Identification and Kinetics Analysis of a Novel Heparin-binding Site (KEDK) in Human Tenascin-C*

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The interaction between tenascin-C (TN-C), a multirepeat extracellular matrix protein, and heparin was examined using a surface plasmon resonance-based technique on a Biacore system. The aims of the present study were to examine the affinity of fibronectin type III repeats of TN-C fragments (TNIII) for heparin, to investigate the role of the TNIIII4 domains in the binding of TN-C to heparin, and to delineate a sequence of amino acids within the TNIII4 domain, which mediates cooperative heparin binding. At a physiological salt concentration, and pH 7.4, TNIII3–5 binds to heparin with high affinity (KD = 30 nM). However, a major heparin-binding site in TNIII5 produces a modest affinity binding at a KD near 4 μM, and a second site in TNIII4 enhances the binding by several orders of magnitude, although it was far too weak to produce an observable binding of TNIII4 by itself. Moreover, mutagenesis of the KEDK sequence in the TNIIII4 domain resulted in the significant reduction of heparin-binding affinity. In addition, residues in the KEDK sequences are conserved in TN-C throughout mammalian evolution. Thus the structure-based sequence alignment, mutagenesis, and sequence conservation data together reveal a KEDK sequence in TNIIII4 suggestive of a minor heparin-binding site. Finally, we demonstrate that TNIIII4 contains binding sites for heparin sulfate proteoglycan and enhances the heparin sulfate proteoglycan-dependent human gingival fibroblast adhesion to TNIIII5, thus providing the biological significance of heparin-binding site of TNIIII4. These results suggest that the heparin-binding sites may traverse TNIIII4–5 and thus require KEDK in TNIIII4 for optimal heparin-binding.

Tenascin-C (TN-C) is a large extracellular matrix glycoprotein composed of a linear arrangement of domains homologous to epidermal growth factor, fibronectin type III domains (FNIII), and fibrinogen (1, 2). TN-C has an oligomeric structure made up of six identical subunits in a radially arranged six-armed structure called a hexabrachion (3). Each subunit exists as a string of small globular domains, including 8–15 FNIII domains (4, 5). FNIII is found in a wide variety of proteins including cell surface receptors and cell adhesion molecules (6). The core structure of the FNIII repeats is conserved forming a β-sandwich with four β-strands on one side and three on the other (7, 8). The third FNIII repeat of TN-C (TNIII3) contains an Arg-Gly-Asp (RGD) tripeptide sequence, has been shown to interact with cells via a variety of integrins, including ovβ3, ovβ6, oβ81, and α2β1 (9–14). In addition, TN-C binds to a number of other cell surface proteins such as CTB-proteoglycan (15), neurocan, phosphacan (16), and syndecan-1 (17). Syndecan-1 and TN-C are co-expressed transiently in the organ primordial at the interface between mesenchyme and epithelium (18, 19). Syndecan-1 binding to TN-C depends on the heparan sulfate side chains of the proteoglycan (17).

Heparin/heparan sulfate and heparan sulfate proteoglycans (HSPGs) are a group of macromolecules that are characterized by long unique carbohydrate chains attached to a protein core (20, 21). They exist as components of the extracellular matrix or as membrane-associated proteins and participate in diverse biochemical and physiological processes via interactions with a variety of proteins including extracellular matrix proteins (22–24). Heparin inhibits the adhesion of peripheral neurites to TN-C in a culture (25, 26). To understand TN-C function, it is important to identify the specific domains of the protein responsible for binding to glycosaminoglycans. Two heparin-binding sites have been identified in TN-C (27, 28), one comprising the fifth FNIII repeat (TNIIII5) and the other comprising the COOH-terminal fibrinogen-like domain.

Heparin/heparan sulfate binding seems to be a common feature of extracellular matrix molecules, including TN-C. TN-C binds to heparin at physiological salt concentrations, although the affinity of TN-C binding to heparin has not been demonstrated specifically until now (29). The present study was undertaken to examine more specifically the interaction of TN-C with heparin using surface plasmon resonance (SPR) and to delineate the putative heparin-binding site that mediates this interaction. Until today, the characterization of binding sites on heparin that interact with the FNIII domains has largely focused on the TNIIII5 domain, although it is not clear whether or not these are the only motifs required for heparin interaction. In this study we investigate the specific role of the TNIIII4 domain in heparin binding by characterizing wild-type and mutant TNIII protein through the use of SPR direct binding studies. Here we present evidences suggesting that the secondary heparin-binding site in TNIIII4 contributes to overall TNIII binding to heparin.

