Characterization of Multiple Adhesive and Counteradhesive Domains in the Extracellular Matrix Protein Cytotactin

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Abstract. The extracellular matrix molecule cytotactin is a multidomain protein that plays a role in cell migration, proliferation, and differentiation during development. To analyze the structure–function relationships of the different domains of this glycoprotein, we have prepared a series of fusion constructs in bacterial expression vectors. Results obtained using a number of adhesion assays suggest that at least four independent cell binding regions are distributed among the various cytotactin domains. Two of these are adhesive; two others appear to be counteradhesive in that they inhibit cell attachment to otherwise favorable substrates. The adhesive regions were mapped to the fibronectin type III repeats II–VI and the fibrinogen domain. The morphology of the cells plated onto these adhesive fragments differed; the cells spread on the fibronectin type III repeats as they do on fibronectin, but remained round on the fibrinogen domain. The counteradhesive properties of the molecule were mapped to the EGF-like repeats and the last two fibronectin type III repeats, VII–VIII. The latter region also contained a cell attachment activity that was observed only after proteolysis of the cells. Several cell types were used in these analyses, including fibroblasts, neurons, and glia, all of which are known to bind to cytotactin. The different domains exert their effects in a concentration-dependent manner and can be inhibited by an excess of the soluble molecule, consistent with the hypothesis that the observed properties are mediated by specific receptors. Moreover, it appears that some of these receptors are restricted to particular cell types. For example, glial cells bound better than neurons to the fibrinogen domain and fibroblasts bound better than glia and neurons to the EGF fragment. These results provide a basis for understanding the multiple activities of cytotactin and a framework for isolating different receptors that mediate the various cellular responses to this molecule.

CYTOTACTIN is an extracellular matrix glycoprotein that has important functions during morphogenesis, histogenesis, and tumor formation. The molecule exhibits a site-restricted distribution during development (15, 59), which may be controlled by other developmentally important genes. The cytotactin promoter region reveals a rich array of regulatory motifs with homology to the DNA binding sequences for homeotic proteins and growth factors (39) and has recently been shown to be an in vitro target of homeodomain-containing proteins (40). The restricted spatiotemporal expression of cytotactin that results from its developmental regulation is tightly linked to a number of cellular primary processes, including adhesion (29), migration (13, 32, 69), proliferation (12, 14), differentiation (50), epithelial–mesenchymal interactions (3, 4), and cell death (74).

Cytotactin, which is also known as tenascin (11), J1 220/200 (43), hexabrachion (21, 31), the glioma-mesenchymal extracellular matrix protein (6), and myotendinous antigen (8), is composed of polypeptides of 190, 200, and 220 kD when isolated from chicken brain (29). Variation in the polypeptide structure arises from alternative splicing of transcripts from a single gene (37, 38, 66). The polypeptides are disulfide-linked to form a multimeric structure (29, 34). EM of the rotary-shadowed molecule has revealed a characteristic six-armed structure, called a hexabrachion (21, 22), in which six polypeptides are linked through disulfide bonds at their aminotermini. The sequence of cytotactin reveals a multidomain structure (38, 66) with homologies to three other protein families. The amino-terminal portion contains the cysteine involved in interchain disulfide bonding, followed by an array of 13 repeats of 31 amino acids in length that resemble those found in EGF. These EGF-like repeats are followed by a variable number of repeats similar to fibronectin type III repeats. In the chicken, cytotactin polypeptides contain between 8 and 11 type III repeats as a consequence of alternative RNA splicing. Different variants have been shown to be expressed preferentially at certain times and anatomical sites during development (59) and they may have different binding or morphogenetic functions (42, 54, 57). The carboxy-terminal portion of cytotactin is homol-

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ogous to the distal domain of the β and γ chains of fibrinogen and contains a putative Ca^{2+} binding site.

Early studies of cell attachment to cytotactin-coated surfaces suggested that multiple modes of binding to the molecule existed. For example, fibroblasts bind both to intact cytotactin and to a chymotryptic fragment derived from the carboxy-terminal end of the protein (24). These binding activities are inhibitable by peptides containing the amino acid sequence RGD and by antibodies to specific regions of the cytotactin protein. In contrast to their rounded cell morphology on intact cytotactin, cells exhibit a spread morphology on the chymotryptic fragment. Using a variety of recombinant fragments of tenascin, a smaller region of the molecule has been identified as a cell binding site, but no spreading was observed (66). In these studies, a fragment in the amino-terminal region containing the EGF domains appeared to prevent cell binding to other substrates. Together, these observations suggested that at least two binding activities are present in intact cytotactin, one in the carboxy-terminal half of the protein, mediating cell attachment and flattening, and one in the amino-terminal portion, responsible for so-called anti-adhesive effects (66) and rounding of cells exposed to the molecule (12, 24). Studies on the effects of cytotactin on neural attachment and neurite outgrowth have suggested at least one additional interactive site on the molecule based on antibody inhibition studies (16, 23, 26, 35, 47, 72).

The remarkable pattern of distribution throughout development and the complex multidomain structure of cytotactin suggest that it may play multiple roles during morphogenesis. That each type of protein domain in cytotactin appears in several different proteins suggests that individual domains may represent independent functional units. These observations have prompted us to search for functions of the various domains. To do this, recombinant protein fragments of cytotactin were made containing specific regions spanning almost the entire molecule. Cell attachment and morphology of cells plated on substrates coated with each of the fragments were then analyzed. Three different classes of cells were examined, fibroblasts, glia, and neurons, all of which have been shown to bind to intact cytotactin in various assays (12, 24, 33, 48). In contrast to previous mapping studies that identified a single cell binding site and a repulsive site (24, 66), at least four nonoverlapping sites on the molecule were found to interact with the cell surface. Cell attachment was sensitive to the method of preparation of the single cell suspension; for example, one cell binding site was seen in cells prepared by trypsin treatment but not in cells prepared with EDTA. The results suggest that multiple sites on cytotactin mediate its adhesive and counteradhesive effects on cells; these effects are likely to be mediated by multiple, specific cell surface receptors.

Materials and Methods

Fusion Protein cDNA Constructs

Seven different constructs containing cytotactin cDNA inserts were made by subcloning cDNA fragments into either the pGEX-2T or -3X vectors in the correct translational reading frame (Pharmacia, Uppsala, Sweden). The fusion proteins all consist of the carboxy-terminal portions of glutathione-S-transferase (GST)[1] fused to the cytotactin regions. As described below, the GST portion was removed by proteolytic cleavage. The different fusion proteins are designated as follows. EGF contains all the EGF repeats; FN1 contains the proximal fibronectin type III repeats (II-VI); FN2 contains fibronectin type III repeats II-VI and includes the three alternatively spliced fibronectin type III repeats (VaVbVc); FN3 contains the last two fibronectin type III repeats with the exception of the 13 amino-terminal amino acids of repeat VIII (VII and VIII); Fg contains the entire fibrinogen domain and includes the 13 amino-terminal amino acids of fibronectin repeat VIII; FNFg1 contains the last two fibronectin type III repeats (VII and VIII) and the first 71 amino acids of the fibrinogen domain; FNFg2 contains the entire fibrinogen domain and the last two fibronectin type III repeats (see Fig. 1).

The numbering of base pairs refers to that given by Jones et al. (38). The EGF fusion protein was made by subcloning a 1.35-kb EcoRI cDNA fragment (Frederick Jones, unpublished observations) isolated from λgt11 bacteriophage into the EcoRI site of pGEX-2T. This cDNA fragment spans all the EGF-like repeats of cytotactin from bp 830 to bp 2,182.

The FN1 cytotactin-GST fusion protein was generated by excising the 1,780-bp cDNA insert from pEC802 with BamHI (37). The ends of this cDNA were filled using Klenow to generate blunt ends. The insert derived from pEC802 was further digested with EcoRI and ligated into EcoRI/SmaI digested pGEX-3X vector. The resulting 1,280-bp insert spans from bp 2,414 (BamHI site) to bp 3,694 (EcoRI).

The FN2 fusion protein construct was made using λgt11 containing a 4.5-kb cytotactin cDNA insert (38). EcoRI digestion generated two fragments of 3.8 and 0.7 kb. The 3.8-kb fragment was subcloned into the EcoRI site of Bluescript KS vector (Stratagene, La Jolla, CA). Plasmid DNA was prepared (51) and digested with BamHI to cut a site in the internal portion of the insert and of the plasmid. The ends of the BamHI fragment were filled using DNA polymerase (Klenow fragment) to generate blunt ends, and subsequently digested with EcoRI. The resulting 2,099-bp cDNA was ligated into EcoRI/SmaI in pGEX-3X vector. The 2,099-bp cDNA spans from the BamHI site at bp 2,414 to the EcoRI site at bp 4,513 and includes the 819-bp of the alternatively spliced region.

The FN3 fusion protein was also made from pEC803 (40). A 500-bp BglII/EcoRI fragment was isolated from pEC803 and the 5′ protruding ends were filled with T4 polymerase. This fragment was subcloned into the SmaI site of pGEX-2T, and the correct orientation was determined by restriction analysis of the clones. This construct spans from bp 4,513 (EcoRI site) to bp 5,013 (BglII site).

The FNFg1 fusion protein was made by excising a 779-bp EcoRI fragment from pEC803 (38) and subcloning it into the EcoRI site of pGEX-2T. This clone spans the cytotactin cDNA sequence from bp 4,513 (EcoRI site) to bp 5,292 (EcoRI site).

The FNFg2 fusion protein was made from pEC803. A 1,549-bp clone was made by ligating the 780-bp EcoRI insert from pEC803 and a 769-bp EcoRI insert obtained from the 3′ most cytotactin cDNA clone (38) into Bluescript KS (pCG2). The insert in pCG2 extends from bp 4,515 (EcoRI) site to bp 6,061.

