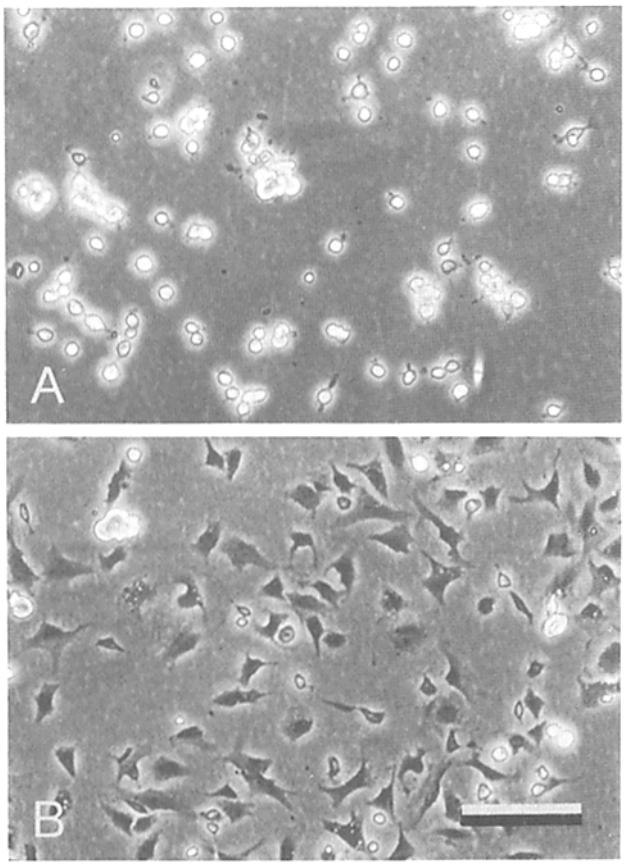


*Figure 2.* Attachment of fibroblasts to cytotactin-coated substrates in the gravity assay. Fibroblasts were attached to substrates coated with cytotactin (*A*) or fibronectin (*B*) as described in Materials and Methods. The concentration of protein solutions used to coat these spots were 100 μg/ml for cytotactin and 10 μg/ml for fibronectin. Bar, 100 μm.

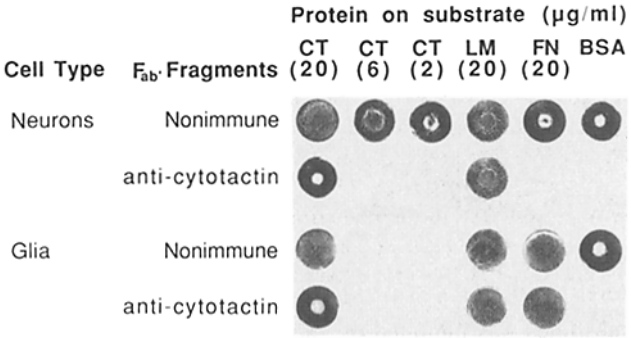


*Figure 3.* Attachment of neurons and glia to cytotactin-coated substrates in the centrifugation assay. Substrates were prepared by incubation with the indicated concentrations of cytotactin (*CT*), laminin (*LM*), fibronectin (*FN*), or with only the BSA-containing blocking buffer (*BSA*). Wells were preincubated for 30 min with medium containing 500 μg/ml of Fab' fragments; cells were then added without a change of medium and the centrifugation cell attachment assay performed as described in Materials and Methods. The anti-cytotactin antibody used in this experiment is the antibody to the 35-kD CNBr fragment of cytotactin characterized below in Fig. 4.

is quantitated either by reversing the direction of centrifugation or by washing the substrates under conditions of defined shear and determining the number of cells that remain attached. The main advantage of this assay is that by performing a series of successively more stringent washes, precise quantitations of adhesive strengths can be made because the biophysical parameters of sedimentation and shear to which the cells are subjected are well defined. Centrifugation assays using U-shaped wells are based on the following observations: (*a*) that cells centrifuged into U-shaped wells coated with nonadhesive proteins form a small pellet at the bottom of the well, and (*b*) that cells centrifuged onto adhesive substrates coat the entire bottom of the well. Presumably, cells that attach to the substrate are not dislodged by the centrifugal force while cells that do not attach are driven to the bottom of the well.

In the current studies, we chose to use a U-shaped well centrifugation assay because it is easier and faster both to do and to score, yet provides a reliable determination of the relative ability of cells to attach to various substrates. It requires only a single centrifugation in contrast to the flat-bottomed well assay which usually requires at least two steps. The U-shaped well assay is reliably scored by visual inspection (see Figs. 3 and 6) as are hemagglutination assays (45). The scoring of flat-bottomed well assays is somewhat more laborious, requiring that the number of cells attached to each well be individually determined.

Using the gravity assay, neither neurons nor glia attached to substrates coated with cytotactin or BSA although they attached well to substrates coated with laminin or fibronectin (Table I). When neurons were incubated for much longer time periods, the number of cells that attached to cytotactin was less than the number that attached to regions treated only with BSA. Moreover, fewer neurons were attached to substrates coated with both cytotactin and fibronectin than were attached to substrates coated with fibronectin alone, suggesting that cytotactin (which also binds to fibronectin [23]) had an apparent inhibitory effect on cell–substrate adhesion in this assay.

Although neurons did not attach to cytotactin-coated substrates in the gravity assay, the earlier observation (23) that cytotactin-coated beads bound to neurons in suspension suggested that these cells do express functioning cell-surface receptors for cytotactin. In support of this idea, dose-dependent attachment of neurons to cytotactin-coated substrates was observed in the centrifugation assay (Fig. 3); in this as-

*Table I. Attachment of Neurons and Glia to Extracellular Matrix Proteins in the Gravity Assay*

| Molecule on substrate | Neurons | Neurons | Glia |
|---|---|---|---|
| | *1 h* | *24 h* | *1 h* |
| Fibronectin | 242 ± 30* | ND | 280 ± 25 |
| Laminin | 226 ± 20 | ND | 310 ± 42 |
| Cytotactin | 2 ± 2 | 4 ± 1 | 10 ± 3 |
| "Background" | 4 ± 2 | 67 ± 12 | 6 ± 5 |
| Fibronectin and cytotactin | 116 ± 37 | ND | ND |

Attachment assays were performed as described in Materials and Methods. Substrates were prepared by incubation with solutions containing 20 μg/ml of fibronectin or laminin, or 100 μg/ml of cytotactin. The substrate coated with both fibronectin and cytotactin was prepared by sequential adsorption in that order. Cells were incubated with substrates for the indicated time periods (1 or 24 h). Background attachment was determined for regions treated with the BSA-containing blocking buffer only.

