Cell Surface Annexin II Is a High Affinity Receptor for the Alternatively Spliced Segment of Tenascin-C

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Abstract. We have investigated the binding of soluble tenascin-C (TN-C) to several cell lines using a radioligand binding assay. Specific binding was demonstrated to U-251MG human glioma cells and to a line of bovine aortic endothelial cells, but hamster fibroblasts showed no specific binding. Recombinant proteins corresponding to specific domains of TN-C were used to map the binding site(s) in TN-C. The alternatively spliced segment (TNfnA-D) inhibited the binding of native TN-C most strongly, and itself bound to glioma and endothelial cells. Scatchard analysis of TNfnA-D binding indicated 2–5 $\times$ 10^5 binding sites per cell, with an apparent 2 nM dissociation constant. The cell surface receptor for TNfnA-D was identified as a 35-kD protein on the basis of blot binding assays and affinity chromatography of membrane extracts on native TN-C and TNfnA-D columns. Protein sequencing indicated that this 35-kD receptor was annexin II. Annexin II is well characterized as a cytoplasmic protein, so it was surprising to find it as a presumably extracellular receptor for TN-C. To confirm that it was the 35-kD receptor, we obtained purified annexin II and demonstrated its binding to TNfnA-D and TN-C at nM concentrations. Antibodies to annexin II prominently stained the external surface of live endothelial cells and blocked the binding of TNfnA-D to the cells. Thus annexin II appears to be a receptor for the alternatively spliced segment of TN-C, and may mediate cellular responses to soluble TN-C in the extracellular matrix.

TENASCIN-C (TN-C)[1] is the first discovered member of the tenascin family, which now includes TN-R and TN-X (9, 25, 29, 52). TN-C was described as a hexabrachion because it exists as a hexamer of 220–320 kD elongated subunits (26). Each subunit of TN-C comprises three types of structural modules: EGF-like domains, FN-III domains, and a terminal knob homologous to the beta- and gamma-chains of fibrinogen, as shown in Fig. 1. The multidomain structure of the TN-C suggests the possibility of multiple-independent functions (27).

TN-C shows a restricted expression pattern during the development. It is selectively present in the mesenchyme surrounding growing epithelia in organs (3). TN-C is prominent in specific layers of developing brain (6, 19) and it is distributed along the principal neural crest cell migration pathways (10, 19, 55, 56). TN-C is prominent in differentiating bone and cartilage (13, 48). Although TN-C is missing from most adult tissues, it reappears at places where active tissue regeneration and cell migration occurs, namely in a large range of tumors (14, 41), in wound healing (17, 44, 47, 60), and in regenerating nerves (20, 30). A very important recent study reported that mice genetically engineered to eliminate TN-C developed normally (57). This has led to much discussion, including suggestions that TN-C (and any other protein) may be expressed in some tissues where it has no function (24), and that its functions are subtle rather than vital. It is clear, however, that TN-C and probably the whole tenascin family are conserved in every vertebrate species, so these molecules must have functions that contribute to survival (25). Experiments in cell culture will continue to provide important clues about possible functions in the living animal.

The possibility of cell surface receptors for TN-C has been investigated so far mainly by cell adhesion assays. Cells bind to TNfn3 (which has an RGD sequence in human and chicken TN-C, but not in other species) via the $\alpha v\beta 3$ integrin, and to TNfbg via a proteoglycan or the $\alpha 2\beta 1$ integrin (4, 36, 54, 58). A positive result in a cell adhesion assay clearly demonstrates a cell surface receptor, but some receptors may not give cell adhesion. To look for additional cell surface receptors for TN-C, we have now assayed directly the binding of soluble TN-C to cells.

There is growing evidence that TN-C may affect cells as a soluble ligand. Native TN-C and a recombinant domain TNfnA-D (see Fig. 1 and caption for nomenclature of domains and recombinant proteins), acting as soluble ligands, are able to provoke the loss of focal adhesions in well spread

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1. *Abbreviations used in this paper*: BSA, bovine serum albumin; D-TBS, 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$; FN, fibronectin; TBP-35, tenascin-binding protein, 35 kD; TN-C, tenascin-C.

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endothelial cells (51), and inhibit adhesion of uterine epithelial cells to matrigel (37). End et al. (22) reported that TN-C has mitogenic activity, distinct from the signaling pathways induced by peptide growth factors. Although the mitogenic activity needs to be further examined (e.g., Crossin reported an inhibition of mitogenesis by TN-C [18]), we believe that TN-C may affect cellular regulation through cell surface receptors. It is therefore important to determine whether domains of TN-C, as soluble ligands, bind to receptors on the cell surface.

In the present study, we have done binding assays using soluble radiolabeled ligands. We first demonstrated the specific binding of native TN-C to endothelial cells and glioma cells. Then, using recombinant segments of TN-C (4), we mapped the major binding site to TNfnA-D, the alternatively spliced segment. A single class of high affinity receptor on endothelial cells has been defined by Scatchard analysis, and biochemical characterization and identification of the receptor are presented.

Materials and Methods

Cells and Cell Cultures

A human glioma cell line U-251MG (clone 3, obtained from Dr. Darell Bigner, Duke University), the bovine endothelial cell line GM7373 (from Coriell Institute for Medical Research, Camden, NJ, (33)), and the hamster fibroblast cell line NIL.8M (from Dr. Richard Hynes, MIT) were grown in DMEM, high glucose supplemented with 10% heat inactivated FCS.

Proteins and Antibodies

TN-C was purified from culture supernatant of U-251MG human glioma cells by gel filtration and mono Q ion exchange chromatography as described by Aukhil et al. (5). Fibronectin (FN) was purified from human plasma or horse serum by gelatin-agarose affinity chromatography (23). Mouse laminin from EHS tumors was a generous gift from Dr. Hynda Kleinman, NIH. Bacterial expression proteins were purified as described by Aukhil et al. (4). TN-C, and TN-C segments TNfnA-D and TNfn3 were iodinated by the Chloramine T method (32) to a specific activity of 3-10 mCi/mg. The integrity of the labeled ligand was ascertained by SDS-PAGE followed by autoradiography. Anti-annexin II antiserum and purified bovine annexin II was provided by Dr. Carl Creutz, University of Virginia. Annexin II was also purified in our lab from bovine lung as described by Drust and Creutz (21). Affinity-purified anti-annexin II antibody was prepared on a column of 0.6 mg annexin II coupled to cyanogen bromide-activated Sepharose. Bound antibody was eluted with 0.1 M glycine, pH 2.6, and neutralized. Affinity-purified anti-spectrin antibody was provided by Dr. Vann Bennett, Duke University.