The Fg was made by excising a 1,020-bp BglII/XmnI insert from pCG2, and the 5′ protruding ends were filled with T4 polymerase and then ligated into the SmaI site of pGEX-2T. This clone spans from bp 5,013 (BglII site) to bp 6,033 (XmnI site).

Preparation, Purification, and Cleavage of Fusion Proteins

The protocol used for induction and purification of the fusion proteins has been reported (52, 53) and is described briefly here. *E. coli* strain NM522 (Stratagene) was transformed with GEX plasmids under selection with ampicillin. An ampicillin-resistant colony was used to inoculate 100 ml of LA-broth (L-broth/ampicillin 50 μg/ml) (51) and was incubated at 37°C, with agitation for 10 h. These cultures were used to inoculate 900 ml of LA-broth (10-fold dilution) which were then further incubated for 3–4 h at 25°C with agitation, until an optical density of 1.0 at 650 nm was reached. Isopropyl β-thiogalactopyranoside (IPTG) (Sigma Chemical Co., St. Louis, MO), was then added to a final concentration of 0.1 mM and the cultures were incubated for an additional 20 h at 25°C with agitation. The bacterial cells were harvested at 9,000 rpm in a GSA rotor for 10 min, and the pellet was resuspended in 50 ml of L-buffer consisting of 50 mM Tris-HCl, pH 7.5, 25% sucrose, 0.5% NP-40 and 5 mM $MgCl_2$.

The resuspended cells were partially lysed by three cycles of freeze thaw-

1. *Abbreviation used in this paper*: GST, glutathione-S-transferase.

ing, and 7-ml aliquots were then sonicated at the tip limit, 150 W, 1 min at 4°C. The sonication was repeated five times for 1 min. The lysate was centrifuged at 10,000 rpm for 20 min in an SS 34 rotor, and the supernatant was rotated at ~6 rpm for 1 h with 14 ml of glutathione-Sepharose 4B beads (Pharmacia) at 4°C. The beads were washed three times with 5 vols of GST-wash buffer consisting of 20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$ and 1 mM DTT (Sigma Chemical Co.).

The fusion protein was eluted serially three times with two bead volumes of GST-elution buffer consisting of 50 mM Tris-HCl, pH 9.6, and 5 mM glutathione (Sigma Chemical Co.). Proteolytic cleavage of the fusion proteins was performed while the proteins were still attached to the glutathione beads. The beads were equilibrated in cleavage buffer consisting of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 or 2.5 mM $CaCl_2$ when thrombin or activated factor Xa were used, respectively. The cleavage was done in one bead volume, using 10 μg of thrombin for the EGF and Fg fusion protein or 10 μg of activated factor Xa for FN1. Factor Xa was activated at 37°C for 5 min, using 1 ng of activating enzyme per 1 μg of factor Xa in a buffer containing 8 mM Tris HCl, pH 8.0, 70 mM NaCl, and 8 mM $CaCl_2$ (65). After cleavage, the beads were pelleted and the proteins eluted as described above. The supernatants and glutathione eluates were dialyzed overnight against H_2O at 4°C and lyophilized. Each fraction was analyzed for purity by SDS-PAGE (44) in 10 or 12% gels, and by Western blotting (71).

Protein Analysis

The amount of protein obtained varied according to the size of the fusion protein; in general, the smaller the protein the larger the amount of protein recovered. For example, fusion proteins of ~70 kD yielded 20–40 mg and those of around 50 kD yielded 80–100 mg. The protein samples were digested overnight in 1 N NaOH/0.02% Triton X-100 and protein concentrations were determined by the Lowry procedure (49). BSA was used as the standard protein and this method was sensitive to as little as 1 μg of protein.

Immunoblotting

To determine that the fusion proteins obtained corresponded to those containing cytotactin sequences, immunoblotting was performed after resolution of the cytotactin fusion proteins on SDS-PAGE and transfer onto nitrocellulose (71). The rabbit anti-cytotactin polyclonal antibodies were previously characterized (34).

Cell Preparations

The following cells were obtained from the American Type Culture Collection (Rockville, MD): neuroblastomas SK-N-SH, IMR-32 and neuro2A; NCTC 929 clone of strain L (L-929); NIH-3T3; myeloma P3X63Ag8U.1; glioma C6. The U251MG glioma cell line was a gift from Dr. Wolfgang Rettig (Memorial Sloan Kettering Cancer Center, New York) and the G2624 glioma (68) was a gift from Dr. Steven E. Pfeiffer (University of Connecticut Health Center, Farmington, CT). Mouse sarcoma 180 cells transfected with L-CAM (S180-L) (55). Primary chicken embryo fibroblasts (CEFs) were prepared as described in (24) and primary chicken glia were obtained from E9 chicken brains as described (27, 28).

All cells were grown at 37°C in a humidified atmosphere of 10% CO_2 in air. For binding assays, cells were harvested in a divalent cation-free buffered medium consisting of HBSS, lacking $MgCl_2$, $MgSO_4$, and $CaCl_2$ (Gibco Laboratories, Grand Island, NY), and including 20 mM Hepes, pH 7.5, and 5 mM EDTA. The cells were incubated with 5 ml of the buffer for 5–20 min depending on the cell type. The cells were recovered, spun in a clinical centrifuge for 3 min at 10,000 rpm and resuspended in 1 ml of binding buffer consisting of 1 × S-MEM (Gibco Laboratories), 20 mM Hepes, pH 7.5, 1 mM $CaCl_2$, and layered over a 9 ml 3.5% BSA gradient, spun in a clinical centrifuge for 3 min at 10,000 rpm, washed twice with binding buffer, resuspended, and counted using a Neubauer chamber. The cells were resuspended in binding buffer to a density of 6×10^5 cells/ml for the gravity assay or 1×10^5 cells/ml for the centrifugation assay and were kept on ice until use.

Different dissociation treatments were used to test the binding of L-929 cells to the cleaved and uncleaved FN3 fragment of cytotactin. These cells were harvested using 0.25% trypsin (2× recrystallized, Cooper Biomedical, Malvern, PA), 5 mM EDTA, a mixture of 5 mM EDTA and 0.25% trypsin, or 5 mM EDTA treatment followed by washing in divalent cation-free buffer and then 0.25% trypsin. All of these preparations were done in divalent cation-free medium. In all of these treatments, the cells were incubated for 5 min at 37°C, washed in 1 × DMEM (Gibco Laboratories) containing 10% calf serum, then washed two more times in binding medium and counted using a hemocytometer.

Centrifugal Cell Attachment Assay

In the centrifugation assay, the cells were briefly spun in a U-shaped well that had been precoated with the protein to be tested and blocked with excess albumin. The equilibrium between the centrifugal force that pulls that cell to the bottom of the well and the force of the cell-substrate adhesion determines the pattern observed in the well. On a nonadhesive substrate, the centrifugal force predominates and the cells are driven to the bottom of the plate into a tight pellet. As the strength of the cell-substrate adhesion increases, the cells become more likely to bind to the substrate as they contact it resulting in a ring pattern around the sides of the well. The diameter of the cell-free area inside this ring is used as a measure of the adhesivity of the substrate.

96-well polystyrene (Falcon 3910; Becton Dickinson and Co., Sunnyvale, CA) 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 blocking buffer containing 20 mg/ml of BSA in PBS, pH 7.5. A volume of 100 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 the ring diameter was measured.

Gravity Cell Attachment Assay

In the gravity assay, a single cell suspension is allowed to settle for a fixed period of time onto a substrate that has been coated with the different proteins to be tested (24). After washing the unbound and loosely bound cells, the attachment and morphology of those cells remaining on the dish were analyzed.

To prepare protein-coated substrates, eight separate drops (1.5 μl each in PBS) containing different concentrations of protein were placed in a circular array near the center of a polystyrene dish (Falcon 1008) in a humid atmosphere to prevent drying. After 30 min, the dish was washed three times with PBS, and incubated with blocking buffer for a minimum of 30 min. After blocking, the dish was rinsed once with PBS, and 250 μl of a cell suspension was added. The cells were incubated at 37°C, typically for 55 min, except where noted, and washed three times in PBS with gentle swirling. The bound cells were fixed with 1% glutaraldehyde, observed by phase-contrast microscopy, and counted using a 10 or 20 × objective and an eyepiece reticle. Cells were counted in four predetermined fields that when 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 was determined visually.

Iodination of Fusion Proteins

The fusion proteins were iodinated using enzymatic iodination with a mixture of lactoperoxidase and glucose oxidase immobilized onto hydrophilic microspheres (Enzymobeads; Bio-Rad Laboratories, Richmond, CA). ~200 μg of fibronectin and 1 mg of fusion proteins were iodinated at a time. To a solution of 10 mg/ml of fusion protein (500 μl) in 0.2 M phosphate buffer, pH 7.5, 350 μl of Enzymobead reagent was added, followed by 125 μl of 2% glucose and 2–3 mCi of Na ^{125}I (New England Nuclear, Boston, MA) (100 mCi/ml). The iodination was allowed to proceed at room temperature for 40 min, and the reaction was terminated by passing the mixture through a gel filtration column PH-19 Sephadex G-25M (Pharmacia). The iodinated protein was eluted with 6 ml of PBS. The first 2 ml of PBS were discarded and the next 4 ml were collected in 1-ml aliquots. The samples were dialyzed against PBS at 4°C for 8 h, with three changes of four liters each. The iodinated proteins were stored at 4°C and the protein concentration was determined by the modified Lowry method as described above to determine the specific activity. The purity of each preparation was assessed by SDS-PAGE using 10–12% gels (44) under reducing conditions, followed by autoradiography.

Determination of Protein Binding to Plastic

The iodinated proteins were diluted using PBS or PBS solutions containing 10, 30, or 100 μg/ml of fibronectin. A volume of 1.5 μl was spotted on polystyrene dishes (four drops per plate), and incubated in a humidified atmosphere for 30 min at room temperature. During the incubation, the diameter of the drop was measured through a 2.5 × lens using a micrometer.

