Identification and Characterization of a Conformational Heparin-binding Site Involving Two Fibronectin Type III Modules of Bovine Tenascin-X*

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Tenascin-X is known as a heparin-binding molecule, but the localization of the heparin-binding site has not been investigated until now. We show here that, unlike tenascin-C, the recombinant fibrinogen-like domain of tenascin-X is not involved in heparin binding. On the other hand, the two contiguous fibronectin type III repeats b10 and b11 have a predicted positive charge at physiological pH, hence a set of recombinant proteins comprising these domains was tested for interaction with heparin. Using solid phase assays and affinity chromatography, we found that interaction with heparin was conformational and involved both domains 10 and 11. Construction of a three-dimensional model of domains 10 and 11 led us to predict exposed residues that were then submitted to site-directed mutagenesis. In this way, we identified the basic residues within each domain that are crucial for this interaction. Blocking experiments using antibodies against domain 10 were performed to test the efficiency of this site within intact tenascin-X. Binding was significantly reduced, arguing for the activity of a heparin-binding site involving domains 10 and 11 in the whole molecule. Finally, the biological significance of this site was tested by cell adhesion studies. Heparan sulfate cell surface receptors are able to interact with proteins bearing domains 10 and 11, suggesting that tenascin-X may activate different signals to regulate cell behavior.

Tenascin-X is an extracellular matrix molecule that belongs to the tenasin family, comprising five members: TN-C, TN-R, TN-X, and the more recently characterized TN-Y and TN-W. All are multi-domain proteins consisting of an N-terminal region involved in oligomerization, a series of epidermal growth factor-like repeats, a variable number of fibronectin type III (FNIII) modules, and a C-terminal domain homologous to fibrinogen (Fbg). This complex structure gives rise to multiple interactions with proteins and carbohydrates. Alternative splicing events involving FNIII domains generate a variety of isoforms whose different interaction properties and functions have been demonstrated for TN-C (1).

Many studies have emphasized the possible role of proteoglycan binding in the functions of members of the tenascin family. TN-C binding to glycosaminoglycans, namely heparin, heparan and chondroitin sulfates is well documented. Two heparin-binding sites have been identified in the fifth FNIII and the Fbg domain of TN-C by the use of recombinant domains produced in bacteria (2, 3). The latter domain contains the active site of the whole molecule (4, 5). Several types of proteoglycans have been shown to interact with TN-C, e.g. brain proteoglycans (6), or chondroitin sulfate proteoglycans derived from smooth muscle cells in culture (7) or from cartilage (8). Perlecan, an extracellular heparan sulfate proteoglycan, interacts preferentially with the small splice variant of TN-C; FNIII domains 3–5 are involved in this interaction (9). Moreover, incorporation of the small variant of TN-C in the fibronectin matrix deposited by cells in vitro is dependent on the presence of heparan sulfate proteoglycans (9). Some data suggest that cell surface heparan sulfates present on fibroblasts (2) or on hematopoietic cells (10) are able to interact with the Fbg domain. Syndecan from embryonic mesenchyme (11) as well as glypican (12), both of which are heparan sulfate receptors, have also been found to interact with TN-C via their glycosaminoglycan (GAG) chains. Other proteoglycans may interact via N-linked oligosaccharides (phosphacan/RPTPζ) or their core proteins (neurocan) (13–15).

Heparin binding seems to be a common feature of molecules of the tenascin family; TN-X and TN-Y are also heparin-binding proteins (16, 17), although the functional significance of this interaction has not been demonstrated until now. Our study is focussed on the localization of heparin-binding domains within the TN-X molecule. TN-X has the typical arrangement of modules characteristic of TNS (16, 18–20). It has a widespread expression during embryonic and adult life, where it appears more specifically in striated muscle, tendon and ligament sheaths, dermis, adventitia of blood vessels, peripheral nerves, and digestive tract (16, 19, 21–23). Few data are available concerning the function of TN-X, but in one clinical case, a patient deficient in TN-X protein, has been reported to present a connective tissue disorder typical of Ehlers Danlos-like syndrome (24). Symptoms consist of skin and joint hyperextensibility, vascular fragility, and poor wound healing. In view of these connective tissue defects, TN-X might be involved in cell-matrix interactions and in matrix network formation.