Binding Assay

The binding assay was done in DMEM/Hepes (N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid, pH 7.4) supplemented with 0.2% bovine serum albumin (BSA) (binding medium). Cells were seeded on 24 well cell culture plates (Nunc, Naperville, IL) at a density of 2×10^5 cells per well. After 24 h, cultures were rinsed two times with PBS (pH 7.4, 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4) and preincubated in a CO_2 incubator with binding medium for 10 min at 37°C, and then incubated with binding medium containing the iodinated ligand for 1 h at 37°C. Preliminary experiments showed that ^{125}I-TN-C bound to glioma cells in a time dependent manner and reached equilibrium in ~30 min at 37°C (not shown). Subsequent assays were done at a 60-min binding time. After incubation, cells were rinsed three times with ice-cold DMEM/Hepes and cell layers were extracted in 4% SDS. The radioactivity of the extracts was counted in a gamma counter.

For binding assay to cells in suspension, cells were incubated with PBS containing 3 mM EDTA and 1 mM PMSF for 5 min at 37°C and harvested by gentle pipetting. Cells were centrifuged and resuspended in DMEM/Hepes, and then recentrifuged. The pellet was resuspended in DMEM/Hepes containing 0.2% BSA, and the cell concentration was determined with a hemocytometer. Cell viability was between 85-95%, determined by the trypan blue exclusion method. Assays were done in 300 μl suspensions of cells (1-3 × 10^5 cells per tube) with iodinated ligand and with or without unlabeled ligand. After 1 h incubation at 37°C with regular shaking, cells were layered onto two-step sucrose gradients in Titertubes (Bio-Rad Labs., Hercules, CA) (bottom: 0.3 ml of 0.7 M sucrose; top: 0.5 ml of 0.3 M sucrose in binding medium). The tubes were spun for 15 min at 2,500 rpm in a CPR 3000 centrifuge (swinging bucket rotor) (Beckman Instrs., Inc., Fullerton, CA). Tubes were frozen in dry ice, the bottoms were cut off, and the radioactivity was counted.

Affinity Chromatography

To identify and isolate the receptor, native TN-C, TNfnA-D, TNfn3, FN, and BSA were immobilized separately on cyanogen bromide-activated Sepharose 4B beads (Sigma Chem. Co., St. Louis, MO). To detect surface proteins only, cell surface proteins were radioiodinated using lactoperoxidase. Approximately, 10^8 cells suspended in 2 ml PBS were iodinated in the presence of 1 mCi ^{125}I-sodium iodide, 0.1 mg/ml of lactoperoxidase and 15 μl of 0.3% H_2O_2. After 10 min incubation on ice, cells were washed three times with column buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$) and lysed in 2 ml of column buffer containing 100 mM octylglucoside and 1 mM PMSF by vortexing and incubating on ice for 20 min. The extracts were centrifuged at 12,000 *g* for 10 min to remove insoluble cytoskeletal and nuclear components. To test if the receptor for TN-C can be solubilized with detergent, proteins in the detergent extract was incorporated into phosphatidylcholine liposomes as described by Mimms et al. (50) and binding assay was done with liposomes. ^{125}I-TNfnA-D specifically bound to liposomes, which indicates that the receptor is detergent-soluble (not shown).

The detergent extract of labeled cells was subjected to affinity chromatography. Cell extract was first preincubated with Sepharose 4B to remove nonspecific binding, and then applied to the affinity matrix that had been equilibrated with column buffer containing 30 mM octylglucoside, 1 mM PMSF, 1 mM NEM (n-ethylmaleimide) (starting buffer). Protein not bound to the column was recovered upon washing the column with 4 ml of starting buffer. Bound components were eluted by washing the column sequentially with 0.3 M NaCl, 1 mg/ml GRGDSP peptide, and 4 M urea in starting buffer. Eluted fractions were subjected to SDS-PAGE analysis and visualized by autoradiography.

The 35-kD receptor for tenascin was purified from bovine lung in a scaled up protocol. Bovine lung (150 g) was homogenized in a blender in 500 ml of 20 mM Hepes, 200 mM sucrose, 2.5 mM EDTA, 1 mM PMSF, and 0.02% sodium azide. The homogenate was further homogenized with a polytron and centrifuged at 16,000 *g* for 45 min. The supernatant was then centrifuged at 110,000 *g* for 2 h and the pellet (membrane fraction) was solubilized with 0.2% Triton X-100 in D-TBS (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$). After centrifuging at 10,000 *g* to remove cytoskeletal proteins, the detergent-soluble extract was loaded onto TN-C or TNfnA-D affinity columns (10 ml column). A 35-kD tenascin-binding protein (TBP-35), identified by Coomassie-blue stained SDS-PAGE, was eluted from both TN-C and TNfnA-D affinity columns with 0.5 M NaCl or 4 M urea.

To raise antibodies against TBP-35, the peak from the affinity column was run on 10% SDS-PAGE and the TBP-35 band was cut, minced, and mixed with a complete Freud's adjuvant. A rabbit was immunized by subcutaneous injections of this mixture, followed by two boosts. The titer and specificity of antiserum were tested with immunoblotting and ELISA.

Amino Acid Sequencing

Partially purified TBP-35 from the bovine lung preparation was concentrated with a Centricon (Amicon, Inc., Beverly, MA) and further purified by gel filtration using a Superose-12 column (1 × 30 cm) in the presence of 0.2% of Triton X-100 in D-TBS. TBP-35 was present in late fractions. Fractions containing TBP-35 were pooled, concentrated, and run on 10% SDS-PAGE. The resolved proteins were transferred to a nitrocellulose sheet (Schleicher and Schuell, Keene, NH) and the blotted membrane was rinsed with Mili-Q water, stained with 0.1% Ponceau S in 1% acetic acid, and briefly rinsed with Mili-Q water. The band was carefully cut out with a scalpel and sent to Dr. William S. Lane, Harvard Microchemistry Facility (Boston, MA). Tryptic digestion, HPLC, and NH_2-terminal sequencing were done as described by Aebersold et al. (1). TBP-35 usually appeared as a doublet band on SDS gels. The smaller protein band was sequenced.

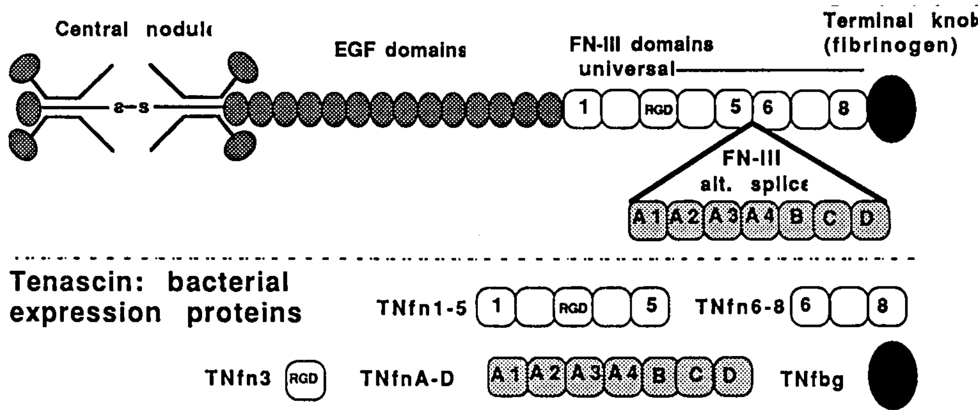


Figure 1. A schematic diagram of the multidomain structure of human TN-C, and the repertoire of bacterial expression proteins we have used. The abbreviations used in the text are indicated on the figure.