After 30 min, the dishes were blocked overnight with blocking buffer. The dishes were rinsed once with PBS, and each dish was cut in four sections, each of which contained an individual radioactively labeled area, and sections were then counted. From the diameter of each drop and the specific activity of each protein, the amount of protein bound per square millimeter was determined. A similar protocol was followed to determine binding to 96-well dishes using 40 μl of protein solutions per well for 30 min. The wells were then blocked for 1 h, washed, cut, and counted.

Results

Expression and Characterization of Cytotactin Fusion Proteins

Seven different fusion proteins, synthesized and named as described in Materials and Methods, together represented almost the entire cytotactin molecule (Fig. 1) with the exception of the disulfide-rich amino-terminal portion and a small portion of the fibronectin type III repeats I and II. The purity of the fragments was assessed by SDS-PAGE (Fig. 2 *A*) and by Western blotting of the fragments (Fig. 2 *B*). The FN3 and Fg fusion proteins appeared as single molecular species. EGF, FN2, and FN1 exhibited multiple species each of which showed antibody reactivity by Western blotting, suggesting that the multiple components arose from proteolysis during the purification procedure. The fusion proteins were cleaved proteolytically as described in Materials and Methods with either thrombin or activated factor Xa. They were further purified to remove the bacterial GST-cleaved portion as shown in Fig. 2 *A* for EGF, FN1, and FN3 before testing them for cell binding activity (see below). After cleavage, the FN2 fragment could not be purified in sufficient yield from the GST protein for further analysis. The fibrinogen fragment was in GEX-2T and the GST portion was cleaved and recovered. Since fibrinogen is a physiological substrate of thrombin, an intact protein was not recovered and therefore this fragment was tested only in its uncleaved state.

The calculated molecular masses of the fusion proteins differ from the apparent molecular masses shown in Fig. 2. The following calculated molecular weights for the uncleaved fusion proteins were used to prepare solutions of the appropriate molarity: FN1, 72.9 kD; FN2, 103.0 kD; EGF, 72.6 kD; FN3, 44.4 kD; FNFg1, 55.1 kD; Fg, 63.3 kD; and FNFg2, 82.6 kD. The contribution of the glutathione-S-transferase portion of the fusion proteins in each fragment is 26 kD, which served as control (CON) in all of the studies described below.

Cell Attachment and Morphology on Surfaces Coated with Cytotactin Fragments

Cell attachment and morphology in the presence of the different cytotactin fragments were determined in the gravity assay (24) (Figs. 3 and 4). Representative cell lines from three different cell types were used: fibroblasts (NIH-3T3, Fig. 3, *3T3* and Fig. 4), neurons (neuro2A, Fig. 3, *N_2A*), and glia (Fig. 3, *U251MG*). The binding of each protein to the plastic culture dishes was quantitated as described in Materials and Methods and was proportional to the coating concentration in each case in which a single molecule was coated on the surface.

All cell types attached well on fibronectin-coated surfaces (Fig. 3, *FN*); NIH-3T3s and U251MGs spread after attachment, and neuro2A cells extended processes. Fewer cells of each cell type bound to cytotactin than to fibronectin, however (Fig. 3, *CT*). The neuro2A cells did not attach at all and, consistent with previous results, NIH-3T3 and U251MG cells attached but remained round (24, 34). When cell attachment to surfaces coated with each of the cytotactin fragments at the same molar concentration was compared, striking differences were observed both among the cell types and among the different fragments. Few cells bound to the EGF fragment (none for neuro2As) (Fig. 3, *EGF*), and the cells that did bind remained round. The NIH-3T3 and U251MG cells attached and spread on fragments FN1 (Fig. 3, *FN1*) and FN2 (Fig. 3, *FN2*); at high fragment concentrations, cells completely covered the area coated with the fragment. The binding of neuro2A cells to these fragments was much lower than that observed on fibronectin, although the bound cells

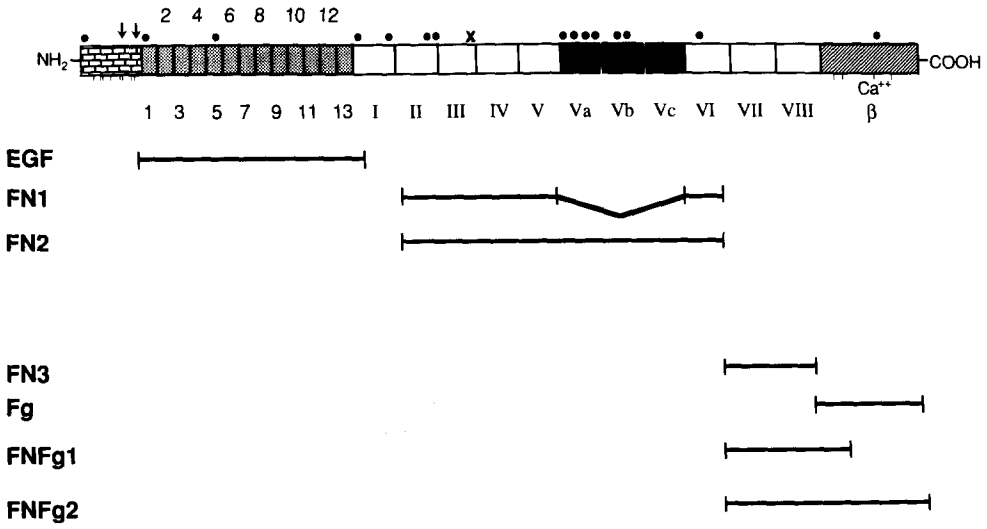

Figure 1. Model of cytotactin and pGEX fusion proteins. The primary structure of cytotactin is shown at the top of the figure (38). (▤) amino-terminal region; (▨) EGF-like repeats; (□) FN type III repeats; (■) alternatively spliced repeats; and (▨) fibrinogen region. The dots above the structure represent potential glycosylation sites, small lines below denote cysteine residues. The arrows are potential glycosaminoglycan addition sites and the RGD site is represented by a cross. The various cytotactin fusion constructs made in pGEX vectors are shown below the primary structure and labeled as presented in the text (see Materials and Methods).

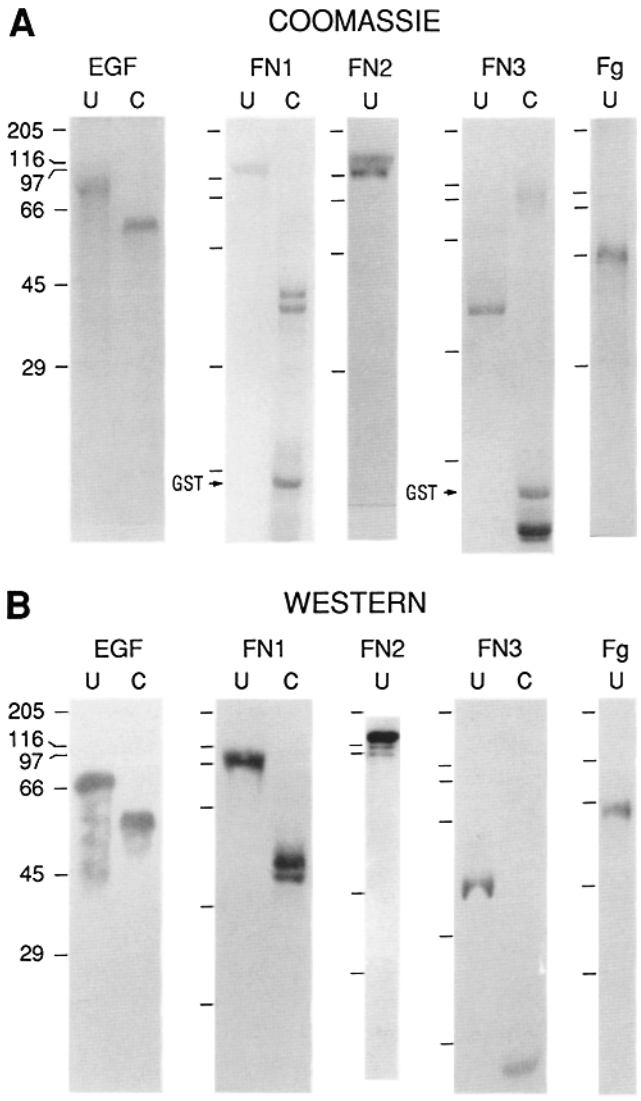

Figure 2. Protein analysis of uncleaved and proteolytically cleaved cytotactin fragments. 10–12% SDS-PAGE gels under reducing conditions were stained using Coomassie blue. EGF, FN1, and FN3 are shown in their uncleaved (*U*) and cleaved (*C*) forms. FN2 and Fg are shown in their uncleaved form. The proteins (0.5–5.0 μg) were loaded in the order indicated in the figure. (*B*) Western blot analysis of the cleaved and uncleaved cytotactin fragments as described above. The fusion proteins and the cleaved proteins show strong immunoreactivity using a polyclonal cytotactin antibody. The GST bacterial portion does not show cross reactivity with this antibody (compare lanes labeled FN1 and FN3 in *A* and *B*). The molecular weights × 10^{-3} of standard proteins are shown at the left of each panel.

started extending processes. None of the cell types tested bound to FN3 (Fig. 3, *FN3*). NIH-3T3 and U251MG cells and a few neuro2A cells bound to fragment Fg (Fig. 3, *Fg*); the cells were essentially round but began to show small processes over the time course of the experiment. Little binding was observed for any of the cell types on the FNFg2 fragment (not shown). This result suggested that the FN3 fragment may antagonize the adhesive effects observed for the Fg fragment (see below).