Because GAGs are likely to be involved in these molecular interactions, we have analyzed the heparin binding properties of TN-X by mapping the heparin-binding site(s) within the molecule using recombinant proteins. In comparison with the localization of the heparin binding site in TN-C, we have pro-

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† The abbreviations used are: FNIII, fibronectin type III; Fbg, fibrinogen; FbgX, fibrinogen-like domain of tenascin-X; FNX, Fibronectin-type III domain of tenascin-X; GAG, glycosaminoglycan; PBS, Phosphate Buffered Saline; TN, tenascin; BSA, bovine serum albumin; PCR, polymerase chain reaction; CHO, Chinese hamster ovary.
duced the recombinant Fbg domain in bacterial or eukaryotic cells and found no interaction with heparin. Sequence analysis of TN-X showed that two successive FN III domains are positively charged at physiological pH (domains 10 and 11). We produced a set of recombinant proteins containing this region and localized one heparin binding site to these domains, the residues crucial for this interaction being identified by site-directed mutagenesis. The efficiency of this site in the context of the whole TN-X molecule was analyzed by blocking experiments using antibodies specific for this region (domain 10). Finally, the physiological significance of this heparin binding site was tested by cell adhesion studies.

MATERIALS AND METHODS

Purification of TN-X from Bovine Embryonic Skin—Bovine TN-X was purified using a procedure previously described (21). Briefly, TN-X was extracted with 0.5 M NaCl and immunopurified on a column prepared with the 4E7 monoclonal antibody. Proteins nonspecifically bound to the column were desorbed with 1 M MgCl₂ and TN-X was then eluted with 0.15 M Na₂CO₃, pH 12.0. After dialysis against phosphate-buffered saline (PBS), purified TN-X was stored at -70 °C.

Production and Purification of TN-X Reombinant Fragments in E. coli—Recombinant proteins encompassing FNIII modules of bovine TN-X are termed FNXn, where n corresponds to the module(s) used. For this study, we used the previously described recombinant fragments FNX9, FNX10, and FNX10-9 (25) and generated new recombinant proteins containing the FNIII b11 repeat, i.e., FNX11, FNX10-11, and FNX9-10-11. The DNA coding for the new proteins was amplified by PCR and cloned in the overproducing plasmid pT7/7-His (25) introduced a restriction site (underlined) at the 3'-end of the DNA and permitted in-frame cloning with the 5'-end of the 5'-bcl restriction site (26). Reversion (5'-TAATCGACGGTTGTCAGGGGATTCTGAGG-3') and FNIII b11-

Restriction site needed for cloning are underlined. Production of TN-X fragments in E. coli strain BL21(DE3) was performed using previously described procedures (25), with the exception that here the IPTG induction was carried out at 37 °C for 3 h instead of overnight at room temperature. The His-tagged proteins were purified in two chromato-
graphic steps. first using a metal affinity column and second using an ion exchange MonoQ (FNX9 and FNX9-10) or Mono-S (FNX10, FNX11, FNX10-11, FNX9-10-11) column (Amersham Pharmacia Biotech). Production and purification of the fibrinogen domain (FbgX) in bacteria has been previously described (25).

Mutations were generated according to the procedure of Kamman et al. (26). Internal mutagenic oligonucleotides are listed in Table I. This procedure consists of two PCR steps. In the first step, one primer contains the mutation, whereas the second is one of two oligonucleotides used to generate the wild type construct. The first PCR fragment, gel purified, is then used as a primer during the second PCR together with the wild type oligonucleotide opposite to that used in PCR1.

Production of Recombinant Fbg Domain in Mammalian Cells—A DNA fragment encoding the Fbg domain was amplified by PCR using Goldstar polymerase (Eurogenetec, Seraing, Belgium) and FbgX as matrix. The sequence of the sense primer (5'-TTTGGGCCACCCCGCGCGG-GTGGGCTGCGGATCCCTTCC-3') introduced a SfiI restriction site (underlined) at the 5'-end of the PCR fragment and allowed in-frame cloning with the Igx-chain leader sequence. The antisense primer (5'-TTTGGGCCCGTCCTCGCGCAGGGCGGTAG-3') introduced an AApI site (underlined) at the 3'-end of the DNA and permitted in-frame cloning with the c-myc and His₆ tags. The PCR fragment was introduced between the SfiI and AApI sites of the mammalian expression vector pSeCTag2/hygro (Invitrogen, Groningen, The Netherlands).

Human embryonic kidney HEK 293 cells were transfected with pSeC-Fbg construct permitting the secretion of recombinant protein harboring c-myc and His₆ tags at the C terminus (Invitrogen). Petri culture dishes (60 mm) with cells at half-confluence were rinsed twice in serum-
free medium and incubated for 4 h at 37 °C in 5 ml of serum-free medium containing 33 μl of Perfect lipid # 8 (Invitrogen) and 11 μg of DNA. After removing the transfection solution, the cells were incubated for 3 h in Eagle’s modified Dulbecco’s medium containing 10% fetal calf serum. Selection was then performed by adding 400 μg/ml hygromycin B in the culture medium. Clones were passaged in 24-well tissue culture plates, and the medium was checked for the presence of the Fbg domain. One milliliter of medium was incubated with 50 μl of Talon resin. The resin was rinsed in Tris-buffered saline and heated in Laemmli sample buffer. The supernatant was analyzed by Western blotting using routine procedures (21), using a monoclonal antibody that recognizes the His₆ tag. This antibody was obtained serendipitously by immunization with recombinant proteins corresponding to SURF modules of sea urchin fibrillar collagen, produced in a bacterial system using the same vector as for the FNX proteins (27).