Flow Cytometric Analysis and Immunofluorescence Staining

Endothelial cells were harvested with 3 mM EDTA in PBS, suspended in D-TBS and cooled to 4°C. 10^6 cells were put in each assay tube. Primary antisera was added with a final dilution of 1:100 for preimmune, annexin II, and TBP-35 antiserum. The affinity-purified spectrin antibody was added to a final concentration of 5 μg/ml. Cells were incubated with the primary antibodies for 1 h at 4°C, washed three times with ice-cold D-TBS, and resuspended with fluorescein-conjugated goat anti-rabbit IgG at a final dilution of 1:100 for 30 min at 4°C. Cells were then washed three times, fixed with 3% freshly made paraformaldehyde (necessary to prevent cell aggregation), and resuspended in 1 ml D-TBS at 4°C. Flow cytometric analysis was done with a Becton-Dickinson model FACScan flow cytometer.

For immunofluorescence staining, endothelial cells were grown in Labtek chamber slides (Nunc), washed with PBS, and fixed for 5 min with freshly made 3% para-formaldehyde in PBS. After washing cells with PBS, remaining formaldehyde was neutralized with 50 mM NH_4Cl. Cells were either directly stained or permeabilized with 0.2% Triton X-100 in PBS for 5 min before staining. Primary antisera were added at a final dilution of 1:100 in PBS, and incubated for 1 h at 4°C. Cells were washed three times with PBS for 15 min and fluorescein or rhodamine-conjugated goat anti-rabbit IgG (Tago, Inc., Burlingame, CA) was added at a final dilution 1:100. The chambers were detached carefully and slides were mounted for observation and photography.

Results

Cell Binding of Labeled TN-C

Three types of cells were tested for the binding of TN-C. U-251MG human glioma cells, which secrete high levels of human TN-C, have shown weak cell adhesion to TN-C substrates (8, 46). A line of transformed bovine aortic endothelial cells, GM7373, was chosen to be tested because soluble TN-C has been shown to induce the loss of focal adhesion in endothelial cells (51) and because these cells adhere to TN-C substrates (36). The hamster fibroblast line NIL.8M was chosen as a third, unrelated cell type which does not adhere to TN-C substrates (46).

The binding of ^{125}I-TN-C to U-251MG and GM7373 cells was significantly inhibited by increasing amounts of unlabeled TN-C, demonstrating the specificity of the binding (Fig. 2 *A*). About 50% of the total binding was specific, meaning it could be competed by an excess of unlabeled TN-C, in both glioma and endothelial cells. The high non-specific binding (~50% of total binding) is commonly observed in

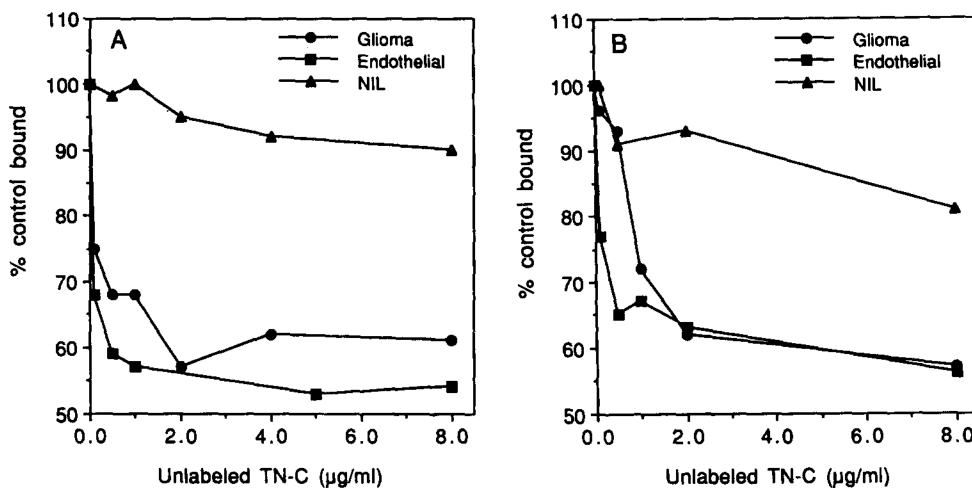


Figure 2. Competition by unlabeled TN-C for ^{125}I-TN-C binding to cells attached to the substrate (*A*) and to cells in suspension (*B*). (*A*) Cells were plated on 24-well tissue culture plates at a density of 1-2 × 10^5 cells per well, and tested 24 h later. Unlabeled TN-C was added in varying amounts together with 100–200 ng/ml of labeled ligands (0.5-1 × 10^6 cpm per μg) to cells. The concentration of unlabeled TN-C for half maximal competition was ~0.7 nM. (*B*) Suspended cells were obtained by the treatment with 3 mM EDTA. 100–200 ng of ^{125}I-TN-C (0.5–2 × 10^6 cpm per μg) was added to suspended cells (5 × 10^5 cells per assay sample) with or without unlabeled TN-C. Each data point is the mean of two measurements. The experiments were repeated three times, showing very similar inhibition. 100% represents the binding in the absence of unlabeled TN-C.

binding studies of large extracellular matrix proteins like fibronectin and laminin. Specific binding was much lower to NIL cells, indicating either a lack of high affinity binding sites or a small number of sites. To assure the integrity of bound TN-C, we compared the ^{125}I-TN-C before and after binding to cells by means of SDS-PAGE. There was no mobility change of TN-C after incubation with cells, indicating no major degradation or modification by cells (not shown).

We tested several possible mechanisms for the non-specific binding. Since TN-C has been reported to bind to several ECM molecules and proteoglycans (4, 15, 28, 34, 43, 49), we tested the binding of TN-C to the ECM. When cells were removed from the substrate by treatment with EDTA, the total binding of labeled TN-C to the residual ECM and plastic was less than 15% of that to the intact cell layer. This implies that most of the binding is to the cells. To determine what fraction of the TN-C might be internalized we treated the cells with trypsin after incubation with ^{125}I-TN-C. About 20% of the total counts remained with the cell layer after trypsin treatment. Thus the 50–60% non-specific binding appears to comprise: 15% to the ECM; 20% internalized by cells; and the remaining 15–25% may be binding to trypsin accessible sites on the cell surface.

Binding of soluble TN-C was also demonstrated to cells in suspension (Fig. 2 *B*). Labeled tenascin bound to suspended glioma and endothelial cells similar to cells attached on substrate, but suspended fibroblasts showed much lower specific binding. Thus, the presence of receptors for TN-C on the surface of endothelial and glioma cells, but not fibroblasts, is indicated by both assays.