To demonstrate that cell attachment activities onto surfaces coated with the different cytotactin fusion proteins did not result from artifacts due to the bacterial portion (GST) of the fusion protein, it was removed from the fragments by proteolytic cleavage as described in Materials and Methods. The cleaved fragments that were then tested presented adhesive properties identical to the uncleaved proteins. When cleaved FN1 was tested for cell attachment and spreading, it showed the same activity as the intact fusion protein (Compare Fig. 3, *FN1* with Fig. 4 *B*). For the cleaved EGF fragment, low levels of adhesion with cell rounding was observed whereas no adhesion was observed for cleaved FN3 (Fig. 4, *A* and *C*). The GST fragment showed no adhesive activity in either the gravity (Fig. 4 *D*) or centrifugation assays (not shown) and served as a control for the experiments in which the uncleaved proteins were used.

Because earlier studies had shown that proteins that did not support high levels of cell attachment in the gravity assay could nevertheless show activity in other assays, we examined the activity of each fragment in the centrifugation assay. This assay allows the observation of interactions that are undetectable in a gravity assay where weaker interactions of cells with the substratum allow the cells to be washed off (24). Using this assay, cell binding was observed for all fragments with the exception of FN3 (Table I). As shown in the table, the binding of a variety of cell types was scored using both assays and found to be qualitatively similar within a given cell type.

Dependence of Cell Attachment on Concentration of Cytotactin Fragments

To determine whether the attachment of the three cell types to the different fusion proteins is concentration dependent and saturable, the binding of NIH-3T3, U251MG, and neuro2As was determined using the centrifugation assay (Fig. 5). To normalize the response of cells to the different fragments, the diameter of the cell-free region within the ring of cells in the centrifugation assay was plotted with respect to moles of protein actually bound to the wells as determined by binding of labeled molecules as described in Materials and Methods.

Cell attachment was proportional to fragment concentration and was saturable for each cell type and each cytotactin fragment tested (Fig. 5). Differences were observed both in the amount of fragment at which the maximal measurable cell binding was obtained and in the maximal binding achieved as measured by the diameter of the ring of cells that adhere along the sides of the well. (These two critical parameters were calculated from the graphs in Fig. 5 and are shown separately in Table II). These are both useful parameters to assess the efficacy of cell attachment to the different proteins. It should be stated, however, that the mechanisms resulting in differences in cell binding cannot be determined from this type of assay alone. Differential cell binding may be the result of differences in receptor affinity or number, or to secondary effects such as those involving the cytoskeleton or second messenger systems.

Cell attachment to FN1 and FN2 was quantitatively similar among the cell types (Fig. 5, *A–C*), but for each cell type, differences were observed between FN1 and FN2. The amount of fragment at which cell binding became saturated and the maximal ring diameter at saturating fragment

WHOLE MOLECULES
CYTOTACTIN FRAGMENTS
FN
CT
EGF
FN1
FN2
FN3
Fg
3T3
N2A
U251MG

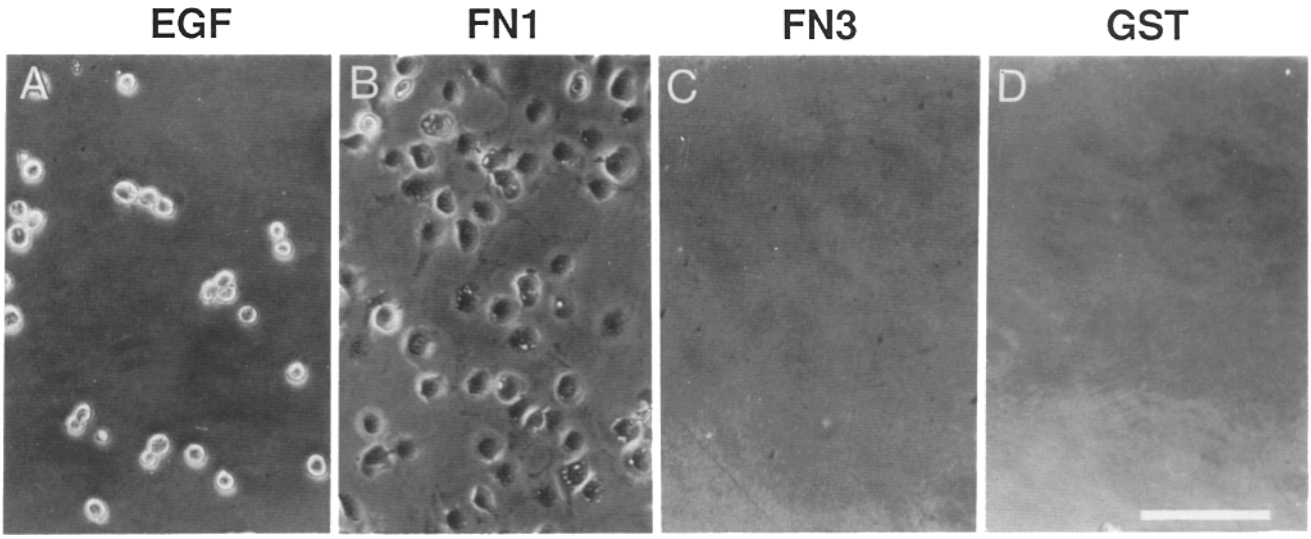

Figure 4. Cell attachment to fusion proteins after removal of GST by proteolysis. NIH–3T3 fibroblasts were allowed to bind in the gravity assay to plastic substrates coated with cytotactin fragments after removal of the GST bacterial portion with activated factor Xa (*FNI*) or thrombin (*EGF* and *FN3*). The coating concentrations of the proteins are: EGF, 12 μM (*A*); FN1, 2.8 μM (*B*); FN3, 11 μM (*C*); and to GST, 11.5 μM (*D*). Bar, 50 μm.

amounts are shown in Table II. Maximal cell attachment of U251MG cells and neuro2A cells was achieved at lower amounts of FN2 than FN1. However, for each cell type, the ring diameter was larger for FN1 than for FN2. These results suggest that the receptors mediating the binding to FN1 and FN2 may not be identical on all cell types.

The adhesive properties of the EGF domain of cytotactin were evident when the centrifugation assay was used (Fig. 5, *D–F*) in contrast to the poor binding observed in the gravity assay (Fig. 4 and Table I). The adhesion of NIH-3T3 cells (Fig. 5 *D*), U251MG cells (Fig. 5 *E*), and neuro2A cells (Fig. 5 *F*) to the EGF domain was approximately half of that observed for the Fg domain. It is notable, however, that the attachment of each of the cell types saturates at a very low amount of the EGF fragment. This may reflect differential receptor number or affinity for this region of the molecule.

The concentration curve for the adhesion of neuro2A cells (Fig. 5 *F*) to the Fg domain was strikingly different from that

*Table I. Quantitation of Binding of Cell Types in Gravity and Centrifugation Assays**

	Gravity					Centrifugation				
	EGF	FN1	FN2	FN3	Fg	EGF	FN1	FN2	FN3	Fg
Fibroblasts										
NIH3T3	+/–	++++	+++	–	+++	1.4	3.4	2.8	0	3.4
CEF	+/–	++++	+++	–	+++	1.2	3.4	2.1	0	3.4
L929	–	+++	++	–	++	0.7	3.4	1.0	0	3.4
Glia										
Primary glia	–	+++	+++	–	+++	0.7	2.1	1.5	0	3.4
U251MG	+/–	+++	++	–	++	0.5	3.4	0.5	0	2.8
G2624	–	+++	++	–	+++	0.5	3.4	1.0	0	3.4
Neurons										
Neuro2A	–	++	+	–	+	0.3	2.4	1.4	0	1.7
SK-N-SH	–	+++	–	–	–	0.3	3.4	1.0	0	2.1
IMR	–	+	–	–	++	0.7	3.4	2.4	0.3	2.8
Other										
S180-L-CAM	–	++	–	–	–	1.0	2.1	1.0	0	1.4
P3U myeloma	–	++	–	–	–	0	1.7	0	0	0

* The symbols represent relative cell attachment to fragment-coated substrates. In a representative experiment the cell number in an area of 0.13 mm^2 would be the following: ++++, 690 cells; +++, 540 cells: ++, 370 cells; +, 185 cells. In the gravity assay the FN1, FN2 and Fg fusion proteins mediated very good cell attachment, EGF poor cell attachment, and FN3 only mediated cell attachment after trypsinization of the cells.

Figure 3. Cell attachment to fibronectin, cytotactin, and cytotactin fusion proteins. 3T3 fibroblasts (*3T3*), neuro2A neuroblastoma cells (N_2A), and U251MG glioma cells (*U251MG*) were allowed to bind in the gravity assay to plastic substrata coated with proteins as described in Materials and Methods. The first two columns are substrata coated with either fibronectin (*FN*) or intact cytotactin (*CT*); the remaining columns are substrata coated with the indicated fusion proteins labeled as in Fig. 1. The coating concentration for each fragment was 12 μM; for fibronectin, 0.3 μM; and for cytotactin, 0.6 μM. Bar, 50 μm.