* Numbers are averages ± mean deviations ($n = 2$) and represent cells attached per 0.38 mm².

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say, neurons also attached to substrates coated with laminin but not to substrates coated with BSA. As expected, anti-cytotactin Fab' fragments inhibited cell attachment to cytotactin-coated substrates but not to laminin-coated substrates (Fig. 3). Consistent with observations from other laboratories that, under certain assay conditions, central neurons attach poorly to fibronectin (35), neurons attached only weakly to fibronectin in the centrifugation assay. Glial cells also attached to cytotactin in the centrifugation assay, and, in addition, attached to fibronectin and laminin but not to BSA (Fig. 3). Again, only cell attachment to cytotactin-coated substrates was inhibited by anti-cytotactin antibodies.

These results indicate that specific attachment can occur when neurons or glial cells are centrifuged onto a cytotactin-coated substrate. Use of the combined assays therefore allowed us to compare the ability of cytotactin and its fragments produced by limited proteolysis to promote the attachment of a variety of cells including fibroblasts, neurons, and glia.

### *Chymotryptic Fragments of Cytotactin*

To map the cell-binding function of cytotactin to its structure, proteolytic fragments of cytotactin were prepared. In preliminary experiments, digestion of cytotactin with trypsin or chymotrypsin under native conditions was found to have little effect on the molecule. However, in the presence of 2 M urea, chymotryptic digestion of cytotactin yielded two classes of fragments that could be separated by gel filtration in guanidine-HCl (Fig. 4 *A*). The material that eluted first, called fraction I, barely entered a 6% polyacrylamide gel under nonreducing conditions (Fig. 4 *B*, lane *1*); when analyzed under reducing conditions, fraction I contained a single major 100-kD component and two minor components of 85 and 75 kD (Fig. 4 *B*, lane *3*). The second class of fragments, called fraction II, contained two prominent components, 90 and 65 kD, whose mobilities were only slightly affected by reduction (Fig. 4 *B*, compare lanes *2* and *4*). Two-dimensional peptide mapping experiments (not shown) indicated that the 90- and 65-kD components in fraction II were very similar in structure.

These results are consistent with the idea that fraction I contains a disulfide-bonded oligomeric fragment of cytotactin while fraction II contains monomeric fragments of cytotactin. To test this hypothesis directly, sedimentation velocity centrifugation was used to estimate the native molecular masses of intact cytotactin, the material in fraction I, and the