To map the cell binding site(s) on TN-C, we tested the activity of recombinant expression proteins to compete with binding of native ^{125}I-TN-C. The strongest competitor was the alternatively spliced segment, TNfnA-D (Fig. 3). The maximal competition by TNfnA-D was almost the same as that of native TN-C, but required a higher concentration on molar basis. Specifically, maximum inhibition was observed at 1 μg/ml for both native TN-C (Fig. 2 *A*, 4 nM in large subunit, based on the 240-kD peptide mass), and TNfnA-D (Fig. 3, *A* and *B*, 14 nM in the 70-kD expression protein). Half maximal inhibition by TNfnA-D was seen at the lowest concentration tested, 0.1 μg/ml or 1.5 nM, which is close to the 2-nM K_D determined by Schatchard analysis (Fig. 5). This result indicates that both glioma and endothelial cells have receptors interacting with the TNfnA-D segment. Monomeric TN-C produced by reduction and alkylation also competed well with radiolabeled hexameric TN-C (not shown). The TNfn3 domain, which has an RGD sequence, showed competition with TN-C in both cells but was less effective than TNfnA-D. TNfn6-8 and TNfbg showed minimal competition even at 200-fold molar excess.

As an alternative approach to test the importance of the TNfnA-D in binding to cells, we tested the two major splice variants of human TN-C, produced in transfected BHK cells (4). HxB.L is the large splice variant, and HxB.S is the small variant, missing the TNfnA-D segment. Both forms are assembled into apparently normal hexabrachions and secreted into the medium (4). HxB.L showed specific binding to substrate-attached endothelial cells that was essentially identical to TN-C (not shown). This result was expected because the human TN-C prepared from U-251MG glioma cells is ~90% the large splice variant. HxB.S showed a significantly lower total binding and no competition by 0.5–5.0 μg/ml cold HxB.S. A higher concentration of HxB.S (10 μg/ml) inhibited binding of radiolabeled HxB.S ~20%, suggesting a small amount of specific binding. This experiment confirms that the specific binding of TN-C to endothelial cells is mediated primarily by the alternatively spliced segment, TNfnA-D.

Because TNfn3 showed substantial inhibition of binding of native TN-C (Fig. 3), we tested its binding directly to cells. TNfn3 showed no significant binding to cells attached to substrate, but did bind to endothelial cells in suspension. This binding was inhibited by TNfn1-5 and by the peptide GRGDSP, but not by GRGESP nor by TNfnA-D (data not shown). A combination of TNfn3 plus TNfnA-D competed more effectively than either protein alone for binding of native TN-C to cells, consistent with the hypothesis that these two domains bind to separate receptors. These experiments are all consistent with the previous conclusion that the $\alpha v \beta 3$ integrin on endothelial cells is a receptor for TNfn3 (36, 58). We therefore focused our efforts on the binding of TNfnA-D,

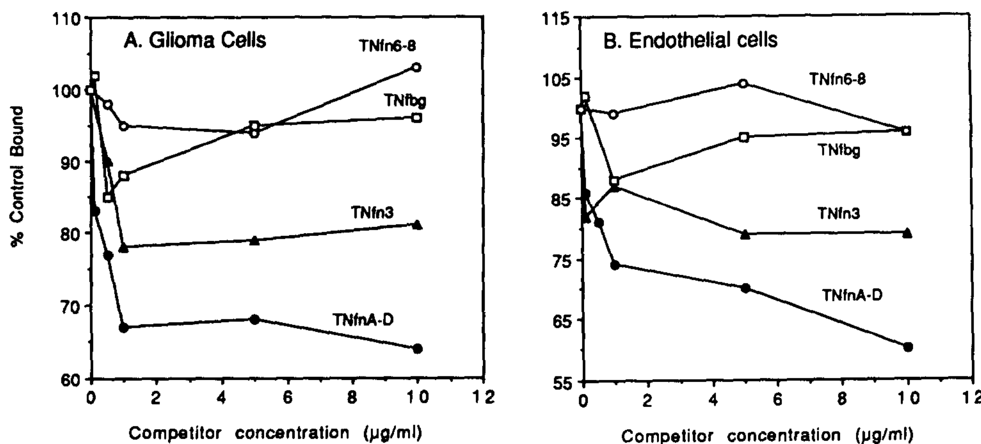


Figure 3. Competition by recombinant TN-C segments for ^{125}I-TN-C binding to cells attached to the substrate. This experiment was similar to that in Fig. 2 *A*, but competitors were TNfnA-D, TNfn3, TNfn6-8, and TNfbg. They were added in varying amounts with ^{125}I-TN-C to cells (2×10^5 cells per well) cultured on a substrate. The total binding of ^{125}I-TN-C in the absence of competitors is referred to as 100%. The concentration for half maximal competition was ~1.5 nM for TNfnA-D and 30 nM for TNfn3. Each data point is the mean of six measurements from three independent experiments.

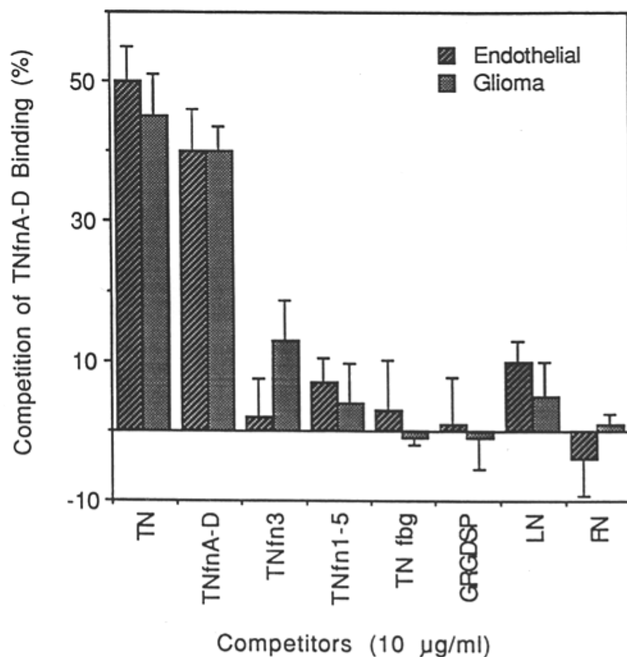


Figure 4. Competition for the binding of ^{125}I-TNfnA-D by other TN-C segments and ECM proteins. Competitors (10 μg/ml each) were added with ^{125}I-TNfnA-D (200 ng/ml) to cells cultured on a substrate. Binding of TNfnA-D in the absence of competitors is indicated as 0% competition. Each data point is the mean of six measurements from three independent experiments. Error bars indicate the standard error of the mean in Figs. 4, 5, and 9.