To produce the recombinant proteins HEK293 cells were cultured in serum-free conditions, and the medium was collected every 48 h. The medium was loaded on to a Talon metal affinity resin equilibrated in Tris-buffered saline, and elution was performed in the same buffer containing 50 mM imidazole, pH 8.0. After dialysis against 50 mM Tris, pH 8.0, the eluate was further purified on a HiTrap Q-Sepharose column (Amersham Pharmacia Biotech). Separation was achieved by elution with a linear NaCl gradient from 0 to 1 M. Fractions containing the Fbg domain were pooled and dialyzed against PBS. Protein purity was checked by SDS-polyacrylamide gel electrophoresis where it was estimated to be greater than 90%. Protein concentration was determined by absorbance at 280 nm.

Solid Phase Assay of Heparin Binding Activity—96-well microtiter plates (Maxisorp, Nunc) were coated overnight at +4 °C with purified bovine TN-X or recombinant proteins diluted in PBS. Wells were saturated with T-PBS-BSA (PBS, 0.05% Tween 20, 1% bovine serum albumin) for 2 h at room temperature and then incubated with heparin-albumin-biotin (Sigma) in T-PBS-BSA for 2 h at room temperature. Wells were rinsed with PBS and incubated for 30 min with peroxidase-conjugated sheep anti-biotin (Sigma) diluted 1:2000. After the incubation of rinses, bound peroxidase was detected with H₂O₂ and 2,2'-azino-bis-(3-ethylthiazoline-6-sulfonic acid), and the absorbance was read at 405 nm. Experiments were repeated three to five times. Each data point represents the mean of duplicate or triplicate determination.

For inhibition experiments, incubation with antibodies was performed for 1 h before adding the heparin-albumin-biotin complex. De-
tection of bound heparin was performed as described above.

Analytical Affinity Chromatography—30 μg of recombinant FNX10-11 fragments dissolved in buffer A (50 mM phosphate buffer, pH 7.2, 100 mM NaCl) were loaded onto a Hi-Trap heparin-Sepharose column (Amersham Pharmacia Biotech) at 0.5 ml/min using a high performance liquid chromatography system (Waters). The column was then washed with buffer A. Retained proteins were then eluted with a linear NaCl gradient (0.1–1 M NaCl in buffer A) with constant monitoring of absorbance at 280 nm.

Cell Adhesion Assay—Parental CHO-K1 and mutant CHO-677 and CHO-T45 cell lines, developed by Esko et al. (28), were purchased from the American Type Cell Culture Collection (Manassas, VA). The cells were cultured in F12K medium supplemented with 10% fetal bovine serum and 1% pen-strep. Cells adhered to microtiter plates was tested according to a previously described procedure (19), except that cells were suspended using a nonenzymatic dissociation solution (Sigma). Inhibition experiments with GAGs were performed by incubating the FNX10-11 substrate for 15 min with heparin or chondroitin sulfate that was diluted in serum-free medium before adding cells to the plates. The data points are expressed as mean of three replicates, and each experiment was repeated a minimum of three times.

Molecular Modeling—A working model of each of the FNIII-like domains b10 and b11 was constructed based on sequence comparison with known three-dimensional structures of FNIII repeats available from the protein data base at RCSB. The program CLUSTAL W (29) was used to align the FNIII repeats as independent domains with the following Protein Data Bank codes: 1fnf, 1ttg, 1fn1, 1fn2, 1fcr, and 1aw7. The alignment was reformatted, and 10 slightly varying three-dimensional models from each domain were calculated with the modeling program MODELLER4 (30) on an ORIGIN2000 work station (Silicon Graphics Inc.) applying molecular dynamics with standard parameters. The 10 models of each domain were visually inspected with the program X-PLOR (32) to obtain a single model of each FNIII domain. Molecular properties of the three working models, such as potential surface or side chain interactions, were visualized with the freely available program "WebLab viewer Light" (Molecular Simulations Inc.) on a standard PC.