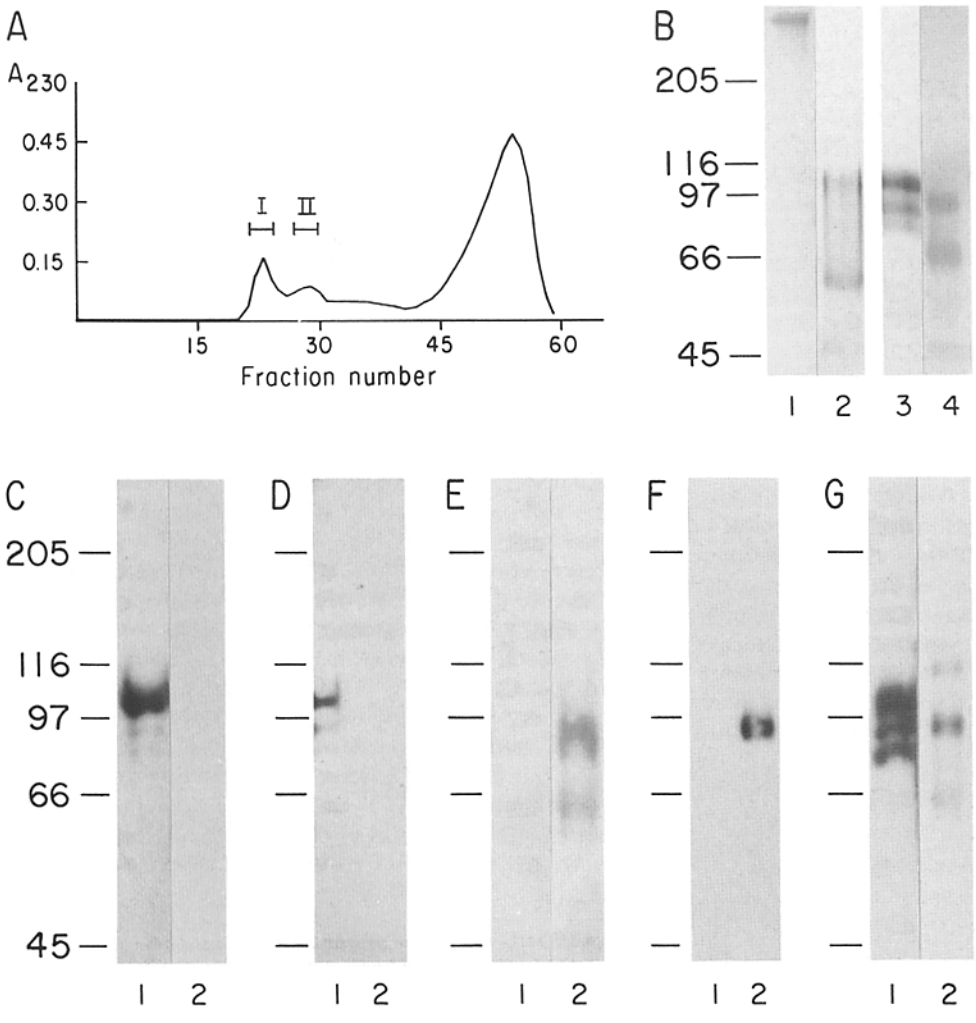


*Figure 4.* Structural properties of cytotactin fragments. Chymotryptic fragments of cytotactin were prepared as described in Materials and Methods, fractionated by gel filtration on Sephacryl S-300 in 4 M guanidine-HCl/0.1 M Tris-HCl (pH 7.6), and resolved by SDS-PAGE on 6% polyacrylamide gels. (*A*) Elution profile from gel filtration column. The $A_{230}$ of the eluate from the gel-filtration column is plotted vs. fraction number. Fractions 22–24 comprise "fraction I" and fractions 27–29 comprise "fraction II." (*B*) Silver-stained gels (33). 2-μg aliquots of fraction I (lanes *1* and *3*) and fraction II (lanes *2* and *4*) were resolved under nonreducing (lanes *1* and *2*) or reducing (lanes *3* and *4*) conditions. (*C–G*) Immunoblots (41). 2-μg aliquots of fraction I (lanes *1*) and fraction II (lanes 2) were resolved under reducing conditions, transferred to nitrocellulose, and incubated with either a polyclonal antibody prepared against a 75-kD CNBr fragment of intact cytotactin (*C*), monoclonal antibody M1 (*D*), a polyclonal antibody prepared against a 35-kD CNBr fragment of intact cytotactin (*E*), monoclonal antibody 1D8 (*F*), or monoclonal antibody HNK-1 (*G*). Immunoblots incubated with monoclonal antibodies were further incubated with appropriate second antibodies (rabbit anti-mouse IgG for M1 and 1D8, rabbit anti-mouse IgM for HNK-1). All immunoblots were finally incubated with $^{125}$I-protein A and the position of this label was detected by autoradiography.

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*Table II. Sedimentation Coefficients and Estimated Molecular Masses for Cytotactin and Fragments of Cytotactin*

| Form of Cytotactin | S Value (Estimated kD) |
|---|---|
| Intact unreduced | 13.2 ± 0.7 (1,450) |
| Reduced | 7.5 ± 0.7 (225) |
| Fraction I unreduced | 9.6 ± 1.3 (450) |
| Reduced | 3.6 ± 0.4 (64) |
| Fraction II unreduced | 3.3 ± 0.4 (58) |
| Reduced | 3.3 ± 0.4 (58) |

S values were determined by ultracentrifugation on glycerol gradients (15). Each gradient contained a radioiodinated form of cytotactin and the following unlabeled standard proteins: laminin, 11.5 S; fibronectin dimer, 10 S; fibronectin monomer, 7.5 S; IgG, 7 S; and BSA, 4.4 S. Gradient fractions were resolved by SDS-PAGE and the positions of standard proteins determined by Coomassie Blue staining and the position of forms of cytotactin by autoradiography.

The molecular masses of forms of cytotactin were estimated from their S values using a conversion curve derived from the known S values (see above) and molecular masses of standard proteins (laminin, $1 \times 10^6$; fibronectin dimer, $4.4 \times 10^5$; fibronectin monomer, $2.2 \times 10^5$; IgG, $1.5 \times 10^5$; BSA, $6.8 \times 10^4$). These should only be considered rough estimates because the conversion curve is derived from data for both fibrous and globular proteins, and because it is uncertain in what class each of the forms of cytotactin should be placed. Nevertheless, a semilog plot of the molecular mass vs. S value for these standard proteins, which include both classes, is a good fit to a straight line.

components in fraction II both before and after reduction and alkylation (Table II). Reduced intact cytotactin had a native molecular mass (225 kD) similar to its apparent molecular mass determined under denaturing conditions by SDS-PAGE (220 kD); the unreduced molecule had a native molecular mass approximately six times as great. These results support the idea that hexabrachions contain six polypeptide chains and further suggest that the formation of hexabrachions requires interchain disulfide bonds.

Comparison of the material in fraction I before and after reduction suggested that it consisted of multiple polypeptide chains. The molecular masses obtained for this fragment of cytotactin under reducing and nonreducing conditions are consistent with the conclusion that it is hexameric; this conclusion must be considered tentative, however, because the estimated native molecular mass for the reduced material (64 kD) is considerably less than its molecular mass as estimated by SDS-PAGE (100 kD). In contrast to fraction I, the polypeptides in fraction II did not change in molecular mass after reduction and apparently do not contain multiple subunits. The apparent native molecular mass of this material is also less (58 kD) than its molecular mass determined on polyacrylamide gels under denaturing conditions (a mixture of 90 and 65 kD components). This relationship is consistent with the idea that the components in both fraction I and fraction II have a rod-like shape.

### *Immunological and Structural Characterization of the Fragments*

To determine the antigenic and structural relationships of the components in fractions I and II and to evaluate whether these fractions were well separated by chromatography, aliquots of fractions I and II were immunoblotted with five different antibodies. These antibodies were a polyclonal antibody (anti-35 kD) prepared against a 35-kD CNBr fragment of cytotactin, a polyclonal antibody (anti-75 kD) prepared against a 75-kD CNBr fragment of cytotactin which migrates as a multimer on SDS gels run under nonreducing conditions, and three monoclonal antibodies: 1D8, which recognizes a polypeptide epitope in cytotactin (26); M1, which binds to an epitope in the proximal part of the arms of the hexabrachion (17, 42); and HNK-1, which recognizes a carbohydrate epitope present in cytotactin isolated from brain but not from other tissues (24). No molecular species common to fractions I and II were detected by immunoblotting (Fig. 4, *C–G*) indicating that these fractions were well resolved by chromatography. Both anti-75 kD and M1 recognized all the components in fraction I and no components in fraction II (Fig. 4, *C* and *D*). Conversely, anti-35 kD and 1D8 recognized components in fraction II and no components in fraction I (Fig. 4, *E* and *F*). Anti-35 kD recognized both major components in fraction II while 1D8 only appeared to recognize the 90-kD component. To confirm these results on native molecules, the antibodies were tested for their ability to recognize fractions I and II in an ELISA. As expected, anti-75 kD and M1 were specific for fraction I while anti-35 kD and 1D8 were specific for fraction II. The anti-carbohydrate antibody, HNK-1, bound to all the components in both fractions I and II, but to a greater extent to the components in fraction I (Fig. 4 *G*). An additional 120-kD component in fraction II was recognized by HNK-1 that was not detected with any other antibody. The fact that this species was also not detected by silver staining suggests that it is present at low levels and raises the possibility that it may be derived from cytotactin molecules that differ structurally from the majority of the cytotactin molecules in the preparation.