EXPERIMENTAL PROCEDURES

Materials—Biotinylated heparin (BiHep) was from Sigma. Biosensor BiacoreX and sensor chip CM5 (disposable sensor surface covered with a carboxymethyl dextran layer for protein immobilization) were from Biacore (Uppsala, Sweden). HBS buffer (10 mM Hepes, pH 7.4, contain-
ing 150 mM NaCl and 0.005% surfactant P20, NHS (N-hydroxysuccinimide), EDC (N-ethyl-N-3-(3-dimethylaminopropyl)carbodiimide), and ethanolamine were from Biorad.

Immobilization of Heparin—In these assays, streptavidin was coupled to a CM5 sensor chip using the amine coupling kit (Biorad) with the procedures specified by the supplier. There are two flow cells/ sensor chip for the BiacoreX system. The streptavidin was immobilized in the first flow cell (FC-1) to 20,000 resonance units. Bovine serum albumin was immobilized in the second flow cell (FC-2) to 950 resonance units as a negative control for background correction. BiHep was immobilized to the streptavidin-coupled CM5 sensor chip according to supplier’s instructions. The flow cell with the sensor surface was kept at 25°C.

Biacore Binding Analysis—Evaluation of kinetic parameters ($k_a$ and $k_d$) was performed by using a SPR-based BiaCoreX biosensor. To determine the binding kinetics of each TN-C protein, a series of samples with varied concentrations was injected during the association phase for 5 min (40 μl/min). The dissociation phase was carried out over a period of 10 min. The flow cell could be regenerated with a 3-min injection of 1.0 M NaCl, which removed the TN-C protein while leaving the heparin intact. The kinetic analysis of sensograms from the interaction of various analytes with the immobilized BiHep was based on the rate equation, $dR/dt = -k_a R_{\text{max}} - k_d C$, where $dR/dt$ is the rate of change of the SPR signal (resonance units) due to analyte interaction with immobilized BiHep at time $t$ seconds, $k_a$ and $k_d$ are the association- and dissociation-rate constants, respectively, $C$ is the concentration of analyte, and $R_{\text{max}}$ is the maximum analyte binding capacity to BiHep in resonance units. Both association- and dissociation-rate constants were determined from the analysis of sensograms using the Biaevaluation software, version 3.0. All binding curves were corrected for background and bulk refractive index contribution by subtraction of the reference flow cells. Models were fitted both globally across the data sets and for a single concentration.

Construction of Expression Vectors and Site-directed Mutagenesis—Human TN-C cDNAs were amplified from human cDNA by employing standard PCR amplification methods. PCR primers were designed to recognize type III modules (TNIII3–6), TN5 forward primers 5′-AGCCATAGTGTCCTCCTCCACACTGACCTG-3′, TN5 reverse primers 5′-GGGAATCCACTGCGGCGCTCCAC-3′, TN3 forward primers 5′-TCGAATTTACAGGGCAGCTTT-3′, and TN3 reverse primers 5′-TCGAATTTACAGGGCAGCTTT-3′. PCR-amplified cDNA products were digested with KpnI and EcoRI, separated using a PCR Purification kit (Qiagen, Chatsworth, CA), and ligated into pBADDHA expression vector (Invitrogen). Site-directed mutagenesis was performed using the QuikChange kit method (Stratagene, La Jolla, CA). The following TN-C residues were individually substituted with glutamine: Lys731, Lys734, Lys1057, and Arg1059. All expression constructions and mutations were verified by DNA sequencing. The recombinant fragments of human TN-C spanning FNIII domains in its interaction with heparin, we prepared a series of recombinant proteins, which contained the primary heparin-binding site alone produces a dramatic effect on the binding of TNIII5 to BiHep at physiological conditions (30 and 40 nM for TNIII3–6 and TNIII4–5, respectively) using BiHep immobilized to the streptavidin-coupled CM5 sensor chip (Fig. 2). As shown in Table I, both TNIII3–6 and TNIII4–5 exhibited a fast association rate with heparin ($k_a$ TNIII3–5, 8.64 × 10^3 M$^{-1}$s$^{-1}$; $k_a$ TNIII4–5, 4.46 × 10^3 M$^{-1}$s$^{-1}$) biosensor surfaces. The resultant complexes between TN-C-heparin were stable as illustrated by slow dissociation rates ($k_d$ TNIII3–5, 0.29 × 10$^{-3}$ s$^{-1}$; $k_d$ TNIII4–5, 0.2 × 10$^{-3}$ s$^{-1}$).