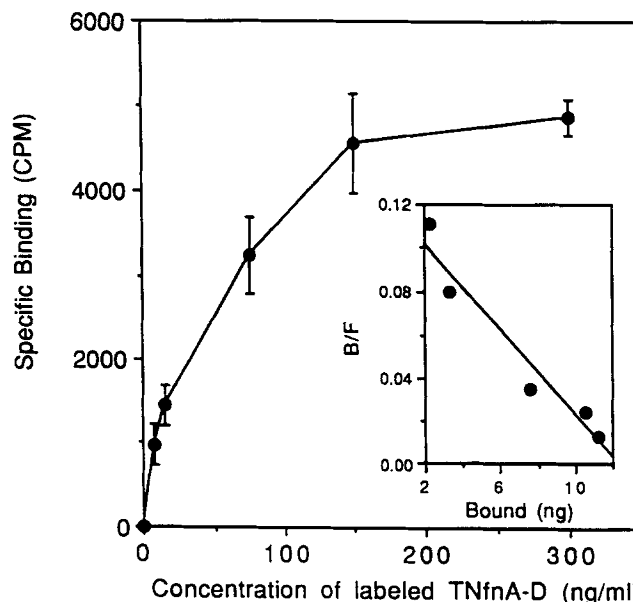


Figure 5. Saturation binding and derived Scatchard analysis (*inset*) for the binding of ^{125}I-TNfnA-D to substrate-attached endothelial cells. Specific binding represents the difference between the binding in the absence and presence of a 100-fold excess of unlabeled ligand. The Scatchard plot of the specific binding data are shown with the ratio bound/free and the amount of bound ligand as ng per 5×10^5 cells. The regression coefficient was $\gamma = 0.94$. Data points are the mean of six measurements.

which appears to be the most important domain for binding TN-C to cells, and which involves a previously unknown receptor.

Cell Binding of Labeled TNfnA-D

To determine if both TNfnA-D and TNfn3 domains bind to the same receptor, we examined the competition between radiolabeled TNfnA-D and other domains and ECM proteins (Fig. 4). Unlabeled TNfnA-D and native TN-C both competed well with ^{125}I-TNfnA-D for binding to both cells, but TNfn3, TNfnl-5, and TNfbg could not displace ^{125}I-TNfnA-D at a 200-fold molar excess. In addition, fibronectin and laminin, major constituents of the ECM, did not compete for the binding of TNfnA-D. Thus, TNfnA-D appears to interact with a receptor on the cell surface that does not interact with other domains of TN-C.

To characterize the receptor for TNfnA-D, we performed a saturation binding assay (Fig. 5). An apparent dissociation constant (K_D) and numbers of binding sites for TNfnA-D were calculated from the Scatchard plot. The saturation binding data from GM7373 cells were fit well by a straight line determined with a linear regression program. This is consistent with a single class of high affinity binding site. The apparent K_D for TNfnA-D binding was 2 nM and the density of receptors was 3×10^5 sites per cell.

Identification of the Cell Surface Receptor

The identification of the receptor was pursued by three techniques: (*a*) blot binding of cell extracts; (*b*) affinity column purification of labeled cell surface receptor from tissue cultures; (*c*) bulk purification from bovine lung.

In the blot binding assay, TN-C selectively bound to three different polypeptides in octylglucoside extracts of cultured cells: 130, 48, and 35 kD (Fig. 6). Two or three minor bands between 48 and 130 kD were sometimes detected on blots. TNfnA-D bound to two of these three polypeptides, 48 and 35 kD. TNfn3 did not bind to any proteins on the blot. Binding of ^{125}I-TNfnA-D to the 35-kD band was blocked by adding unlabeled TNfnA-D but binding to the 48- and 130-kD was not inhibited (these two bands are weaker in Fig. 6 *B* than in 6 *A*, and are the same intensity with and without excess TNfnA-D). The same three polypeptides were labeled in detergent extracts of both endothelial cells and glioma cells.

We next used affinity chromatography to isolate the TN-C receptor(s). From endothelial cell extract, three major polypeptides (140, 125, and 110 kD) were eluted with GRGDSP peptide from the TN-C affinity column. These proteins, which are probably subunits of integrins, were absent in fractions eluted from TNfnA-D column. A major doublet band around 35 kD and a minor population of high molecular weight proteins were eluted in the 4 M urea fractions from both the TN-C and TNfnA-D affinity columns (Fig. 7 *A*). In other experiments, the same 35-kD doublet was eluted from both affinity columns with 0.5 M NaCl. In the 0.5 M NaCl eluted fractions, high molecular weight proteins were not found in the autoradiogram. This 35-kD protein was also found in chromatography of glioma cell extracts (Fig. 7 *B*). Affinity columns of TNfn3, FN, and BSA did not bind the 35-kD protein. In some experiments, the 35-kD protein was further purified by gel filtration on Superose-12 column in the presence of Triton X-100. The 35-kD protein was eluted in the late fractions from Superose-12 column. We referred

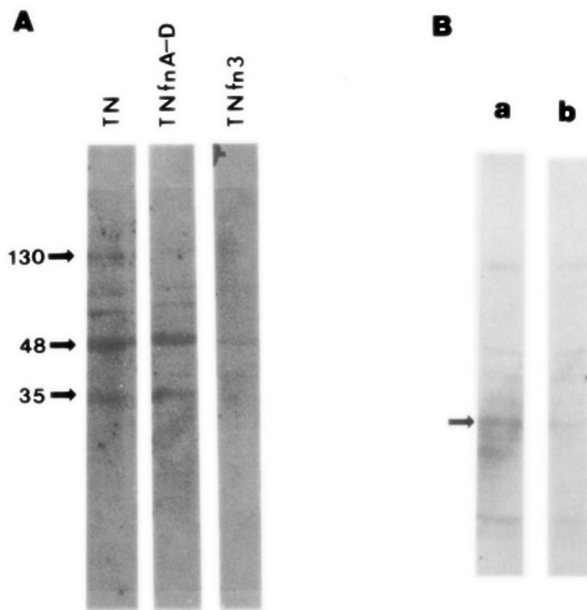


Figure 6. (*A*) Blot binding of ^{125}I-TN-C or ^{125}I-TNfnA-D to proteins of endothelial cells. (*A*) Proteins were extracted from cultured cells with 100 mM octylglucoside, run on SDS-PAGE (10% gel), and transferred to a PVDF membrane. Remaining binding sites on the membrane were blocked with 5% dry milk, and then incubated with 300 ng of ^{125}I-TN-C (10^6 cpm), ^{125}I-TNfnA-D (0.5×10^6 cpm), or ^{125}I-TNfn3 (0.5×10^6 cpm) in 10 ml of D-TBS containing 5% heat-denatured BSA. After incubation for 2 h at room temperature, the membrane was washed with D-TBS for 20 min with four changes and dried for autoradiography. Arrows indicate labeled bands of 130, 48, and 35 kD. (*B*) Another blot binding of ^{125}I-TNfnA-D to proteins of endothelial cells in the absence (*a*) or in the presence (*b*) of unlabeled TNfnA-D (20 μg/ml). Arrow indicates the 35-kD band.