RESULTS

Production and Purification of Recombinant Proteins—After two successive purifications by metal affinity resin and ion exchange chromatography, purified proteins were analyzed by SDS-polyacrylamide gel electrophoresis, confirming the excellent level of purity for each protein (Fig. 1). The apparent
molecular masses of the recombinant FbgX domain produced in E. coli and in 293 cells were slightly different. The higher mass of the protein expressed in mammalian cells was due to (i) the additional c-myc tag introduced by the pSeeTag2/Hyg expression vector and (ii) the glycosylation that occurred in eukaryotic cells. Indeed, the analysis of the primary sequence of bovine TN-X allowed us to predict a N-glycosylation site in the Fbg domain, namely the NIS sequence located at residues 76–78 from this domain (19). The presence of these additional sugars was confirmed by staining gels with Schiff’s reagent (data not shown).

The Fbg Domain of TN-X Is Not Responsible for Interaction with Heparin—Prior to the mapping of the heparin binding site, we tested the ability of TN-X, purified from embryonic bovine skin, to interact with heparin. As shown in the solid phase assay (Fig. 2), the heparin-biotin complex was retained in the wells coated with TN-X in a dose-dependent manner, with a saturation limit. We produced the individual C-terminal fibrinogen domain in a bacterial system (FbgX) and tested its interaction with heparin under the same conditions as intact TN-X. In this case, no interaction could be observed. Because this recombinant domain was recovered from the bacterial pellet after urea extraction and renaturation by dialysis, it is possible that this domain was not correctly folded and that we missed a conformational binding site present in this region. To circumvent this problem, we produced the same domain in mammalian cell cultures. As before, results in the solid phase heparin-binding test were negative, confirming that the fibrinogen domain was not responsible for the interaction of TN-X with heparin.

Localization of a Heparin-binding Site in FNX10-11—To identify a heparin-binding site within TN-X, we tested the binding of recombinant domains spanning FNIII domains 9–11. This region was chosen because sequence analysis of both domains 10 and 11 predicted a positive charge at physiological pH. We tested the recombinant proteins in the solid phase assay, where it clearly appeared that the two domains 10 and 11 were necessary for interaction with heparin (Fig. 3). Only the recombinant proteins FNX10-11 and FNX9-10-11 exhibited heparin binding activity, whereas the individual modules in FNX10, FNX11, or FNX9-10 did not.

Interaction with heparin was also tested using soluble recombinant proteins loaded on to a heparin-Sepharose column (Fig. 4). As expected, FNX10, FNX11, and FNX9-10 were found in the unbound fraction, and FNX10-11 and FNX9-10-11 were retained in the column. By elution with a linear NaCl gradient, both proteins were eluted as a single peak whose position corresponded to a concentration of 0.33 M NaCl. The same apparent affinity was observed when whole TN-X, purified from skin, was loaded on the heparin-affinity column (data not shown). When all recombinant proteins were loaded together in the column, the same result was obtained, suggesting that domains 10 and 11 must be contiguous in the same protein to generate a fully active heparin-binding site. A more careful examination of chromatograms in the unbound fractions suggested that domain 11 had an higher affinity for heparin than domain 10. Indeed, FNX11 protein was found in the late fractions during column washing (Fig. 4A).

Identification of Basic Residues Involved in Heparin Binding—To further map the heparin binding activity, we decided to produce recombinant FNX10-11 proteins bearing single or double mutations replacing basic residues Arg and Lys by Gln and Ser, respectively. First, we examined the primary structure of the two modules 10 and 11. One BBXB sequence, which had been determined to be a potential heparin binding site, was present in these two modules, but this sequence was found at a similar position in 13 of the 30 FNIII-like domains characterized in bovine TN-X (19). Thus, this sequence was unlikely to be a major binding site in our system. In agreement with this hypothesis, our data clearly demonstrated that the site was conformational and involved both domains 10 and 11.

Mutations were dictated by the predicted accessibility of positively charged residues according to our three-dimensional models of the isolated FNIII domains. The contributions of Arg3, Arg4, Lys12, Arg13, Lys16, and Arg18 located in the region connecting both domains 10 and 11 were tested. As shown in Fig. 5, a cluster of basic residues was predicted in domain 11 that comprised Lys12, Arg13, and Arg16. An additional residue
Arg<sup>18</sup> was mutated as a control. In domain 10, the adjacent residues Arg<sup>84</sup>, Lys<sup>85</sup>, and Arg<sup>86</sup> that projected outwards in the three-dimensional model, located in a loop between the F and G β-sheets, were considered as good candidates for the interaction. These residues, together with Arg<sup>45</sup>, a control located on the other side of the module, were mutated. Mutation sites within domains 10 or 11 of FNX10-11 are summarized in Table I (see “Materials and Methods”).