Interestingly, TNII5 exhibited a decreased affinity ($K_D$ = 4.25 μM) for heparin compared with TNIII3–5 and TNIII4–5 (160- and 160-fold, respectively). However, both TN3 and TN4 bind heparin biosensor surfaces so poorly that association and dissociation kinetics could not be quantitated (Table I). An important new finding in the present study is that the heparin binding of an expression protein containing only the TNIII5 domain was always much weaker than the binding of TNIII3–6, even if it were far too weak to produce an observable binding of TNII4 by itself.
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Fig. 2. Biacore analysis of TNIII3–5, TNIII4–5, and TNII5 binding to immobilized heparin. Biosensorsgrams are shown for the interaction of TNIII3–5 (A), TNIII4–5 (B), and TNII5 (C) with immobilized heparin as described under “Experimental Procedures.” The apparent equilibrium dissociation constants estimated from these data are summarized in Table I.

TABLE I
Results of the Biacore binding studies with immobilized heparin and tenasin-C

| Analyte     | $k_a$       | $k_d$       | $K_D$     |
|-------------|-------------|-------------|-----------|
| TNIII3–5    | $2.99 	imes 10^{-2}$ | $8.64$       | $0.03$    |
| TNIII4–5    | $0.24$      | $4.46$      | $0.04$    |
| TNII5       | $0.82$      | $0.19$      | $4.25$    |
| TNII4       | NB          | NB          | NB        |
| TNII5       | NB          | NB          | NB        |

Fig. 3. Structure-based sequence alignment of TN-C type III 3–5 repeats and FN type III 10 domain. A, residues in green are identical in all four repeats, and those in yellow are identical in at least two repeats. RGD sequences are in red. B, the collection of TNII4 sequences across species Homo sapiens (human), Mus musculus (mouse), Gallus gallus (chicken), and Sus scrofa (pig).

Role of Heparin-binding Domain of TNIII4 in Cell Adhesion—To investigate the biological significance of the heparin-binding domain of TNIII4, we explored whether a heparin-binding property was relevant to the process of cell adhesion. HGFs were plated on dishes coated with a series of TNIII fragments. When HGF cell adhesion to a TNIII fragment—coated dish was quantitated by the crystal violet staining of fixed cells, TNIII4–5 exhibited significantly higher adhesion activity than TNIII5. Notably, the cell binding to TNIII4–5 exhibited significantly higher adhesive activity than TNIII5 (70-fold). As expected, mutations of the KRGR sequence in the TNII5 domain (TNIII4–5 K751Q and TNIII4–5 K1057Q) exhibited a reduced affinity (KD = 29.2 μM) for heparin compared with TNIII4–5 (70-fold). Based on these data, we conclude that the TNII5 domain is the primary heparin-binding site, and residues at Arg1059 and Lys1057 in this domain are of critical importance in the interaction. The fact that mutations of the KEDK sequence in the TNIII4 domain reduced affinity for heparin strongly indicates that the complete heparin binding in TN-C relies on secondary heparin binding formed by KEDK residues. These results suggest that the KEDK residues in the loop region between F and G strands contribute to an optimal heparin binding activity of TN-C, and heparin-binding sites in TN-C may span both of the TNII4 and TNII5 domains.

KEDK Residues in TNII4 Contribute to Heparin Binding Activity of TNII5—The heparin binding activity of TNII4–5 mutants was assessed by SPR-based Biacore technology. The heparin binding activity of the TNII4–5 mutants in the putative heparin-binding sites was compared with the TNIII4–5 wild-type protein. The results from SPR-based Biacore are summarized in Table II. The KD for recombinant TNIII4–5 K751Q/K754Q exhibited a reduced affinity (KD = 29.2 μM) for heparin compared with TNIII4–5 (70-fold). To further investigate the loop regions of the alignment most often contain hydrophilic amino acids, because they are positioned on the surface of the protein. The sequence KRGRHKKSPARVK in TNII5 has been implicated in heparin binding activity previously (28). These six positively charged residues (contributed from a loop between F and G strands) project into the solvent. Thus, we focused on the loop regions in TNII4 for a potential role in binding to heparin. With the above information taken collectively, the KEDK (amino acids 751–754) sequence possesses a predominantly positive surface charge that makes its relevance to heparin binding activity previously (28). These results suggest that prior occupancy of the heparin-binding sites by heparin strongly indicates that the complete heparin binding in TN-C relies on secondary heparin binding formed by KEDK residues. These results suggest that the KEDK residues in the loop region between F and G strands contribute to an optimal heparin binding activity of TN-C, and heparin-binding sites in TN-C may span both of the TNII4 and TNII5 domains.