To evaluate further the extent of structural overlap, if any, between the polypeptides in fractions I and II, the 100-kD fragment in fraction I and the 90-kD fragment in fraction II were prepared from radioiodinated cytotactin. After fractionation by SDS-PAGE under reducing conditions, gel slices containing the fragments were treated with *Staph. aureus* V8 protease (Fig. 5) or proteinase K (data not shown) and the resulting subfragments resolved. After treatment with either enzyme, the digestion products derived from the 90-kD polypeptide were all of 10–30 kD (Fig. 5); almost no undigested material remained. In contrast, almost all of the fraction I fragment migrated with an apparent molecular mass close to 100 kD after protease treatment; even when enzyme concentrations were increased fivefold, no further digestion was observed (not shown). These expriments indicate that the sites in the 90-kD polypeptide that are susceptible to cleavage with V8 protease in SDS either are not present in the 100-kD polypeptide or are not accessible. Thus, it is likely that the 90- and 100-kD polypeptides correspond to different nonoverlapping parts of cytotactin. The combined antigenic and structural data suggest that the fragment of cytotactin in fraction I is derived from the central core of the hexabrachion and extends somewhat into each arm of this structure, and that the species in fraction II are generated from the remaining distal portions of each arm of cytotactin.

### *Cell Attachment to Substrates Coated with Chymotryptic Fragments of Cytotactin*

The cell-binding properties of these chymotryptic fragments of cytotactin were then examined using the gravity assay for fibroblasts (Table III, *A*) and the centrifugation assay for neu-

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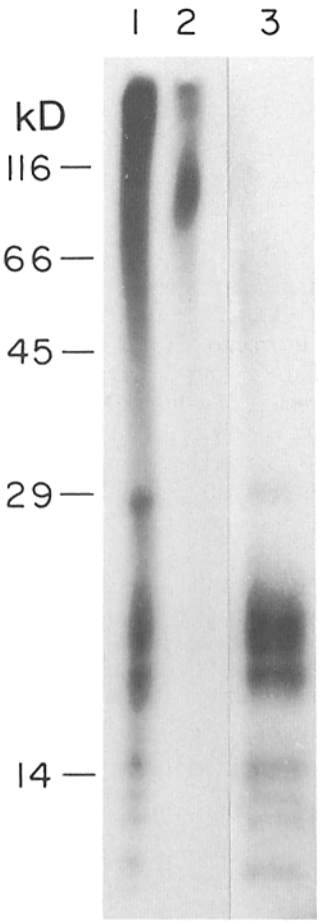


*Figure 5.* Comparison of proteolytic fragments of intact cytotactin, fraction I, and fraction II. Cytotactin was radioiodinated using chloramine T and fractions I and II were prepared from this material. Radioiodinated cytotactin, fraction I, and fraction II were resolved by SDS-PAGE under reducing conditions and the positions of the 220-kD component of intact cytotactin, the 100-kD component of fraction I, and 90-kD component of fraction II were determined by autoradiography. The pieces of polyacrylamide containing those polypeptides were cut from the gel, their contents digested with 0.5 μg *Staph. aureus* V8 protease (7), and the digestion products resolved by SDS-PAGE on a 15% polyacrylamide gel. Lane *1*, intact cytotactin; lane *2*, fraction I; lane *3*, fraction II. Note that essentially all the fragments produced by digestion of intact cytotactin appear in the digests of either fraction I or II. The migrations of standard proteins are indicated on the left.

rons and glia (Fig. 6). In all cases, cells attached to substrates coated with the material in fraction II but not to substrates coated with the material in fraction I. A greater percentage of attached fibroblasts spread on material in fraction II than on intact cytotactin, but the extent of spreading was still much less than on fibronectin (Table III, *A*). Two very different experiments confirm the idea that fraction II contains a specific cell-binding region while fraction I does not. (*a*) Fab′ fragments of the anti–35 kD antibody (which recognizes only fraction II) strongly inhibited the attachment of cells to substrates coated with intact cytotactin or fraction II, while Fab′ fragments of the anti–75 kD antibody (specific for fraction I) had no effect (Table III, *A*; and Fig. 6). As expected, the anti–35 kD antibody did not affect the attachment of fibroblasts to fibronectin (Table III, *A*) or neurons to laminin (Fig. 3). (*b*) Soluble fraction II was found to inhibit the attachment of fibroblasts to a substrate coated with intact cytotactin while fraction I had no effect (Table III, *B*).

Because the sequence Arg-Gly-Asp (RGD) is a functional part of the cell-binding region of fibronectin and other extracellular proteins (25, 37) and is present in the primary structures of cytotactin (26), the effect of RGD-containing peptides on the attachment of cells to substrates coated with cytotactin or fraction II was examined. In the gravity assay, RGD-containing peptides inhibited the attachment of fibroblasts to substrates coated with cytotactin or fraction II even more effectively than they inhibited the attachment of fibroblasts to fibronectin (Table III, *A*). Peptides containing the closely related sequence RGE had no effect on the attachment of fibroblasts to either cytotactin or fibronectin. These results suggest that RGD or a functionally related sequence is part of a cell-binding region in cytotactin. Nevertheless, in the centrifugation assay, RGD-containing peptides had no effect on the attachment of neurons or fibroblasts to substrates coated with cytotactin or fraction II (Fig. 6). This result is not likely to be a methodological artifact resulting from the use of the centrifugation assay because RGD-containing peptides were able to inhibit the attachment of fibroblasts to fibronectin in this assay (Fig. 6). Therefore, the results raise the possibility that fraction II of cytotactin may