All mutants were tested by heparin-Sepharose chromatography, and their elution positions were compared with that of the wild type FNX10-11 protein. As shown in Table II, the most pronounced effect of a single mutation was observed for Lys<sup>86</sup> of domain 11; this mutant was found in the unbound fraction of the heparin column. All other single mutations realized within this domain had negligible effects. Double mutants 12-13, 12-90, and 13-90 showed a very moderate decrease (from 63 to 77%) in heparin affinity, confirming that these residues were not crucial for interaction with heparin. In domain 10, mutation of Arg<sup>86</sup> resulted in strong inhibition of heparin binding because the protein was eluted at only 30% retention time compared with the wild type. Moreover, the cluster of basic residues 84–86 appeared important for heparin interaction because the double mutants 85-86 and 84-86 were not retained in the column.

**Efficiency of the FNIII 10–11 Heparin-binding Site in the Whole TN-X Molecule—**To analyze the accessibility of this site in the native TN-X molecule, we performed inhibition studies using polyclonal and monoclonal antibodies. The polyclonal antibody was prepared by immunizing guinea pigs with the FNX9-10 protein. Because these two domains showed only a moderate sequence identity with other FNIII domains, one can assume that the polyclonal antibody is specific to the region of domains 9 and 10 in the intact TN-X. Moreover, when the reactivity of this antibody was tested on FNX recombinant proteins by enzyme-linked immunosorbent assay, we found a strong affinity for FNX9, FNX10, FNX9-10, and FNX9-10-11, with negligible affinity for FNX11 (data not shown), even though sequence identity between domain 11 and domains 9 and 10 is 62 and 58%, respectively. To confirm the inhibition data, we also used a monoclonal antibody whose epitope is located within domain 10 (clone14G5) and control monoclonal antibodies (clones 15F12 and 19D1) that recognize the region encompassing FNIII domains 12–15. Localization of epitopes was determined by reactivity with clones derived from the cDNA expression library from bovine embryonic skin (19) and by enzyme-linked immunosorbent assay with the set of recombinant FNX proteins. Clone 14G5 reacted only with proteins bearing domain 10, whereas 15F12 and 19D1 clones were negative with any of the proteins derived from domains 9–11 (data not shown).

When polyclonal anti-FNX9-10 or monoclonal 14G5 antibodies were incubated with the proteins before adding heparin, an inhibition of interaction was observed for both FNX10-11 and intact TN-X purified from bovine skin (Fig. 5). A maximum of 93% inhibition was obtained using purified polyclonal antibodies and FNX10-11 protein, whereas with intact TN-X, inhibition was only 67%. Monoclonal antibody 14G5 was also able to inhibit this interaction but to a lesser extent, 61 and 52% for FNX10-11 and TN-X, respectively. Control incubations using buffer, nonimmune guinea pig IgG, 15F12, or 19D1 clones did not give a significant inhibition of heparin binding. Taken together, these results confirm first that the FNIII domain 10 was involved in interaction with heparin and second that the site located within domains 10 and 11 that we described above was functional in the whole TN-X molecule.
FNX 10

| FNIII domain | Mutated residue | Retention % |
|--------------|-----------------|-------------|
| Arg<sup>18</sup> | 87 | |
| Lys<sup>84</sup> | 67 | |
| Arg<sup>25</sup> | 59 | |
| Arg<sup>46</sup> | 30 | |
| Lys<sup>84</sup> and Arg<sup>45</sup> | 52 | |
| Arg<sup>6</sup> and Arg<sup>26</sup> | 0 | |
| Lys<sup>84</sup> and Arg<sup>46</sup> | 0 | |

FNX 11

| FNIII domain | Mutation(s) | Retention % |
|--------------|-------------|-------------|
| Arg<sup>7</sup> | 86 | |
| Arg<sup>6</sup> | 92 | |
| Lys<sup>12</sup> | 81 | |
| Arg<sup>13</sup> | 90 | |
| Lys<sup>16</sup> | 0 | |
| Arg<sup>18</sup> | 92 | |
| Arg<sup>20</sup> | 82 | |
| Lys<sup>12</sup> and Arg<sup>13</sup> | 63 | |
| Arg<sup>13</sup> and Arg<sup>20</sup> | 77 | |
| Arg<sup>18</sup> and Arg<sup>20</sup> | 75 | |