TABLE II
Biacore binding studies with immobilized heparin and tenasin-C mutants

| Analyte         | $k_a$       | $k_d$       | $K_D$     |
|-----------------|-------------|-------------|-----------|
| K751Q/K754Q (KEDK) | 0.01        | 1.38        | 29.2      |
| K1057Q (KGR)    | NB*         | NB          | NB        |
| R1059Q (KGR)    | NB          | NB          | NB        |

*NB, no binding.
enhances the adhesive activity of TNIII5 to HSPGs through a cooperative mechanism.

**DISCUSSION**

In the present study we used SPR-based Biacore technology to define the influence of the KEDK sequence of the TNIII4 domain upon the interaction of TN-C with heparin. In agreement with a previous finding, we observed that the TNIII5 fragment containing a major heparin-binding site binds to heparin under conditions of physiological pH and ionic strength. However, the affinity of TNIII5 alone for heparin is significantly lower than that of fragments containing both TNIII4 and TNIII5 domains. Therefore, the presence of the TNIII4 domain may enhance the heparin-binding affinity of TNIII5 by some cooperative mechanism. The simplest model would postulate two binding sites, one in TNIII4 and another in TNIII5, which can bind simultaneously to heparin. It is of interest to note that the cooperative contribution of the adjacent FNIII repeat(s) is often found in maximum heparin binding. A similar example of this cooperative heparin-binding with the adjacent FNIII repeat can be seen with the primary heparin-binding site in the 13th type III repeat of fibronectin (FNIII13) and a putative minor site ~60 Å away in the 14th type III repeat of fibronectin (FNIII14) (30). An additional similar example comes from the two contiguous heparin-binding sites in the 10th and 11th type III repeat of tenascin-X (TN-X) (33).

The overall architecture of FNIII repeat molecules may adopt a variety of shapes depending on the tilted angles of inter-repeats and the length of these inter-repeat regions. The previous study (7) proposed that the TNIII4 and TNIII5 repeats are tightly packed resulting in an extended and relatively straight strand. Our analysis of the structure-based sequence alignment of the TNIII repeats and FNIII repeat structure indicates that the sequence KEDK in the loop region between the F and G β-sheets of TNIII4 is on the same face of the molecules as the sequence KGR in the loop region of TNIII4, although two TNIII repeats are concatenated in a slight spiral. Interestingly, the heparin-binding site in TN-X has been predicted to reside in this loop region between the F and G β-sheets of the FNIII10 module and a linker region between FNIII10 and FNIII11. However, because of the linker-domain region, TN-X has more a flexible orientation between the FNIII10 and FNIII11, producing a cationic pocket.

Heparin-protein interactions are based on ionic contacts between positively charged residues (Arg and Lys) and the negatively charged groups of heparin (sulfate groups of glucosamine or the carboxylate group of iduronic acid) (34). Residues Lys751 and Lys754 in the TNIII4 domain were found to constitute the minor heparin-binding domain, with a double mutation of K751Q/K754Q resulting in the significant reduction of heparin-binding affinity. In addition, residues in the KEDK sequences are conserved in TN-X between different species ranging from a chick to a human. Thus the structure-based sequence alignment, mutagenesis, and sequence conservation data together suggest that the KEDK sequence in TNIII4 is essential for the high affinity binding to heparin. Because the KEDK sequence in TNIII4 is on the same face of the molecules as the GR sequence in TNIII5, heparin-binding sites may traverse TNIII4–5 and thus require the residues KEDK from the TNIII4 for optimal heparin binding.

The sequence KGKKKPARKKVK in TNIII5 has been implicated in heparin binding activity previously (28). In this study, we found that mutagenesis of Lys1057 and Arg1059 reduces heparin binding drastically. However, other basic residues cannot be excluded for the heparin-binding site. The simplest model would postulate two binding sites, one in TNIII4 and another in TNIII5, which can bind simultaneously to heparin. We have now confirmed that the secondary low affinity heparin-binding site in the TNIII4 domain is conserved ranging in chick, mouse, pig, and human. The conservation of this activity over the 300-million years that separate chickens and humans suggests that it is biologically important for the functioning of TN-C in tissues. Thus, we conclude that the TNIII4 domain contributes to optimal heparin binding and the KEDK sequence in TNIII4 is minor heparin-binding site.

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