*Table III. Attachment of Fibroblasts to Cytotactin and Fraction II in the Gravity Assay*

*A. Inhibition by Specific Antibodies and Peptides in Solution*

| Molecule on substrate | Fab′ fragments | | | Peptides | |
|---|---|---|---|---|---|
| | Unimmunized rabbit | Anti-35 kD§ | Anti-75kD§ | GRGDS | GRGESP |
| Cytotactin | 184 ± 6* (0)‡ | 4 ± 2 | 189 ± 10 | 23 ± 7 | 155 ± 20 |
| Fraction II | 217 ± 5 (69 ± 22) | 6 ± 2 | 205 ± 8 | 8 ± 3 | 206 ± 27 |
| Fraction I | 6 ± 3 (0) | ND | ND | ND | ND |
| Fibronectin | 174 ± 33 (145 ± 34) | 166 ± 33 | 191 ± 11 | 90 ± 23 | 200 ± 1 |
| – | 5 ± 2 | 7 ± 2 | 11 ± 1 | 11 ± 4 | 5 ± 1 |

*B. Inhibition by Fragments of Cytotactin*

| Molecule on substrate | Molecule in solution | Bound cells |
|---|---|---|
| Cytotactin | – | 223 ± 50 |
| Cytotactin | Fraction II | 83 ± 28 |
| Cytotactin | Fraction I | 230 ± 15 |
| – | – | 35 ± 1 |

Attachment assays were performed as described in Materials and Methods. Substrates were prepared by incubation with solutions containing 100 μg/ml of cytotactin, 5 μg/ml of fraction II, or 10 μg/ml of fraction I or fibronectin. For antibody perturbation experiments, dishes were preincubated for 30 min with 50 μl of medium containing 500 μg/ml of the appropriate Fab′ fragments; cells were then added without a change of medium. In other perturbation experiments, synthetic peptides (Peninsula Laboratories, Inc.; GRGDS, Code 9137; or GRGESP, Code 9135) at a concentration of 1 mg/ml or fractions I or II at a concentration of 2 μg/ml were preincubated with cells in medium for 30 min before addition of the mixtures to the dishes.

* Numbers are averages ± mean deviations ($n$ = 2) and represent cells attached per 0.38 mm$^2$.
‡ Number of spread cells.
§ Anti-cytotactin antibodies.

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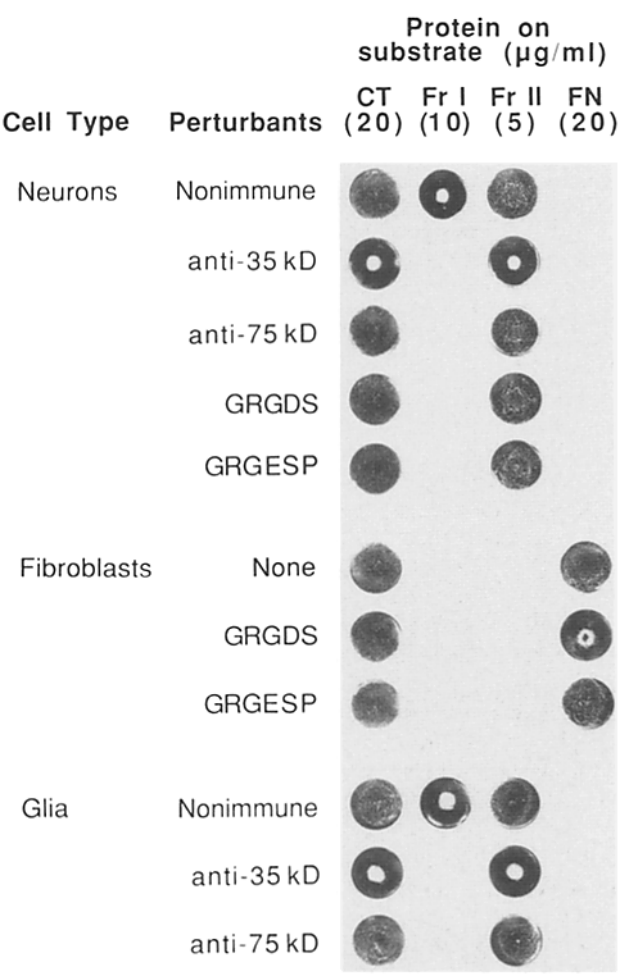


*Figure 6.* Inhibition of cell attachment in the centrifugation assay by region-specific antibodies and synthetic peptides. Substrates were prepared by incubation with the indicated concentrations of cytotactin (*CT*), the proteolytic fragments of cytotactin in fraction I (*Fr I*) or fraction II (*Fr II*), or fibronectin (*FN*). For antibody perturbation experiments, wells were preincubated for 30 min with medium containing 500 μg/ml of Fab′ fragments; cells were then added without a change of medium. For peptide perturbation experiments, GRGDS or GRGESP (see Table III) at a concentration of 500 μg/ml were incubated with cells in medium for 30 min before addition of the mixtures to the wells. The centrifugation cell attachment assay was then performed as described in Materials and Methods.

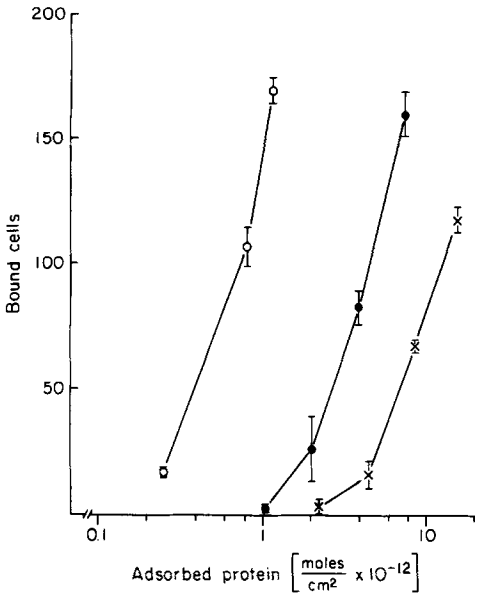


*Figure 7.* Specific binding activities of cytotactin and fraction II. Increasing amounts of unreduced cytotactin (×), reduced cytotactin (●), and fraction II (○) were adsorbed to the substrate. Replicate spots bearing adsorbed protein were used either in fibroblast attachment experiments (gravity assay) or in ELISA assays to estimate the amount of adsorbed protein as described in Materials and Methods. Adsorbed protein is expressed in moles of polypeptide per $cm^2$. Points represent averages ($n$ = 2) of cells bound per 0.38 $mm^2$; and bar half-lengths are mean deviations.

contain separate binding sites involved in RGD-sensitive and RGD-insensitive cell attachment.