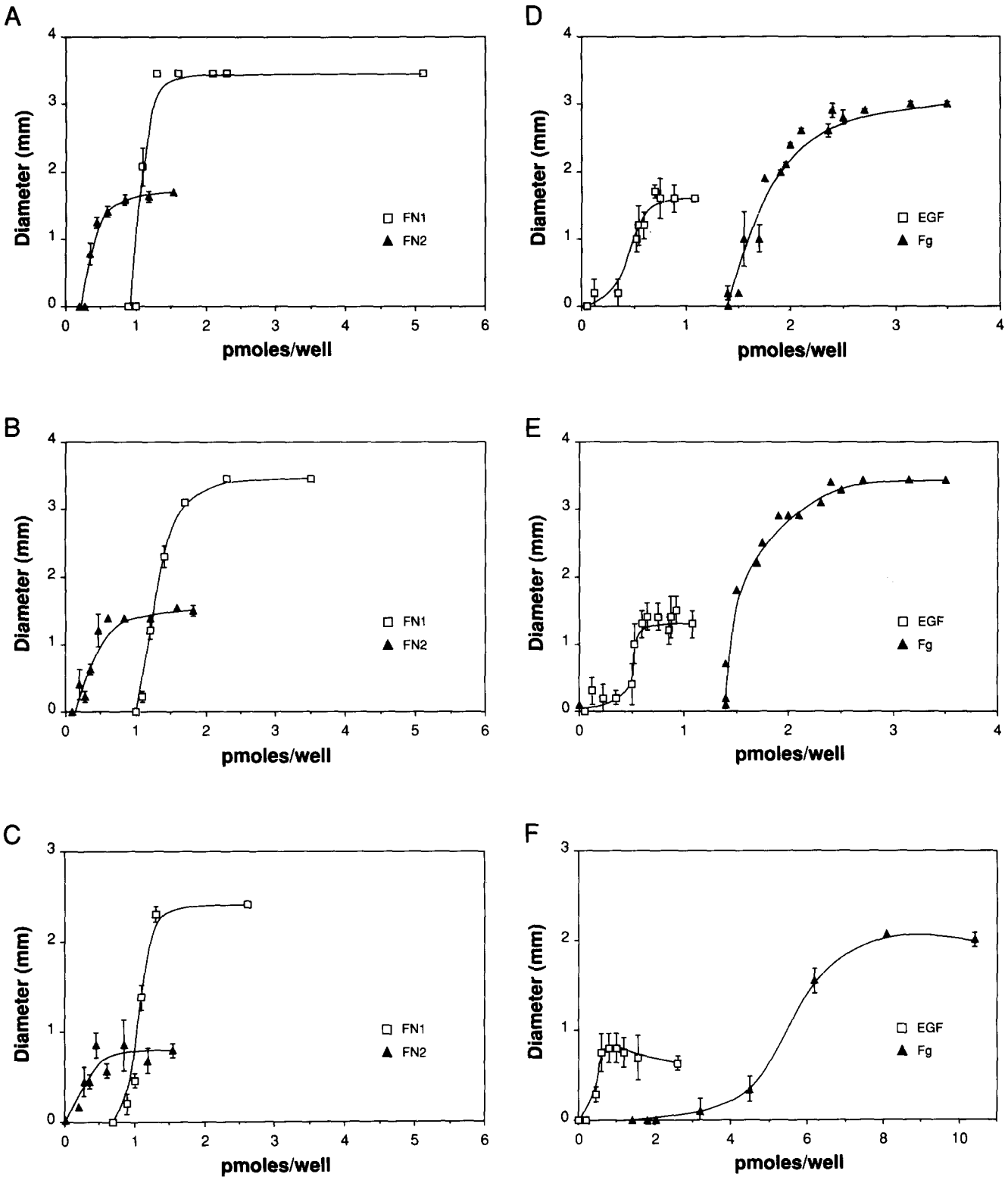

Figure 5. Quantitation of cell attachment to cytotactin fusion proteins. 96 well dishes were coated with decreasing concentrations of fusion proteins and incubated either with NIH-3T3 cells (*A* and *D*), U251MG cells (*B* and *E*) and neuro2A (*C* and *F*). The ring diameter in the centrifugation assay is plotted versus the amount of the fragment that bound to the plate as determined by binding of labeled molecules to parallel dishes; (*A–C*) (□) FN1; and (▲) FN2. (*D–F*) (□) EGF; and (▲) Fg. Bars represent SEM.

*Table II. Maximal Values for Cell Binding in the Centrifugation Assay**

Fragment	NIH 3T3 Cells		U251MG Cells		N2A Cells	
	pmoles of fragment	Ring diameter	pmoles of fragment	Ring diameter	pmoles of fragment	Ring diameter
FN1	1.3	3.6	2.2	3.3	1.3	2.3
FN2	0.7	1.7	0.7	1.4	0.5	0.8
Fg	2.2	2.6	2.3	3.2	7.6	2.3
EGF	0.7	1.6	0.4	1.3	0.8	2.0

The pmoles of fragment and the ring diameter at saturation of binding in the centrifugation assay are shown for ease of comparison between fragments and between cell types.
* Extracted from Fig. 5.

obtained for fibroblasts and glial cells. The amount of fibrinogen fusion protein required for cell attachment was approximately six times higher for the neuro2A cells than for the fibroblasts and glial cells.

Trypsinization Uncovers Cell Attachment Activity to FN3

The observations that fragment FN3 showed no adhesive activity in the gravity or centrifugation assays contrasted with earlier results of Spring et al. (66) who had proposed that a cell binding site was localized within this region on the basis of tests on trypsinized cells. To investigate whether the differences between their study and the present results were due to the cell type used, the type of fusion protein, or to the method by which the cells were prepared and assayed, the experiment was repeated with cytotactic fragments following four protocols, including that of Spring et al. (66) and with the cell line L929 as used previously (66). Four different methods of cell dissociation were tested (Fig. 6): 0.25% trypsin alone (Fig. 6, *A–C*), 5 mM EDTA alone (Fig. 6 *D*), 5 mM EDTA followed by washing and subsequent treatment with 0.25% trypsin (Fig. 6 *E*), a mixture of 5 mM EDTA

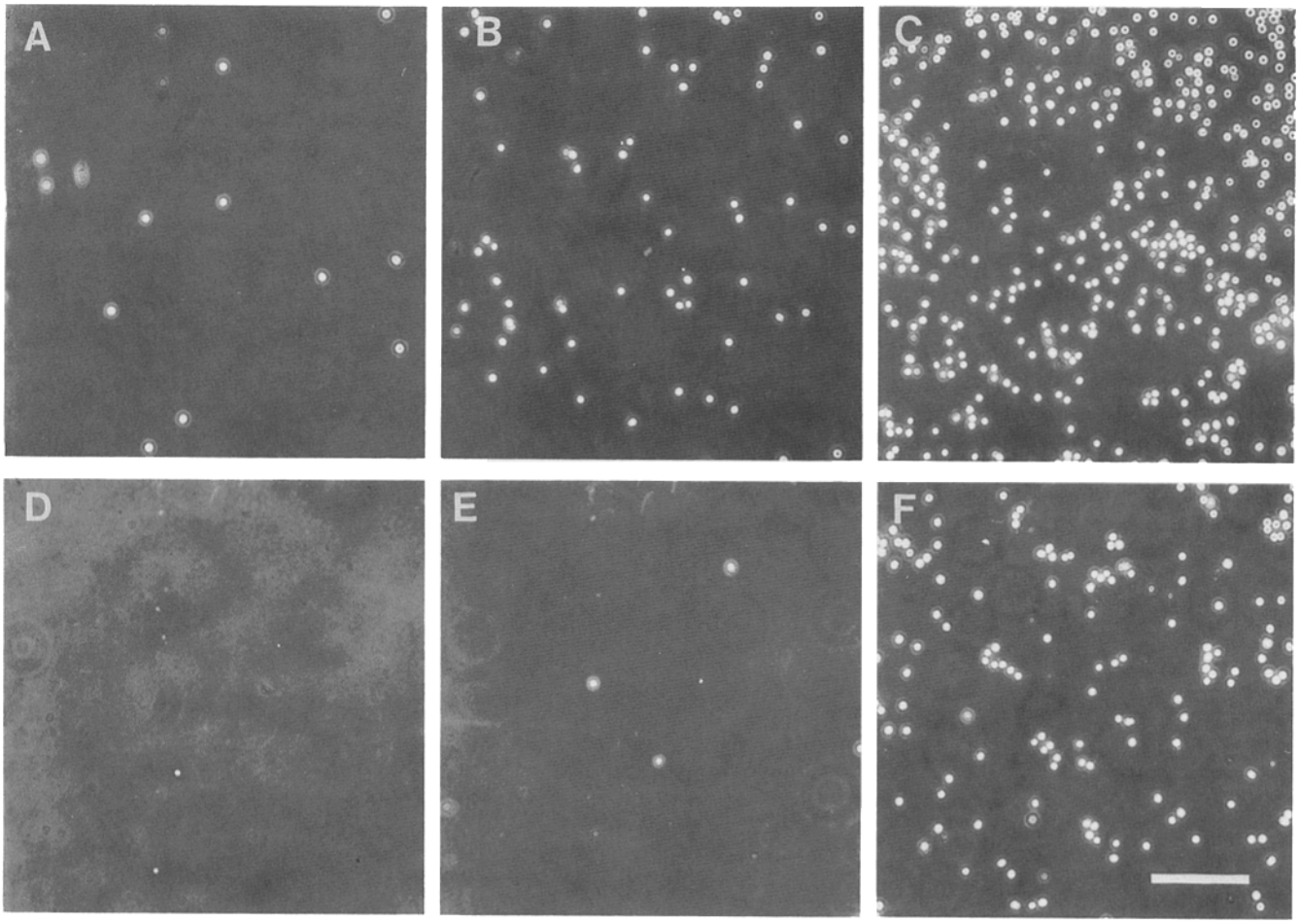

Figure 6. Trypsin treatment reveals biding to FN3. L929 cells initially dissociated with 0.25% trypsin (*A–C*) were plated onto tissue culture plastic dishes and coated with solutions containing decreasing concentrations of FN3; 12 μM (*A*); 4 μM (*B*); 1.3 μM (*C*). Cells dissociated with 5 mM EDTA (*D*), 5 mM EDTA followed by 0.25% trypsin (*E*), or a mixture of 5 mM EDTA and 0.25% trypsin (*F*) were plated onto substrates coated with 1.3 μM FN3. Bar, 100 μm.

and 0.25% trypsin (Fig. 6 *F*). After each of these treatments, the cells were further treated as described in Materials and Methods, resuspended in binding medium containing calcium, and tested on the substrates coated with each of the fusion proteins and with fibronectin.