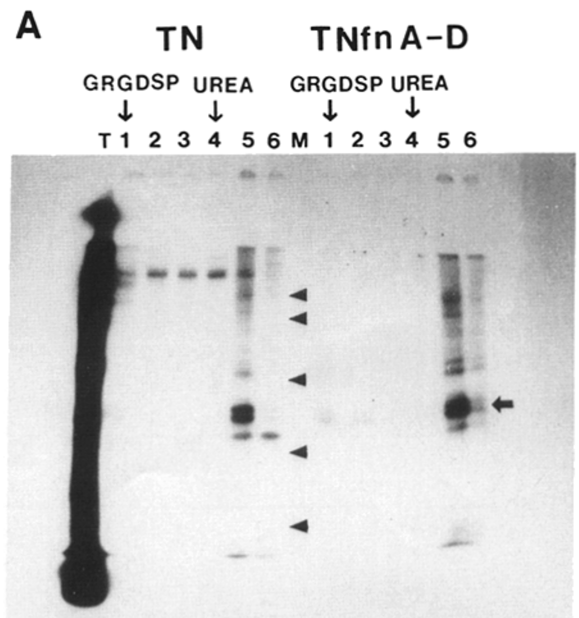
to this putative receptor as the 35-kD tenascin-binding protein (TBP-35).

Interestingly, two polypeptides (130 and 48 kD) identified in the ligand-blot (Fig. 6) were not major components in the autoradiogram from the affinity column. However, these two polypeptides were found in silver-stained gels of cell extracts, so they may be relatively abundant cytoskeletal proteins.

Because tissue culture cells are expensive to produce in large quantity, we tried to purify the TBP-35 protein from bovine lung, which should be a rich source of endothelial cells. Lung membrane extract was made by differential centrifugation, the membrane fraction was solubilized with 0.2% Triton X-100, and the extract was loaded onto affinity columns. A TBP-35 protein, identified by Coomassie blue–stained SDS-PAGE, was eluted from both TN-C and TN-fnA-D affinity columns with 0.5 M NaCl or urea (Fig. 8 *A*). We tested the specificity of this protein by the blot binding assay. TNfnA-D bound to several proteins including the TBP-35 protein in the lung membrane extract (Fig. 8 *B*). The binding of ^{125}I-TNfnA-D to those proteins was blocked by the presence of excess soluble TNfnA-D. TNfnA-D also bound to the partially purified TBP-35.

Annexin II: the Receptor for Tenascin

To determine whether TBP-35 was a new protein or a protein



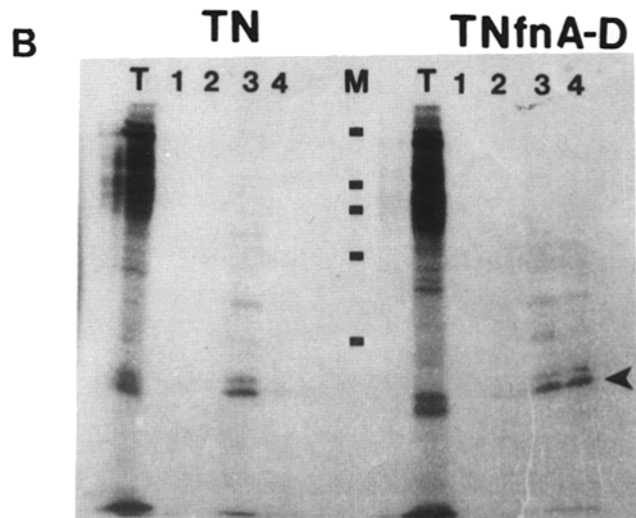


Figure 7. Endothelial (*A*) and glioma (*B*) cell extracts affinity purified on native TN-C and TNfnA-D affinity columns. Glioma and endothelial cells (10^8 cells) were surface labeled with ^{125}I by using lactoperoxidase and extracted with 100 mM octylglucoside. The extract (3 ml, lane *T*) was loaded onto the affinity column (2 ml). Fractions (1 ml each) were collected and 100 μl of each fraction was analyzed by SDS-PAGE (8%) under reducing condition. (*A*) Where indicated by arrows, 1 mg/ml of the synthetic peptide GRG-DSP and 4 M urea was added to the elution buffer. (*B*) Lanes *1* and *2*, fractions eluted with 1 mg/ml of GRGDSP peptide; lanes *3* and *4*, fractions eluted with 4 M urea. Molecular weight markers (lane *M*) in *A* were phosphorylase B, 97.4 kD; bovine serum albumin, 66.2 kD; ovalbumin, 43 kD; carbonic anhydrase, 31 kD; soybean trypsin inhibitor, 21.5 kD. Molecular weight markers in *B* were myosin, 200 kD; b-galactosidase, 116 kD; phosphorylase b, 97.4 kD; bovine serum albumin, 66.2 kD; ovalbumin, 45 kD.

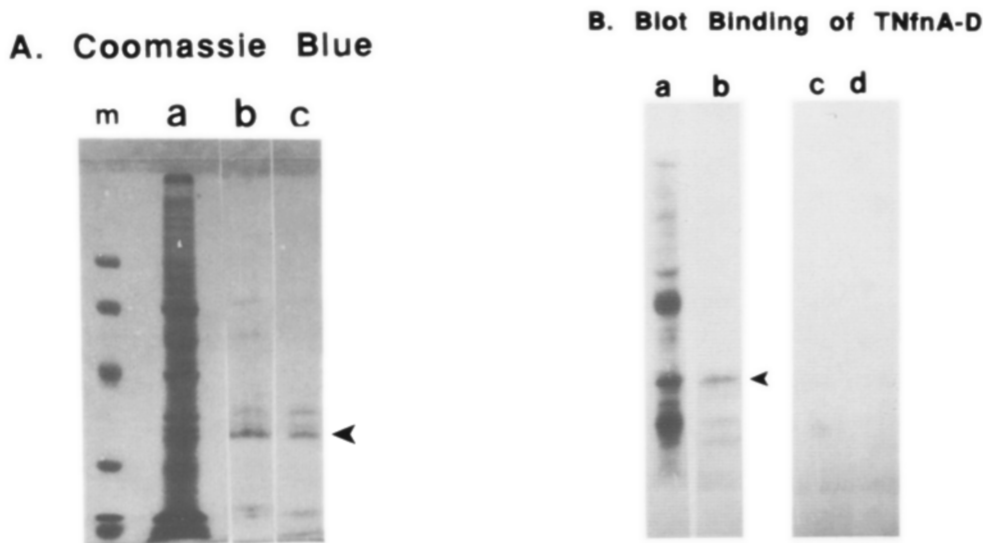


Figure 8. (*A*) Coomassie blue staining of SDS-PAGE of (*a*) lung membrane extract, (*b*) protein eluted from the TN-C affinity column by 0.5 M NaCl, and (*c*) further purified by gel filtration on a Superose 6 column. Lane *m* shows molecular weight markers, as in Fig. 7. (*B*) Blot binding assay with ^{125}I-TNfnA-D (5 × 10^5 cpm): *a* and *c*, lung membrane extract; *b* and *d*, 35-kD protein after purification on Superose-6. For *c* and *d*, the blot binding assay was done in the presence of 20 μg/ml unlabeled TNfnA-D. The arrow head indicates the 35-kD receptor.

already known, the 35-kD band was separated by SDS-PAGE and sent to Harvard Microchemistry Facility for amino acid sequence analysis. Three internal sequences were obtained from tryptic peptides of TBP-35. The sequences were run on a database and two amino acid sequences, SLYYYIQQDTK and EVDMLK, were identical to sequences in bovine annexin II. Annexin II is a 35-kD protein, thus matching the size of our TBP-35 band. Another sequence, FGDGYNGYGG, was identical to the sequence of small ribonuclear protein, which is also a 35-kD protein. This sequence is apparently from a contaminating nuclear protein in our TBP-35 preparation.