When the specific activities of intact cytotactin and fraction II were compared on a molar basis, fraction II was found to promote cell attachment at 20-fold lower concentrations than did intact cytotactin (Fig. 7). Cytotactin monomers (i.e., reduced cytotactin) were also somewhat more active than hexabrachions in supporting cell–substrate adhesion. The apparent low specific activity of intact cytotactin relative to fraction II may result from the effects of steric hindrance on the availability of cell-binding sites when hexabrachions adsorb to plastic. Alternatively, the binding of fraction II to cells may, in fact, be more avid than the binding of intact cytotactin due to the release of conformational restraints near the binding sites.

### *Localization of Binding Sites in Cytotactin for CTB Proteoglycan and Fibronectin*

Previous observations suggested that CTB proteoglycan binds to cytotactin thereby inhibiting its ability to bind to cells (23). We therefore examined the effects of the proteoglycan on the attachment of fibroblasts to substrates coated with cytotactin or fraction II (Table IV). Soluble CTB proteolgycan inhibited the attachment of fibroblasts to fraction II by over 80%, to intact cytotactin by ~40%, but had little effect on the attachment of cells to fibronectin. Chondroitin sulfate did not affect cell attachment to cytotactin or its fragment, indicating that the inhibition caused by CTB proteoglycan was not simply due to effects from the negative charge of the chondroitin sulfate in the proteoglycan. To distinguish whether the inhibition of binding to the cytotactin fragment was due to binding of the proteoglycan to the fragment itself or to cells, CTB proteoglycan was preincubated either with fraction II–coated substrate or with the cells and then washed out before the incubation of the cells with the substrate. Preincubation of the substrate with CTB proteoglycan strongly inhibited the attachment of fibroblasts to fraction II while preincubation of the cells with CTB proteoglycan had little effect (Table V). These experiments are consistent with the notion that CTB proteoglycan inhibits the attachment of fibroblasts to cytotactin-coated substrates by binding to a domain in cytotactin that, like the cell-binding domain, is located within fraction II.

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*Table IV. Effects of CTB Proteoglycan on the Attachment of Fibroblasts to Cytotactin in the Gravity Assay*

| Molecule on substrate | Soluble molecule | | |
|---|---|---|---|
| | None | CTB proteoglycan | Chondroitin 6-sulfate |
| Cytotactin | 201 ± 22* | 122 ± 10 | 243 ± 45 |
| Fraction II | 310 ± 17 | 51 ± 7 | 281 ± 32 |
| Fibronectin | 404 ± 89 | 365 ± 34 | 412 ± 31 |
| – | 16 ± 7 | 12 ± 2 | 8 ± 3 |

Attachment assays were performed as described in Materials and Methods. Substrates were prepared by incubation with solutions containing 100 μg/ml of cytotactin, 5 μg/ml of fraction II, or 10 μg/ml of fibronectin. 50-μl drops of medium containing CTB proteoglycan (5 μg) or chondroitin 6-sulfate (5 μg) were incubated in dishes for 15 min; cells were then added without a change of medium.
* Numbers are averages ± mean deviations ($n$ = 2) and represent cells bound per 0.38 mm$^2$.

To confirm the localization of the binding sites for CTB proteoglycan and fibronectin in the cytotactin molecule, the four antibodies specific for either fraction I or II were tested for their ability to perturb the binding of cytotactin-coated beads to either CTB proteoglycan–coated beads or fibronectin-coated beads (Table VI). Both intermolecular binding mechanisms were strongly inhibited by low doses of Fab' fragments of the anti–35 kD antibody while even fivefold higher doses of the anti–75 kD antibody had no effect. Monoclonal antibodies 1D8 and M1 had little or no effect on binding. Therefore, these results indicate that the binding sites in cytotactin for cells, CTB proteoglycan, and fibronectin are all in the same general region of the molecule. However, given the large stretch of polypeptide that might be blocked by an antibody molecule, it cannot be concluded that all three binding sites are located within the 35-kD CNBr fragment of cytotactin. It will be necessary to use higher resolution methods to determine the exact physical relationships among these three binding sites.

## Discussion

In the present study, we have investigated the effects of cytotactin on cell–substrate adhesion and cell shape, and have characterized a fragment of the molecule that contains the cell-binding, proteoglycan-binding, and fibronectin-binding sites present in the intact molecule. The results on the behavior of cells after attaching to cytotactin-coated substrates have reconciled various disparate observations made in previous studies. They are consistent with the notion that the effects of cytotactin on cells in various contexts in vivo will depend on the local composition of the extracellular matrix; i.e., the presence of varying amounts of such cytotactin-binding molecules as fibronectin and CTB proteoglycan. The data on the binding properties permit the proposal of a model of cytotactin relating its structure to its binding functions.

The present studies show that cytotactin mediates cell attachment but, as noted previously (3, 40), it does not promote cell spreading. In contrast, cell attachment to fibronectin substrates is routinely followed by the energy-dependent processes, cell spreading and formation of focal contacts (8, 19, 29). Cell attachment to fibronectin, however, can be dissociated from these latter steps. Cells attach but spread poorly either on low concentrations of fibronectin or on higher concentrations of fibronectin in the presence of high concentrations of collagen (20, 34). Cells attach and spread on substrates coated with fibronectin fragments that include the Arg-Gly-Asp recognition sequence. These cells will not form focal contacts, however, unless the fibronectin fragments also include the heparin-binding domain of the molecule which interacts with integral cell-surface heparin sulfate proteoglycans (29, 43). Therefore, while cytotactin and fibronectin have distinct effects on cell behavior in vitro, their effects in vivo may overlap depending on the molecular context provided by the local composition of the extracellular matrix and by the specific receptors available at the cell surface. Moreover, various modes of intermolecular binding between cytotactin, fibronectin, and other matrix components may differentially modulate the effects of these molecules on cell behavior (23).