When cells were prepared by different methods, dramatic differences were found in cell attachment to FN3 (Fig. 6) but not to other fragments (not shown). Cells treated with trypsin alone attached well to the surface coated with FN3 (Fig. 6, *A–C*) and remained round. Cells attached to the FN1 coated area in the same dishes were spread suggesting that the round morphology on FN3 did not result from the method of cell preparation. Cells prepared with EDTA alone did not attach to FN3-coated plastic (Fig. 6 *D*), similar to the results described above for other cell types. Cells treated with EDTA and then with trypsin (Fig. 6 *E*) exhibited low binding similar to that in cells treated with EDTA alone. Cells prepared by treatment with trypsin together with EDTA (Fig. 6 *F*), however, bound almost as well as cells prepared by trypsin treatment alone. These results suggest that trypsin treatment reveals a cell binding activity for FN3. Moreover, the molecules coated on both tissue culture plastic dishes and polystyrene dishes also gave similar results, suggesting that cell attachment to FN3 is not specific for the particular type of plastic substrate.

When cell attachment was compared on surfaces coated with decreasing concentrations of the FN3 fragment (Fig. 6, *A–C*), the highest cell binding was found on the area coated with the lowest concentration of fragment (Fig. 6 *C*). The adjoining uncoated area of the dish was completely devoid of cells. This inverse proportionality between cell attachment and fragment concentration suggests that an activity which prevents attachment exists in this domain and is revealed at high fragment concentration (see below).

Effect of Cytotactin Fragments Mixed with the Permissive Substrate Fibronectin

The observation that a cytotactin domain can prevent cell attachment and flattening is consistent with previous reports (34, 66). To determine whether any of the different cytotactin fragments modify cell binding to a positive substrate, mixtures containing a constant concentration of fibronectin (0.3 μM) and either of two concentrations of the fragments (12 and 1.3 μM), were tested using the gravity assay (Fig. 7).

Among the cytotactin fragments shown in Fig. 1, three were found to decrease cell attachment significantly when mixed with fibronectin, relative to the attachment observed on fibronectin alone (Fig. 7). The decrease in cell attachment was ~30% in mixtures of fibronectin with FN2 and ~50% when either EGF or FN3 were present in the mixture at high concentration. This effect was concentration dependent; at a fragment concentration of 1.3 μM the number of attached cells increased for each fragment; although for the EGF and FN3 fragments, the binding was still significantly lower than the control.

The inhibition by FN2 was unexpected, given that FN2 promoted cell attachment and spreading in the gravity assay in the absence of fibronectin (Fig. 3). In this experiment, at high concentrations of FN2, cells were significantly more aggregated than in the presence of any of the other fragments. This made the accurate counting of the bound cells difficult, possibly accounting for the lower numbers of attached cells.

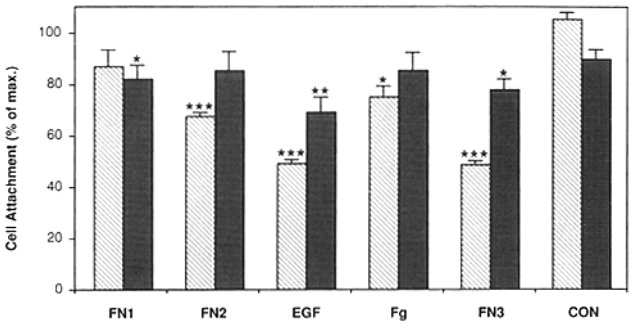

Figure 7. Cell attachment to fibronectin and cytotactin fragment mixtures. NIH-3T3 cells were plated onto polystyrene-coated dishes with solutions containing mixtures of 12 μM (□) or 1.3 μM (□) cytotactin-fusion proteins and 0.3 μM fibronectin. Fibronectin was mixed with BSA to determine the maximal attachment for individual plates. The cells attached in four different fields were counted and averaged. The error bars represent SEM. The asterisks above each bar indicate the level of significance compared with the fibronectin control as judged by the *t* test; no asterisk indicates not significant. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

Counteradhesive Properties of the EGF and FN3 Domains of Cytotactin

The previous experiments suggested a counteradhesive activity within both the EGF and FN3 fragments. As mentioned above, the amount of protein adsorbed to the plastic substrate is proportional to the concentration of the coating solution. In mixture experiments, the presence of one protein might alter in some way the binding of the other. To address this possibility, mixtures of fibronectin and FN3 or EGF were prepared and cell attachment was correlated with the concentrations of proteins bound to the plastic (Fig. 8). Mixtures of 30 μg/ml of fibronectin and varying concentrations (1–300 μg/ml) of EGF (Fig. 8 *A*) or FN3 (Fig. 8 *B*) were tested. Each fragment inhibited cell attachment when high concentrations were mixed with fibronectin. At the highest fragment concentrations, cell attachment to fibronectin in the presence of the EGF fragment was 40% of maximal (Fig. 8 *A*, ■, left axis) and attachment in the presence of the FN3 fragment was only 10% of maximal (Fig. 8 *B*, ■, left axis).

When a constant amount of iodinated fibronectin (30 μg/ml) was mixed with increasing concentrations of the EGF fragment, the amount of labeled fibronectin bound to the polystyrene remained constant over the concentration range from 1–300 μg/ml of the EGF fragment (Fig. 8 *A*, △, right axis). Similarly, the same amount of iodinated EGF fragment bound to the polystyrene when it was mixed with either 10, 30, or 100 μg/ml of unlabeled fibronectin (not shown). Thus at a constant amount of fibronectin (30 μg/ml), increasing concentrations of EGF fragment do not decrease the amount of fibronectin bound to the plastic. Similar results were observed with mixtures of fibronectin with FN1, FN2, and Fg fragments. This indicates that the decrease in cell attachment is due directly to the presence of the EGF fragment and not to changes in the amount of fibronectin available for cell attachment.

A different result was found with the FN3 fragment, however. At increasing concentrations of FN3, decreasing concentrations of fibronectin bound to the plastic (Fig. 8 *B*, △, right axis). Conversely, increasing the concentration of fibronectin resulted in decreased binding of FN3 to the plastic

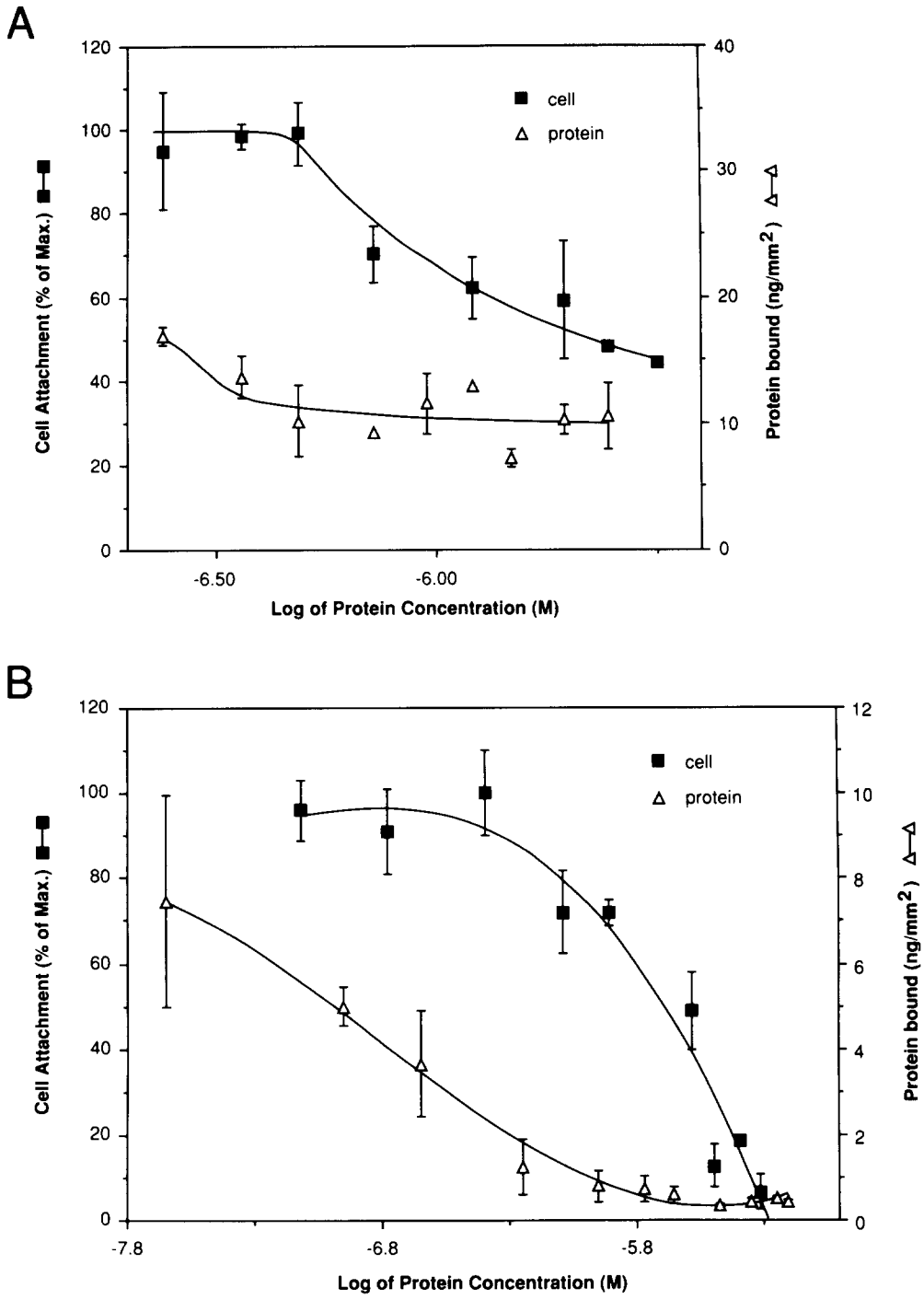

Figure 8. Concentration-dependent inhibition of cell attachment to fibronectin by mixture with EGF and FN3 cytotactin fusion proteins. Mixtures of 30 μg/ml of fibronectin and concentrations ranging from 0.3 to 7 μM of either EGF (*A*, ■, left axis) or FN3 (*B*, ■, left axis) were coated on polystyrene dishes and the percent of the maximum cell attachment of NIH-3T3 cells was measured in the gravity assay. On the right y axis the amount of iodinated fibronectin (ng/mm^2) (△ in *A* and *B*, right axis) bound to the plastic at each coating concentration of fusion protein is shown. Error bars represent SEM.