To test whether annexin II could indeed function as a receptor for TNfnA-D, we obtained some purified annexin II, and a polyclonal antibody against it, from Dr. Carl Creutz, University of Virginia. The purified annexin II bound to the TNfnA-D affinity column and was eluted with 0.5 M NaCl. In Western blots the antibody to annexin II and our anti-TBP-35 both stained purified annexin II and the 35-kD band in membrane extracts (not shown). Finally, the affinity purified polyclonal antibody against purified annexin II was tested for its ability to block binding of ^{125}I-TNfnA-D to endothelial cells. As seen in Fig. 9, this antibody blocked binding in a concentration dependent manner, and the maximum inhibition by the antibody was approximately the same as by native TN-C. Our anti-TBP-35 antibody also blocked this binding (not shown).

The binding of purified annexin II to TN-C was further tested in a solid phase binding assay. TN-C was coated on plastic, and incubated with ^{125}I-labeled annexin II plus increasing amounts of unlabeled annexin II (Fig. 10). Labeled annexin II was displaced as the unlabeled annexin II increased from 0.15 to 0.6 μg/ml (4–17 nM). The affinity of purified annexin II for TN-C in this assay is thus comparable to the 2 nM K_D determined for binding of TN-C to endothelial cells.

Is Annexin II on the Cell Surface?

If annexin II is the receptor for TN-C, it must be exposed on the external cell surface. This implication was at first surprising since annexin II is well characterized as a cytoplasmic protein, and it has no hydrophobic signal sequence for secretion. It is unlikely that annexin II on the cell surface could have come from the fetal calf serum; annexin II has never been reported in serum, and our own Western blots of serum failed to detect it (not shown). Thus, the annexin II exposed on the cell surface was apparently synthesized by the endothelial cells and somehow translocated to the outside

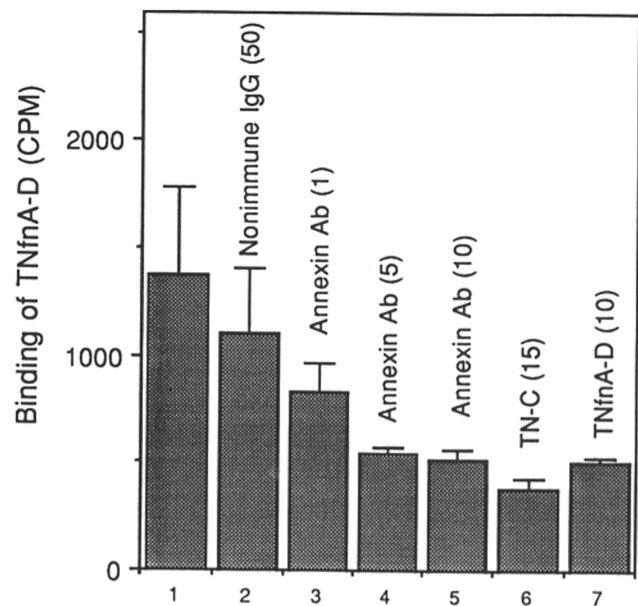


Figure 9. Inhibition of ^{125}I-TNfnA-D binding to endothelial cells by affinity-purified annexin II antibody. Endothelial cells (5 × 10^5 cells per tube) were harvested with 3 mM EDTA, washed with DMEM/Hepes two times, and incubated with ^{125}I-TNfnA-D in the absence or presence of various competitors and affinity purified anti-annexin II antibody (1, 5, and 10 μg per ml) for 1 h at 37°C. After incubation, cells were loaded onto a discontinuous sucrose gradient and pelleted as described in Materials and Methods. Each data point is the mean of three measurements. The first bar shows total binding of TNfnA-D in the absence of inhibitor. Inhibitors are indicated above the bars and the concentration of inhibitors in μg/ml is in parenthesis.

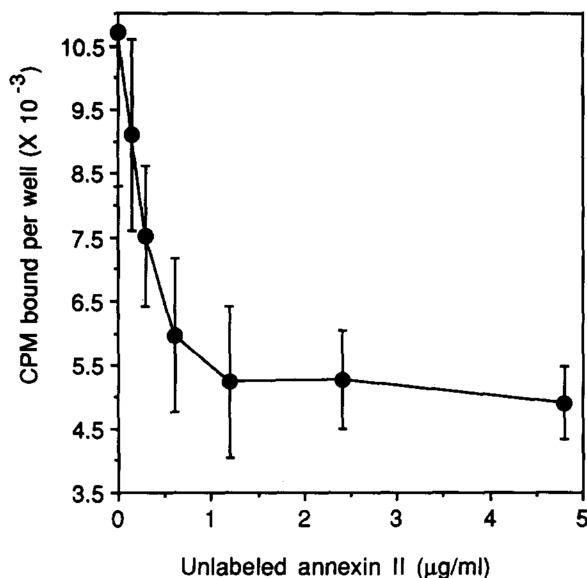


Figure 10. Binding of annexin II to tenascin. Tenascin (10 μg per well) was coated on a plastic 96 well plate and the remaining binding sites were blocked with 5% dry milk. ^{125}I-annexin II (120 ng per well, 10^5 cpm), 100 μg/ml BSA, and increasing amounts of unlabeled annexin II were added to each well and incubated for 2 h at room temperature. The plate was washed with D-TBS and extracted with 2% SDS for determination of annexin binding.

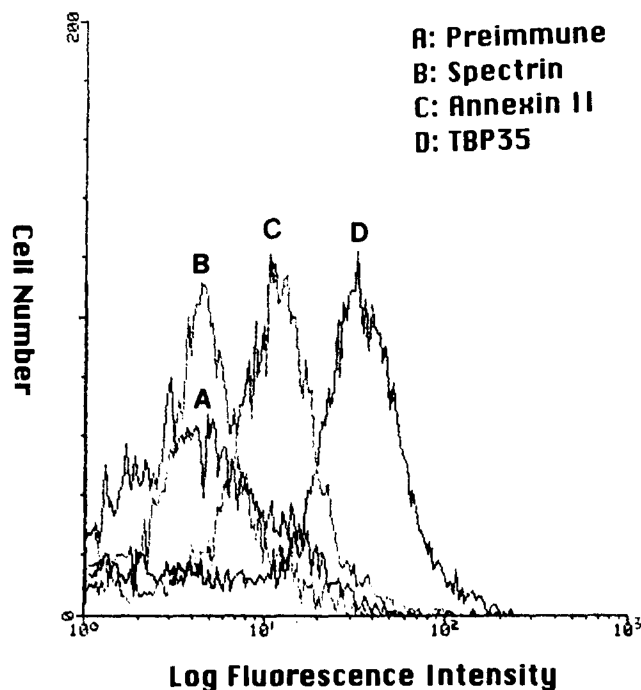


Figure 11. Flow cytometric analysis of expression of the annexin II on endothelial cell surface. One million cells were labeled with four different antibodies. Preimmune antibody (1:200 dilution) and affinity-purified spectrin antibody (5 μg/ml) were used as a negative control. TBP-35 and annexin II antiserum were also added at a final dilution 1:200.

surface. To examine whether annexin II was actually exposed on the surface of the endothelial cells, we used flow cytometric analysis. Living cells were stained with antibodies and subsequently fixed and subjected to flow cytometric analysis. Fig. 11 demonstrates that cells stained with preimmune or with spectrin antibodies gave minimal fluorescence, while cells stained with antibodies against annexin II, or our own antibody against TBP-35, gave prominent staining.