The data on the effects of cytotactin on the adhesion of neurons and glia to substrates emphasize the potential differences in results that may be obtained depending on the choice of adhesion assay used. In an assay in which contact between cells and the substrate is initiated by gravity and may be stabilized by cell spreading, fibroblasts attached to cytotactin-coated substrates but remained rounded while neurons and glia did not attach to the substrate. Indeed, cytotactin inhibited their attachment to other protein-coated substrates. However, neurons and glia did attach to cytotactin-coated substrates in an assay in which cell–substrate contact was driven by centrifugation. It appears, therefore, that these

*Table V. Reversibility of the Effects of CTB Proteoglycan on the Attachment of Fibroblasts to Fraction II in the Gravity Assay*

| Molecule on substrate | Soluble molecule | Attached cells* |
|---|---|---|
| Fraction II | – | 272 ± 28 |
| Fraction II | CTB proteoglycan | 58 ± 15 |
| Fraction II | CTB proteoglycan, incubated with dish and washed out | 104 ± 32 |
| Fraction II | CTB proteoglycan, incubated with cells and washed out | 214 ± 18 |
| – | – | 9 ± 1 |

Attachment assays were performed as described in Materials and Methods. Substrates were prepared by incubation with solutions containing 5 μg/ml of fraction II. 50-μl drops of medium alone or medium containing CTB proteoglycan (5 μg) were incubated in dishes for 15 min before introduction of cells. The indicated dishes were washed by changing the medium five times. Cells were preincubated for 15 min in medium alone or in medium containing 100 μg/ml of CTB proteoglycan, and were then washed three times by centrifugation in medium.
* Numbers are averages ± mean deviations ($n$ = 2).

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*Table VI. Antibody Inhibition of Cytotactin–CTB Proteoglycan and Cytotactin–Fibronectin Interactions*

| Antibody | Cytotactin-coated beads plus CTB proteoglycan-coated beads | | Cytotactin-coated beads plus fibronectin-coated beads | |
|---|---|---|---|---|
| | Superthreshold particles* | Inhibition (%) | Superthreshold particles* | Inhibition (%) |
| Nonimmune | 16,900 | – | 18,100 | – |
| Anti-35 kD | 4,400 | 74 | 1,100 | 94 |
| Anti-75 kD | 16,100 | 5 | 17,900 | 1 |
| 1D8 | 16,600 | 2 | 16,500 | 9 |
| M1 | 15,900 | 6 | 16,300 | 10 |

Binding assays monitoring the coaggregation of microscopic beads (Covaspheres) coated with cytotactin with Covaspheres coated with other proteins were performed as previously described (23, 24). Forms and amounts of antibodies present during incubations were as follows: Fab' fragments of rabbit IgG–nonimmune, 50 μg; anti-35 kD, 10 μg; and anti-75 kD, 50 μg; Monoclonal IgG-1D8, 25 μg; M1, 25 μg.
* Average of duplicate points. In all cases, the mean deviation was <2,000 superthreshold particles. Each species of bead, when incubated alone, resulted in <1,000 superthreshold particles.

cells contain functional cell-surface receptors for cytotactin. The apparent failure of these cells to attach to cytotactin-coated substrates may be due to a transient binding of the molecule to these cells which results in global cell-surface modulation (11) and an inhibition of cell spreading. In turn, the failure to spread may block the further stabilization of cell attachment and, in the case of neural crest cells (40), cause the slowing or inhibition of cell migration. The inhibitory effect of cytotactin on cell adhesion in in vitro assays may not have a precise counterpart in vivo. Cells that adhere to cytotactin in vivo may, for example, be able to spread because they interact with other extracellular matrix components and other cells in three dimensions. Nonetheless, if cytotactin causes cells to round up in vivo, it is likely that it will also inhibit in vivo cell migration at least to some degree.

The functional effects described above are obviously dependent on structural factors including the location and steric properties of the binding sites, the valence, and the shape of the molecule. It is, therefore, particularly important to locate the binding sites in the overall structure. Cytotactin is known to have a distinctive structure, appearing in electron microscopic images as a figure known as a hexabrachion with six arms emanating from a central core. The integrity of this structure requires intact interchain disulfide bonds (see Table II). On the basis of this and other studies, we have formulated a model that summarizes our knowledge of the structure of cytotactin polypeptide chains and their orientation in the hexabrachion (Fig. 8). In view of the findings that the molecular mass of a hexabrachion is approximately six times that of a single chain of cytotactin and that hexabrachions have six indistinguishable arms in electron microscopic images, we assume that each polypeptide chain contributes to a single arm of the hexabrachion. In the proposed model, we have also assumed that each polypeptide in the hexabrachion is identical and has a molecular mass of 220 kD, the predominant species in the cytotactin preparations used in these studies. Given the possibility that various cytotactins may arise from alternative RNA splicing events, it is possible that other cytotactin polypeptides are, in fact, a structural component of some hexabrachions.