(not shown). The presence of FN3 interferes with fibronectin binding to a plastic substratum. At the highest concentration of EGF fragment, the concentration of fibronectin was reduced 20-fold. It cannot be concluded from the mixture experiments with FN3, therefore, that FN3 inhibits cell binding to fibronectin. It is nevertheless clear from the results using trypsinized cells described above and from competition experiments (see below) that this fragment has counteradhesive activity.

Inhibition of Cell Attachment by Soluble Cytotactin Fragments

To substantiate further that the counteradhesive fragments acted by binding to the cell surface, a number of competition experiments were done. In contrast to cells preincubated with the control protein, BSA, (Fig. 9 *A*), preincubation of NIH-3T3 cells with cytotactin fusion proteins EGF (Fig. 9 *B*), FN3 (Fig. 9 *C*), or with cytotactin itself (not shown) all decreased cell attachment to fibronectin-coated surfaces. It appears therefore that the interaction of cytotactin fragments with the cell surface is sufficient to inhibit cell attachment and spreading on fibronectin.

Competition experiments were also used to determine whether the preincubation of cells with FN1, FN2, or intact cytotactin could alter the ability of the cell to attach to substrates coated with FN1. After preincubation, the cells were

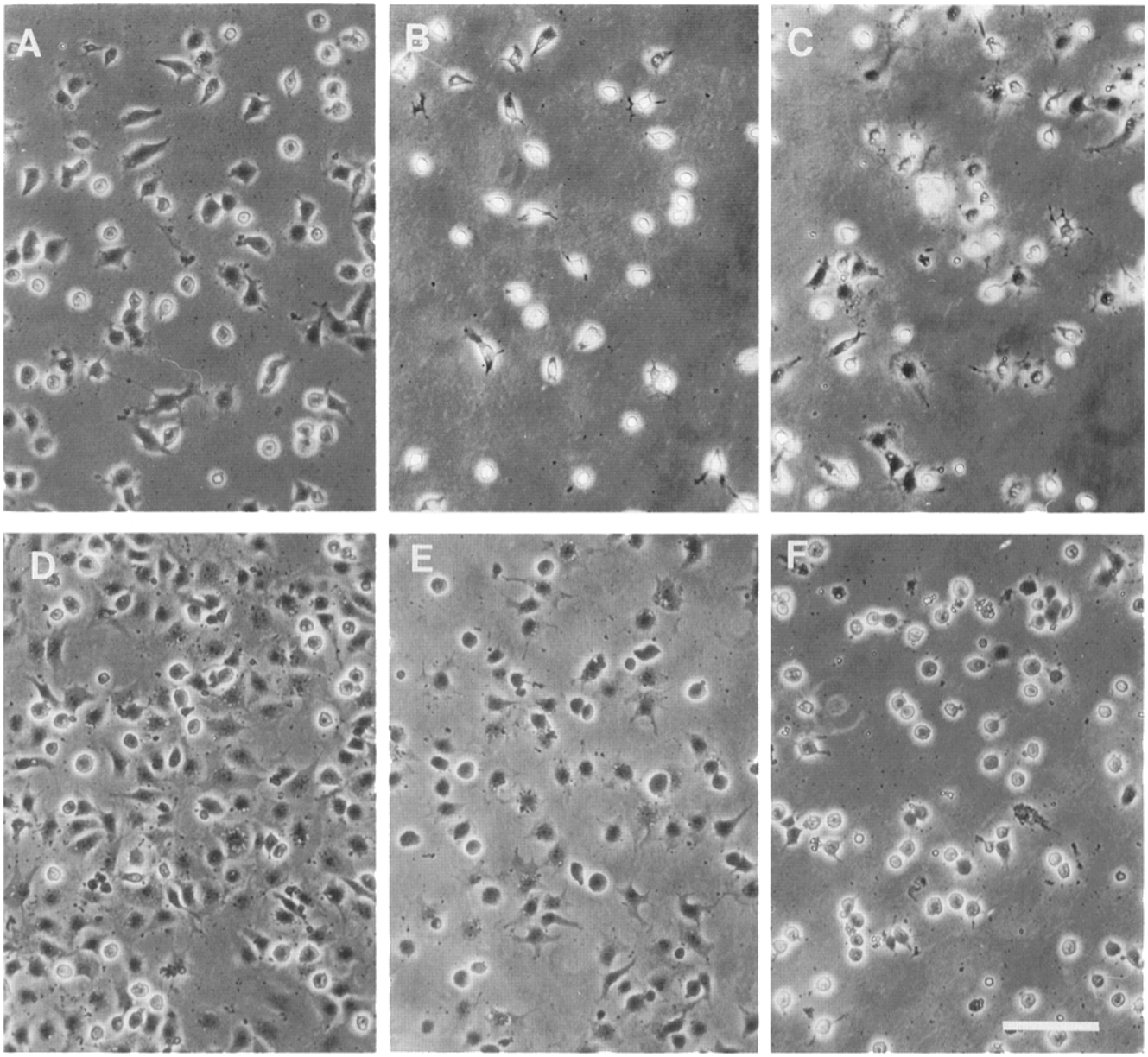

Figure 9. Inhibition of cell attachment to substrates by cell preincubation with soluble molecules. (*A–C*) Polystyrene dishes coated with a solution of 100 μg/ml of fibronectin were incubated with cells that had been preincubated with 2 mg/ml of BSA (*A*); EGF (*B*); or FN3 (*C*). The cells that had been preincubated with EGF and FN3 show a rounded morphology when compared with those preincubated with albumin (*A*). (*D–F*) Polystyrene dishes coated with 12 μM FN1 were incubated with cells that had been preincubated with 2 mg/ml of BSA (*D*), FN1 (*E*), or intact cytotactin (*F*). Fewer cells were observed when the cells were preincubated either with FN1 (*E*) or with cytotactin (*F*). In addition, a dramatic change in morphology was observed in cells preincubated with cytotactin (*F*). Bar, 50 μm.

washed twice to remove the excess proteins. As shown in Fig. 9, cells preincubated with albumin attached and spread on FN1 (Fig. 9 *D*) in a fashion similar to unincubated cells (compare with Fig. 3). Cell binding and spreading were both reduced when the cells were preincubated with 30 μM FN1 (Fig. 9 *E*) or with 10 nM purified cytotactin (Fig. 9 *F*). Preincubation of the cells with cytotactin had a most dramatic effect on cell number and morphology; fewer cells attached to the substrate and the cells that did attach remained round (Fig. 9, compare panels *D* and *F*).

These results were quantitated using different concentrations of FN1 coated on the substrate (Fig. 10). When cells were preincubated with 30 μM FN1 or 20 μM FN2, cell binding was reduced to 10–40% of maximal at FN1 coating concentrations lower than 7 μM, as compared with control cells preincubated with albumin. At higher coating concentrations of FN1, soluble FN2 was a less effective inhibitor than FN1. The fact that soluble molecules inhibit cell attachment to the immobilized molecule suggests that the binding of NIH-3T3 cells to FN1 occurs through specific cell surface receptors.

Discussion

Using fusion proteins generated in bacterial expression vectors, we have identified and characterized at least four dis-

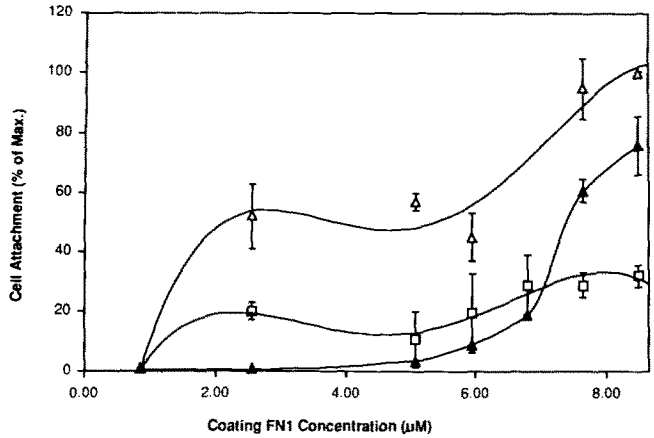

Figure 10. Inhibition of cell attachment to FN1 by cell preincubation with soluble BSA, FN1, and FN2 fragments is concentration dependent. Polystyrene dishes were coated with concentrations of FN1 ranging from 1 to 9 μM. The cells were preincubated with 2 mg/ml of soluble BSA (△), FN1 (□), or FN2 (▲). The maximum number of cells that attached to the highest concentration of FN1 is defined as 100%. The relative cell number in the presence of each soluble protein in plotted versus the concentration of the FN1 that was used to coat the plastic. Error bars represent SEM.

tinct, nonoverlapping regions in cytotactin that affected the cell attachment and morphology of three cell types, fibroblasts, glia, and neurons. Fragments FN1, FN2, and Fg (see Fig. 1) promoted cell attachment when coated onto substrates. Different cellular morphologies were observed after binding on these fragments; FN1 and FN2 allowed cells to spread whereas cells plated on Fg remained round. The EGF and FN3 regions were also able to inhibit cell attachment and spreading on fibronectin, and thus represent counteradhesive substrates. Different methods were required to establish these findings. For example, binding to the EGF domain was revealed in centrifugation assays and cell attachment to FN3 was uncovered by trypsinization of the cells and was also demonstrated in competition assays. Quantitative studies showed that cell attachment to cytotactin domains is concentration dependent and saturable. These data are summarized in Table III and they are consistent with the hypothesis that independent cell surface receptors interact with the different protein regions of the molecule. Given the identical biological activities of the cleaved and uncleaved proteins, as well as their similarity to proteolytic fragments from the same regions of the native molecule, it is likely that these properties represent functions of the intact protein. This is supported by preliminary results of the analysis of the secondary structure of the fusion proteins by circular dichroism. The results obtained indicated that both cleaved and uncleaved FN3 and uncleaved FN1 have significant β-pleated sheet structure that would be expected for fibronectin type III repeats. This is in contrast with the results obtained for Fg and EGF that showed a lower percentage of β-structure. It should also be noted that fusion proteins made in bacterial expression systems such as those used here and by Spring et al. (66) lack the normal carbohydrates present in the native molecule. Given that native chicken cytotactin contains 17 potential glycosylation sites as well as two sites for the potential addition of chondroitin sulfate (33, 39), it is possible that the presence of appropriately linked carbohydrate may also be important in cytotactin functions.