**Cell Interaction with FNX10-11 Protein**—To analyze the possible interaction of heparan sulfate receptors in cell adhesion to TN-X, we performed adhesion studies on the recombinant protein FNX10-11. CHO cells were chosen firstly because they do not express α<sub>1</sub>β<sub>3</sub>, an integrin that might interfere with the adhesion tests on recombinant FNX proteins; indeed, we have previously shown that this integrin is a ligand for the RGD site of domain 10 (25). Secondly, the availability of mutant CHO cell lines deficient in GAG synthesis allowed us to analyze the GAG class involved in the interaction. CHO-K1 cells were able to adhere the FNX10-11 protein in a dose-dependent manner (Fig. 7A). Two mutant cell lines derived from CHO-K1 were tested in adhesion experiments: CHO-77, deficient in heparan sulfate but synthesizing more chondroitin sulfate, and CHO-745, which lacks both heparan and chondroitin sulfates. These two mutant cells exhibited a very low interaction with FNX10-11 protein, corresponding to ~25–30%, compared with the parental CHO-K1 cells (Fig. 7A). As a confirmation of these results, inhibition of cell interaction by incubating the FNX10-11 substrate with GAGs was performed. As shown in Fig. 7B, heparin was a potent inhibitor of the interaction between FNX10-11 and CHO-K1 cells, whereas chondroitin sulfate had no significant effect. These data indicated that cell interaction with FNX10-11 and consequently with TN-X may be mediated by heparan sulfate receptors.

**DISCUSSION**

Since the early studies, TN-X, has been identified as a heparin-binding molecule (16), but the localization of this activity has not been further investigated. Considering that in TN-C the Fbg domain is responsible for this activity and in view of the high level of sequence identity of Fbg within the family of TN molecules, it has been postulated that this domain is involved in the heparin binding properties of TN-X (16). Our first set of experiments was designed to test this hypothesis using solid phase assays. We found that whole TN-X is able to bind heparin, whereas the recombinant Fbg domain, produced either in bacteria or in mammalian cells, is not. In TN-C, the heparin binding activity, mapped to the Fbg domain (2, 5), has not been further investigated. Considering that in TN-C the Fbg domain is responsible for this activity and in view of the high level of sequence identity of Fbg within the family of TN molecules, it has been postulated that this domain is involved in the heparin binding properties of TN-X (16). Our first set of experiments was designed to test this hypothesis using solid phase assays. We found that whole TN-X is able to bind heparin, whereas the recombinant Fbg domain, produced either in bacteria or in mammalian cells, is not. In TN-C, the heparin binding activity, mapped to the Fbg domain (2, 5), has been shown, by mild tryptic digestion, to be located within 10 kDa from the C terminus of the domain. Two possible regions comprising clusters of basic residues were candidates for this interaction: the sequence AKTRYRLRV (amino acids 1702–1710 from the chicken sequence) and the extreme C-terminal sequence GRRKRA (amino acids 1803–1808) (5). It is quite remarkable that these basic residues are conserved in TN-C from different species, whereas these sequences are changed...
Arg86 in domain 10 and Lys 16 in domain 11 as crucial for sulfates.

Chondroitin sulfate, and CHO-745 lacks both heparan and chondroitin cell line, CHO-677 is deficient in heparan sulfate and synthesizes more different CHO cell lines adhering to FNX10-11. CHO-K1 is the control

Interaction with heparin (HEP) was performed by preincubating the wells and diluting the CHO-K1 cell suspension with the indicated concentration of GAG. Results are expressed as the means of triplicate experiments and standard error of the mean.

respectively to DSADEYYRLHL and GRGG in bovine TN-X. These sequential sites are not responsible for the heparin binding properties of TN-X. Moreover, we can also exclude the presence of another conformational binding site within the Fbg domain, because the recombinant domain prepared from mammalian cell expression was not effective in heparin binding. Thus, the mechanisms of interaction of TN-X with heparin are different from those of TN-C.

To map further the heparin binding activity on TN-X, FNIII domains were also checked for this property. In other extracellular matrix proteins such as TN-C (3) or fibronectin (34) some of these domains are involved in heparin-binding. Although other amino acids may participate in the binding, stretches of basic residues that are positively charged at physiological pH, namely Arg and Lys, are good candidates for electrostatic interactions with negatively charged GAG chains. Sequence analyses indicate that the two contiguous FNIII domains 10 and 11 of TN-X are positively charged and that these domains were tested for heparin binding activity. Results obtained with solid phase assays and analytical chromatography on heparin-Sepharose both led to the same conclusion that an heparin-binding site, involving both domains 10 and 11, is present in this region. The data also indicate that the relative orientation of the two domains is important because mixtures of FNX10 and FNX11 did not bind heparin in affinity chromatography (Fig. 4) and solid phase (data not shown). Thus, our results indicate the presence of one conformational binding site whose nature might be either a mutivalent site involving two separate binding regions in each domain or a unique site located in a pocket between the two domains.