These results were confirmed by immunofluorescence staining (not shown). Affinity purified spectrin antibodies stained cells only when they were permeabilized. In contrast, annexin II antibodies gave prominent staining of unpermeabilized cells, as well as of permeabilized cells. These results confirm that there is a substantial amount of annexin II exposed on the cell surface.

Discussion

The biological significance of the alternatively spliced region of TN-C has been emphasized by several reports showing that the large TN-C splice variant is expressed in the onset of important cellular processes that need active cell migration or tissue remodeling. Oyama et al. (53) showed that transformed fibroblasts and fetal lung tissue expressed more TN-C mRNA with the A-D domains than normal cells and adult tissue. The expression of the largest chicken TN-C variant is correlated with cell migration into the developing cornea (38). The large TN-C variant was absent in normal adult mouse skin, but reappeared in healing skin and again disappeared after wound healing (17). Thus, this segment may have a stimulatory effect on cell migration and tissue remodeling, possibly through a receptor-mediated cell signaling mechanism. Murphy-Ullrich et al. (51) showed that soluble TN-C, or the segment TNfnA-D, induced the loss of focal adhesions in well-spread endothelial cells, and Julian et al. (37) found that TNfnA-D inhibited the adhesion of uterine epithelial cells to matrigel. The receptor that we have identified here may be the one that initiates these responses.

In this study, we have found a protein or pair of proteins with molecular weight around 35 kD as a candidate receptor for the alternatively spliced region of tenascin. Purified TBP-35 from cells or lung was usually seen as a doublet band in SDS-PAGE. In the blot assay, labeled ligand usually bound to only one or two of the ~35-kD bands. In the most freshly prepared membrane extracts, we frequently see a single band of the 35-kD protein, whereas two lower molecular weight bands appear at later times. This implies a proteolytic cleavage, which is consistent with the identification of the receptor as annexin II. Annexin II has two protease cleavage sites near the NH_2 terminus, which are likely to produce three closely spaced bands on SDS-PAGE.

We identified the 35-kD receptor to be annexin II, which is a member of the annexin family of calcium-dependent phospholipid-binding proteins (11). Although the precise functions of these proteins are not known, they have been reported to play roles in the regulation of membrane fusion in exocytosis (2), control of the activity of phospholipase A_2 (42), and signal transduction by serving as a substrate for the tyrosine kinase encoded by the *src* oncogene (31). Annexins lack a hydrophobic signal sequence and are well characterized as intracellular proteins. Since the receptor for

TNfnA-D should be an extracellular or integral membrane protein, we undertook the extensive analysis reported in Figs. 9–11 to demonstrate that annexin II was present on the external cell surface and that it was indeed the receptor for TNfnA-D.

In addition to our own analysis, there are several recent studies demonstrating that annexins can be secreted or exposed on the external cell surface. Christmas et al. (16) found that human seminal plasma contained a high concentration of annexin I secreted from the prostate gland. The annexin I present in seminal plasma does not originate from cell lysis because the concentration of annexin I in plasma is much higher than in the prostate gland itself. Annexin V was reported to be secreted from chick embryo fibroblasts without cell lysis (40). There are also previous reports of annexins binding to extracellular matrix molecules. Annexin V binds to type II and X collagen and appears to be localized in the extracellular matrix of calcifying cartilage (39). Heterogeneous expression of annexins (I–VI) on the external cell surface of human and rodent tumor cells has been reported (62). The mechanism of secretion of annexins is still completely unknown, but the fact that they are exported from the cytoplasm, and that they bind to the cell surface and to extracellular matrix molecules, is becoming well established.

The recent study of Tressler et al. (59) is particularly relevant to our present results. Annexin II was found to be prominently expressed on the external surface of a lymphoma cell line, and it mediated adhesion of these lymphoma cells to hepatic sinusoid endothelial cells. The role of endothelial cells appears to be different in their study and ours. In our study the membrane-bound and exposed annexin II was on endothelial cells, and it bound to the extracellular protein TN-C. In their study the annexin II was on the lymphoma cells, and it bound to an unknown receptor on the endothelial cells. The binding to this endothelial cell receptor is similar to the binding TN-C in that it is reversed by 0.5 M NaCl. Tressler et al. did not analyze their endothelial cells for exposed annexin II, but another recent study reported annexin II on human umbilical vein endothelial cells (61). In this context the annexin II served as a receptor for cytomegalovirus. The affinity of annexin II for cytomegalovirus was 57 nM, about ten times weaker than the affinity for TNfnA-D.

The concentration of TN-C has been estimated at about 1 mg/ml in tumor matrix and in the most concentrated extracellular clefts of brain (45), giving a concentration of 2.4×10^{-6} M in the 240-kD subunits. This is 1,000 times higher than the K_D for dissociation from annexin II, meaning that all extracellular annexin II should have bound TN-C in these tissues.

What is the possible physiological significance of TN-C binding to cell surface annexin II? One possibility is that annexin II associates directly with and activates cell surface receptors on the same cell, this association being modulated by the binding of the TNfnA-D ligand. A most interesting possibility is that annexin II may be directly involved in signal transduction. The crystal structure of annexin V shows a central channel that has been implicated as a possible calcium channel (35). Annexin V demonstrates calcium channel activity when absorbed onto lipid membranes, and the selectivity of the channel is altered by a mutation in the putative channel (7). Annexin II has a similar structure (12) and may have similar channel activity. The mechanisms by which annexins are bound on the external surface of a membrane, and how they might allow passage of ions through the membranes, are completely unknown, but the possible channel activity of these proteins could allow them to operate directly in signal transduction. It will be interesting to test whether binding of TNfnA-D, the highest affinity protein ligand for annexin II, can modulate its channel activity.

We thank Dr. Carl Creutz, University of Virginia, for supplying the antiserum against bovine annexin II, and a sample of purified annexin II.

This work was supported by National Institutes of Health grant R37-CA47056 to Harold Erickson.

Received for publication 16 July 1993 and in revised form 25 April 1994.

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