The fragments FN1 and FN2 that contain the proximal fibronectin type III (II–VI) repeats mediated cell attachment and spreading of most cell types tested, as did fibronectin. At decreasing concentrations of these fragments, the proportion of round to spread cells increased; this phenomenon has also been observed for fibronectin (75). Chicken cytotactin contains a single arginine-glycine-aspartate (RGD) tripeptide, located in the third fibronectin type III repeat, which is present in both FN1 and FN2. RGD tripeptides have well-characterized binding activity to the integrin family of cell surface receptors (36). The present results are consistent with previous reports of RGD-dependent binding of cells to cytotactin (5, 24) and with recent reports that cytotactin binds to two members of the integrin family, $\alpha\beta1$ (a novel alpha subunit) (Mendler, M., A. Priest, and M. A. Bourdon. 1991. *J. Cell Biol.* 115:137a) and $\alpha_v\beta3$ (Joshi, P., I. Aukhil, and H. P. Erikson. 1991. *J. Cell Biol.* 115:134a; Mendler, M., A. Priest, and M. A. Bourdon. 1991. *J. Cell Biol.* 115: 137a) and that the binding can be inhibited by peptides containing the RGD sequence. Preliminary results (A. L. Prieto and K. L. Crossin, unpublished results) using the peptide GRGDS and its control GRADS showed that the cell spreading onto FN1 was clearly reduced by preincubation of cells with the active peptide, but not with the control. It should be noted, however, that the RGD sequence in cytotactin is not conserved among species. It is absent from the mouse (73) and newt (58) sequences, but is present in the human (31) and chicken (38, 66) sequences. Additional experiments using smaller fusion proteins will be required to determine

Table III. Summary of Cell Attachment Activities of Fibroblasts for Each of the Cytotactin Fragments

Fragment	Binding, gravity	Morphology	Binding, centrifugation	Inhibition of attachment to FN
			mm	
EGF	+/−	R	1.4	yes
FN1	++++	S	3.4	no
FN2	+++	S	2.8	no
FN3	−(++)*	−(R)*	0	yes
Fg	+++	R	3.4	no

As summarized in this table, two cytotactin fragments, FN1 and FN2, promoted cell spreading (*S*) on substrates coated with them. Two sustained a rounded (*R*) cell morphology, EGF and Fg. FN3 only promoted attachment and cell rounding after trypsinization of the cells. Using the more sensitive centrifugation assay, cell attachment to the EGF but not to the FN3 fusion proteins was observed. Both EGF and FN3 were able to inhibit cell attachment to fibronectin-coated substrates. The binding and centrifugation assays were scored as described in Tables I and II.
* Only after trypsinization.

whether the RGD included in FN1 and FN2 is responsible for the cell attachment and/or spreading of these fragments. Given the recent observation of disruption of focal contacts by the alternatively spliced type III repeats in human tenascin (57), additional cell binding sites may be present in FN2. This is also suggested by several of our binding analyses showing differential responses to FN1 and FN2. Given that the FN1 sequence is completely contained within FN2, the differential responses to FN1 and FN2 suggest additional sites and possible cooperative effects.

More than one activity was found in fragment FN3, which spans the VII and VIII fibronectin type III repeats. In cells dissociated with EDTA, no cell attachment to this region was detected by either the gravity or centrifugation assays. Cell attachment was uncovered, however, after the cells were dissociated with trypsin. The same results were obtained using the cleaved FN3 molecule. This result is consistent with the possibility of integrin-mediated cell attachment, inasmuch as integrins have been shown to be highly trypsin resistant in the presence of divalent cations (70). A large number of integrins bind proteins other than fibronectin (36, 60, 67), many of which, like FN3, lack an RGD sequence (17, 25, 30) and regions in fibronectin other than the RGD sequence have been shown to be important in integrin-mediated cell attachment (2). A cell attachment activity is located in the proximal (II–VI) fibronectin type III repeats and cell attachment to the distal (VII–VIII) fibronectin type III repeats was observed only in trypsinized cells. This multiplicity of variables that affects cell binding activity may explain differences among several previous reports (5, 12, 24, 66; Joshi, P., I. Aukhil, and H. P. Erikson. 1991. *J. Cell Biol.* 115:134a; Mendler, M., A. Priest, and M. A. Bourdon. 1991. *J. Cell Biol.* 115:137a) as to whether cells bind to intact cytotactin and whether this binding is sensitive to inhibition by RGD-containing peptides.

A counteradhesive activity was definitely present in FN3 since attachment was only observed at the lowest concentrations of FN3. Moreover, preincubation of cells with soluble FN3 also inhibited cell attachment and spreading on fibronectin. Thus, two activities are present in this fragment, one that promotes cell attachment of trypsinized cells and one that is counteradhesive and that prevents cell attachment on fibronectin.

The EGF and FN3 fragments have activities that counteract the adhesive effects of a permissive substrate such as fibronectin, as previously described for intact cytotactin (66). The effect of the FN3 region was difficult to quantitate since this molecule displaced fibronectin from a plastic substrate when the two molecules were mixed. The possibility that FN3 interacts with fibronectin to cause the displacement is under investigation. The EGF fragment allowed cell attachment in the centrifugation assay and weak attachment in the gravity assay; identical results were obtained for the cleaved and purified fragment. Competition experiments established that both EGF and FN3 inhibit cell attachment and spreading on fibronectin by binding to the cell surface. The inhibition of cell attachment by soluble fragments supports the hypothesis that receptor-mediated changes on the cell surface, rather than direct interference with fibronectin on the substratum, as suggested previously (46), are responsible for these counteradhesive effects. We refer to this effect as counteradhesive to stress that it is surface mediated, very likely through independent surface receptors, and not likely to be mediated directly by competition for adhesive receptors.

Early studies demonstrate that fibroblasts bind both to intact cytotactin and to a chymotryptic fragment derived from the carboxy-terminal end of the protein (24). A previous report using cytotactin fusion proteins generated in a bacterial system (66) has assigned binding activities to two regions of cytotactin. A cell binding region was localized to repeats equivalent to the FN3 fragment and an "antiadhesive" region was localized to the EGF-like repeats. These authors observed decreased attachment of L-cells on tissue culture plastic coated with a fusion protein containing the EGF-like region of cytotactin. Surprisingly, a second report (9) using proteolytic fragments failed to observe inhibition of binding to fibronectin-coated substrates by an 80-kD proteolytic fragment of authentic tenascin that contained the EGF region, but also contained one or more fibronectin type III repeats. In the same study (9), a 60-kD fragment containing the three distal-most type III repeats and the terminal fibrinogen knob was reported to have an antiadhesive effect in contrast to the earlier report that localized a cell binding site to this region (66). We propose that this effect is the same one detected here in the distal FN-type III repeats. These authors have also reported that a mAb Tn68 inhibits the antispreading effect of cytotactin and have localized the epitope of Tn68 by EM to the last two fibronectin type three repeats (12). It is also significant that these investigators found that intact cytotactin has greater inhibitory activity than the 60-kD proteolytic fragment, suggesting that additional inhibitory activity was removed by this treatment. This supports the idea that another inhibitory activity exists amino-terminal to this fragment, consistent with both the present observations and those of a counteradhesive activity within the EGF domain (66).

Several extracellular matrix proteins have now been shown to exhibit counteradhesive effects, including cytotactin/tenascin (12, 24), thrombospondin (45, 57), and SPARC (secreted protein acidic and rich in cysteine), also called osteonectin and BM-40 (62) and recently laminin (7 and for reviews see 10, 61). These proteins have been shown to inhibit cell attachment and spreading, promote rounded cell shapes, and, in the case of cytotactin and SPARC, decrease the number of focal contacts (57). No common sequence, structure, or receptors have so far been found among these proteins to account for similarities in some of their activities. These phenomena clearly represent cases of cell surface modulation (18). The possibility that all such effects are mediated by a common mechanism involving the cytoskeleton or second messenger systems is an important question for future study of each of these extracellular proteins.

The present results suggest a model in which four different activities that modulate cell shape and cell adhesion can be assigned to specific regions within the cytotactin molecule; a fifth site is uncovered by proteolysis of the cell surface (Table III). Proteases are known to be activated at sites of cell migration, wounding, and tumors (1, 63, 64), sites at which cytotactin expression is also prominent (20). Evidence has been presented (54), for example, that cytotactin polypeptides containing the alternatively spliced repeats are more sensitive to proteolysis than those lacking these repeats. The

possibility that proteolytic events affecting cytotactin or its receptors may actually have physiological relevance therefore deserves further consideration.

The present results also prompt the search for receptors that mediate these effects on cell attachment and morphology and provide a basis for that search. Clearly, differential expression of domain-specific receptors for cytotactin might alter the responses of cells to the presence of the molecule. The activity of the intact cytotactin molecule would represent a summation of its adhesive and counteradhesive activities, providing a rationale for its observed amphitropic (19) properties. Further analysis of the cell interactions that mediate these effects will lead to a better understanding of how cell–substrate interactions and subsequent cell surface modulations regulate developmentally important cellular processes such as proliferation, migration, neurite extension, and differentiation.

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