In the attempt to map the binding functions of the molecule, two groups of cytotactin fragments have been characterized: disulfide-bonded oligomers consisting primarily of

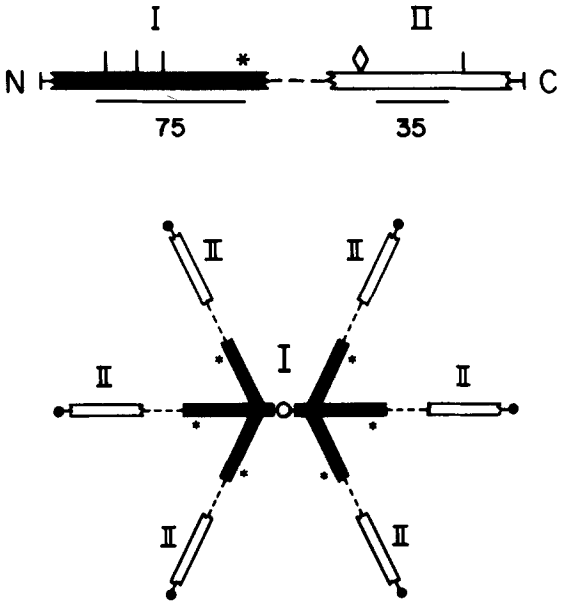


*Figure 8.* Proposed orientation of the fragments of cytotactin in a linear map of the molecule (top) and in a hexabrachion (bottom). Fraction I (solid boxes) includes the portion of the molecule where the polypeptide chains meet to form a hexabrachion and are linked by disulfide bonds. In addition, fraction I extends up the arms of the hexabrachion at least to the site (*) where monoclonal antibody M1 binds (17, 42). Fraction II (*open boxes*) is located in the distal portion of the arms of the hexabrachion and contains a cell-binding site, a CTB proteoglycan–binding site, and a fibronectin-binding site. Fractions I and II are drawn to scale and are drawn with jagged ends to indicate that their precise locations in the molecule have not yet been determined. The 35-kD and 75-kD CNBr fragments of cytotactin (which have also been drawn to scale), the site of the 1D8 epitope (◇), and the sites of the HNK-1 epitopes (vertical bars) have been placed at arbitrary positions within fractions I and II because their precise localizations within these structures have not yet been determined. For clarity, the positions of these features and the amino (*N*) and carboxyl (*C*) termini of the molecule have only been indicated on the linear model. The central globular region of the hexabrachion seen in electron micrographs has been left empty to indicate the possibility that fraction I may not extend into this structure.

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100-kD polypeptides (fraction I), and a mixture of closely related 90- and 65-kD polypeptides (fraction II) that do not contain interchain disulfide bonds and that migrate as monomers during ultracentrifugation under nondenaturing conditions. Immunological and structural analyses suggested that the components in each fraction were related to the other components in the same fraction but not to the components in the other fraction. Because fraction I contains interchain disulfide bonds, it should include regions in the central portion of the molecule (Fig. 8) where the polypeptide chains meet. Consistent with this idea, the components in fraction I cross-reacted with a polyclonal antibody prepared against a 75-kD CNBr fragment of cytotactin that is disulfide-bonded under native conditions. In addition, the material in fraction I must extend into the arms of the hexabrachion because it was recognized by a monoclonal antibody, M1, known to bind to the proximal portion of the arms (17, 42). Fraction I also probably includes the majority of the carbohydrate epitopes recognized by monoclonal antibody HNK-1. In contrast, polypeptides in fraction II are monomeric and probably correspond to portions of the free arms of the molecule (Fig. 8). Both components in this fraction were recognized by a polyclonal antibody prepared against a 35-kD CNBr fragment of cytotactin and the 90-kD component was recognized by monoclonal antibody 1D8; neither of these antibodies react with fraction I. Recent amino acid sequence analyses (Jones, F. S., S. Hoffman, B. A. Cunningham, and G. M. Edelman, unpublished observations) have suggested that the components in fraction I are amino-terminal to the components in fraction II.

The proposed structural model of cytotactin and the results of the binding assays performed here allow an initial localization of various functional regions in cytotactin. Several results indicate that a cell-binding region in cytotactin is located within fraction II and is, therefore, present in the arms of the hexabrachion. The data also suggest that a binding site for CTB proteoglycan is present in fraction II, and that when CTB proteoglycan is bound to fraction II, cell binding is inhibited. These conclusions drawn from experiments involving purified fraction II are supported and extended by the results of antibody perturbation experiments. The anti-35 kD antibody cross-reacts with fraction II and not fraction I and completely inhibits the binding of cytotactin to cells, CTB proteoglycan, and fibronectin. In contrast, the anti-75 kD antibody which specifically recognizes fraction I had no effect on any of these binding mechanisms. Given the relatively large stretch of polypeptide that might be blocked by attachment of an antibody molecule, it should not be concluded that all these binding sites are necessarily located on this single CNBr fragment of cytotactin. However, all three of these binding sites are likely to be located in the same general region of the molecule; i.e., the distal portion of the arms of the hexabrachion. In agreement with our results, a monoclonal antibody that inhibited the binding of tenascin to fibronectin was recently shown to bind to the ends of the arms of the hexabrachion in electron microscopic images (3). Indirect experiments also suggested that this antibody blocks a cell-binding site on tenascin.

Recently, we have sequenced a cDNA clone coding for a contiguous sequence of 933 amino acids in cytotactin, or approximately one-half the molecule (26). A directly determined amino-terminal sequence of a 22-kD CNBr fragment of the molecule recognized by monoclonal antibody 1D8 was found in the sequence deduced from the clone (26). Therefore, the 90-kD component of fraction II, which is also recognized by 1D8, must overlap at least part of this 22-kD CNBr fragment. The deduced sequence of cytotactin (26) also contained the sequence Arg-Gly-Asp, which forms part of a cell-binding site in fibronectin and several other extracellular proteins (25, 37). The present observation that RGD-containing peptides inhibit the binding of fibroblasts to cytotactin or to fraction II suggests that a cell-binding site in fraction II of cytotactin contains an RGD sequence. Nevertheless, this conclusion must remain provisional inasmuch as the available data are still not completely sufficient to prove that the known RGD sequence in cytotactin is present in the components in fraction II.

The accumulated results on the structure and function of cytotactin suggest that this protein may be a natural mediator of global cell-surface modulation (11); i.e., cytotactin, through multivalent interactions with its receptors, may elicit a transmembrane response that affects the status of the cytoskeleton, the mobility of cell surface proteins, and ultimately, cell behavior. The interesting possibility arises that cytotactin may affect fundamental processes of morphogenesis and pattern formation by means of this mechanism.

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