Using site-directed mutagenesis, we identified Lys85 and Arg66 in domain 10 and Lys16 in domain 11 as crucial for interaction with heparin. None of these residues were located in sequences matching those previously determined by Cardin and Weintraub (33) as consensus sequences for heparin binding such as XBBXBX or XBBBXXBB, where B is a basic residue and X a nonbasic, frequently hydrophobic amino acid. The mutagenesis results thus confirmed the conformational nature of the site. Accessibility of the residues involved in the interaction was predicted from our three-dimensional working model of isolated domains 10 and 11 (Fig. 5). Residue Arg86 of domain 10 is located in a region predicted as a loop between the F and G β-sheets of the FNIII module and Lys16 in domain 11 is located in the region linking domains 10 and 11. Our model was not able to predict the relative orientation of domains 10 and 11, a parameter that may be important in the binding site that we have defined. In the model proposed by Leahy et al. (35), the adjacent FNIII domains of TN-C are tightly packed to make an extended filament. The tilted orientation of domains 10 and 11 in TN-X may bring together the basic residues involved in the interaction to create a cationic pocket. The crystallization data obtained by Sharma et al. (36), dealing with repeated arrays of FNIII domains of fibronectin, led to the conclusion that the inter-repeat interface varies from 340 to 660 Å²; some interfaces are thus flexible, whereas others are relatively rigid units. In TN-X, the inter-domain sequences have a variable size but an unusual length when compared with those of fibronectin or TN-C; we have suggested that the highly flexible appearance of TN-X in electron microscopy is due to the length of these inter-domain regions (19). It should be emphasized that in the region comprising domains 10 and 11, these extensions are relatively short. This property may favor a closer orientation between domains 10 and 11 and thus preserve the efficiency of the heparin-binding site.

In TN-C, two heparin-binding regions, i.e. FNIII domain 5 and the Fbg domain, were identified using isolated recombinant domains (2, 3), but only the Fbg domain was found to be functional in the intact TN-C molecule (5). To examine the efficiency of heparin-binding of domains 10 and 11 within intact TN-X, we carried out inhibition studies using antibodies specific for this region. The antibodies were able to decrease specifically the interaction between TN-X and heparin, thus indicating that the conformational binding site that we detected using recombinant proteins is accessible within the intact molecule. However, because inhibition was weaker for the intact molecule than for the recombinant proteins, we cannot exclude the possibility that an additional heparin-binding site might exist in TN-X. In TNs, the FNIII domain region is subjected to alternative splicing events that may modulate the interaction properties of these proteins (1). Such a mechanism might be involved in the interaction of TN-X with heparin or glycosaminoglycans. From studies of mouse TN-X cDNA, in the region corresponding to bovine domains 10 and 11, no alternative transcripts could be detected (20). In contrast to these studies, our analyses of human TN-X transcripts from MG63 cells and human fetal tissues revealed isoforms lacking the human domain 8, which corresponds to the bovine domain 10. The existence of these isoforms, probably deficient in heparin binding, may be important for the spatio-temporal regulation of TN-X functions.

The interaction of TN-X with heparin-related GAGs may be necessary for interaction with extracellular matrix and/or cell surface proteoglycans. Heparan sulfate receptors are involved in numerous cellular events and function as coreceptors, acting in concert with integrins or growth factor receptors (37). In previous studies, we have shown that the interaction of cells with TN-X is partially inhibited by heparin, suggesting that
heparan sulfate receptors may be involved in this interaction (19). In this study, we have tested the ability of different CHO cell lines to interact with a recombinant fragment bearing the heparin-binding site. The results demonstrate that CHO cells interact with the fragment via GAG chains of cell surface proteoglycans and that the interaction is mediated by heparan and not chondroitin sulfate chains. Because we have previously shown that integrin receptors are also involved in the interaction of TN-X with cells (25), we suggest that the combined effects of both signals induced by integrins and by heparan sulfate receptors may be involved in this interaction with a TN-X substrate.

REFERENCES

1. Jones, F. S., and Jones, P. L. (2000) Dev. Dyn. 218, 235–259
2. Aukhil, I., Joshi, P., Yan, Y., and Erickson, H. P. (1993) J. Biol. Chem. 268, 2542–2553
3. Weber, P., Zimmermann, D. R., Winterhalter, K. H., and Vaughan, L. (1995) J. Biol. Chem. 270, 4619–4623
4. Joshi, P., Chung, C. Y., Aukhil, I., and Erickson, H. P. (1993) J. Cell Sci. 106, 389–400
5. Fischer, D., Chiquet-Ehrismann, R., Bernasconi, C., and Chiquet, M. (1995) J. Biol. Chem. 270, 3378–3384
6. Hoffman, S., and Edelman, G. M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2523–2527
7. Chiquet, M., and Fambrough, D. M. (1984) J. Cell Biol. 98, 1926–1936
8. Vaughan, L., Huber, S., Chiquet, M., and Winterhalter, K. H. (1987) EMBO J. 6, 349–353
9. Chung, C. Y., and Erickson, H. P. (1997) J. Cell Sci. 110, 1413–1419
10. Seifert, M., Beck, S. C., Schermutski, F., Muller, C. A., Erickson, H. P., and Klein, G. (1998) Matrix Biol. 17, 47–63
11. Salmivirta, M., Elenius, K., Vainio, S., Faissner, A., and Chiquet-Ehrismann, R., Theileff, I., and Jalkanen, M. (1991) J. Biol. Chem. 266, 7733–7739
12. Vaughan, L., Zisch, A. H., Weber, P., D’Allessandrini, L., Ferber, P., David, G., Zimmermann, D. R., and Winterhalter, K. H. (1994) Contrib. Nephrol. 107, 80–84
13. Barnea, G., Grumet, M., Milev, P., Silvennoinen, O., Levy, J. B., Sap, J., and Schlessinger, J. (1994) J. Biol. Chem. 269, 14349–14352
14. Milev, P., Fischer, D., Haring, M., Schuhbess, T., Margolis, R. K., Chiquet-Ehrismann, R., and Margolis, R. U. (1997) J. Biol. Chem. 272, 15561–15569
15. Rauch, U., Clement, A., Retzler, C., Frohlich, L., Fassler, R., Gohring, W., and Faisner, A. (1997) J. Biol. Chem. 272, 26905–26912
16. Matsumoto, K., Sago, Y., Ikemura, T., Sakakura, T., and Chiquet-Ehrismann, R. (1994) J. Cell Biol. 125, 483–493
17. Hagios, C., Koch, M., Spring, J., Chiquet, M., and Chiquet-Ehrismann, R. (1996) J. Cell Biol. 134, 1499–1512
18. Bristow, J., B., M. K., Gitelman, S. E., Mellon, S. H., and Miller, W. L. (1993) J. Cell Biol. 122, 265–278
19. Elefteriou, F., Exposito, J. Y., Garrone, R., and Lethias, C. (1997) J. Biol. Chem. 272, 22866–22874
20. Bristow, J., Tee, M. K., Gitelman, S. E., Mellon, S. H., and Miller, W. L., and Bristow, J. (1995) Dev. Dyn. 203, 491–504
21. Geffrotin, C., Garrido, J. J., Tremet, L., and Vaiman, M. (1995) Eur. J. Biochem. 231, 85–92
22. Burch, G. H., Bedolli, M. A., McDonough, S., Rosenthal, S. M., and Bristow, J. (1997) J. Cell Biol. 136, 104–108
23. Lethias, C., Descollonges, Y., Boutilier, M. M., and Garrone, R. (1996) Matrix Biol. 15, 19–26
24. Burch, G. H., Gong, Y., Liu, W., Dettman, R. W., Curly, C. J., Smith, L., Miller, W. L., and Bristow, J. (1997) Nat. Genet. 17, 104–108
25. Elefteriou, F., Exposito, J. Y., Garonne, R., and Lethias, C. (1999) Eur. J. Biochem. 263, 840–848
26. Kamman, M., Laufs, J., Schell, J., and Gronenborn, B. (1989) Nucleic Acids Res. 17, 5404
27. Lethias, C., Exposito, J. Y., and Garonne, R. (1997) Eur. J. Biochem. 245, 434–440
28. Esco, J. D., Stewart, T. E., and Taylor, W. H. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3197–3201
29. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680
30. Sali, A., and Blundell, T. L. (1993) J. Mol. Biol. 234, 779–815
31. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard. (1991) Acta Crystallogr. Sect. A 47, 110–119
32. Bruger, A. T., Krukowsky, A., and Erickson, J. W. (1990) Acta Crystallogr. Sect. A 46, 585–593
33. Cardin, A. D., and Weintraub, H. J. (1989) Arteriosclerosis 9, 21–32
34. Busby, T. F., Argraves, W. S., Brew, S. A., Pechik, I., Miller, W. L., and Bristow, J. (1997) Eur. J. Biochem. 264, 483–493
35. Leahy, D. J., Hendrickson, W. A., Aukhil, I., and Erickson, H. P. (1991) Science 250, 587–591
36. Sharma, A., Askari, J. A., Humphries, M. J., Jones, E. Y., and Stuart, D. I. (1999) EMBO J. 18, 1468–1479
37. Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Liniecum, J., and Zako, M. (1999) Annu. Rev. Biochem. 68, 729–777
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