The Plasminogen Binding Site of the C-Type Lectin Tetranectin Is Located in the Carbohydrate Recognition Domain, and Binding Is Sensitive to Both Calcium and Lysine

(Received for publication, June 26, 1998, and in revised form, August 13, 1998)

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Tetranectin (TN) is a 68-kDa plasminogen-binding protein occurring in plasma at an approximate concentration of 10 mg/liter (1) and is also found to be deposited extracellularly in various tissues. In particular, TN is deposited in the tumor-surrounding stroma of breast (2), colon (3), and ovarian (4) tumors and is found co-localized with plasmin/plasminogen at the invasive front of cutaneous melanoma lesions (5). TN has also been inferred to play a role in the pathophysiology of rheumatoid arthritis (6) and in bone mineralization during osteogenesis (7, 8). It is not known whether TN deposited in tumor stroma is recruited from plasma or is expressed locally.

TN appears to be expressed by a variety of cells, including fibroblasts (9), monocytes (10), neutrophils (11), and osteoblasts (7). TN mRNA has been detected in the placenta, heart, spleen, and lung and, in low amounts, also in the liver and kidney (12).

TN is a trimeric protein (13) assembled from three identical polypeptide chains of 181 amino acid residues (14). The molecular architecture of TN reflects the genomic organization (12) in that the three exons correspond to three functional domains: (i) an NH2-terminal domain of about 20 amino acid residues; (ii) a triple α-helix coiled coil-forming domain of approximately 30 amino acid residues; and (iii) a long-form carbohydrate recognition domain (CRD) characteristic of the C-type lectin protein superfamily of approximately 130 amino acid residues.

TN binds to plasminogen (Plg) by a specific and lysine-sensitive interaction with the kringle 4 domain (Plg K4), and binding facilitates the proteolytic activation of Plg to plasmin by the tissue-type plasminogen activator (1). In addition to Plg, TN has been reported to bind apolipoprotein(a) and fibrin (15, 16).

TN is also known to bind calcium as well as a number of complex sulfated polysaccharides including heparin/heparan sulfate, chondroitin, and fucoidan (17).

The three-dimensional structure of tetranectin has been determined by x-ray crystallographic analysis of crystals of full-length recombinant tetranectin, rTN (18), and the long-form CRD, rTN3 (19). The structural analyses revealed a highly conserved fold shared between TN and the neck region and CRD of the human and rat mannose-binding proteins (MBPs) of the collectin protein family. Many secondary structural elements as well as the number and location of calcium ion binding sites are conserved among all three proteins.

Calcium binding site 1 is highly conserved. Although two of the five coordinating residues in rat MBP are substituted in TN (i.e. Asp-188 to Gly-147 and Asp-194 to Asn-151, respectively), both the coordination geometry and position of calcium ion 1 are very similar.

Coordination geometry at calcium binding site 2 in TN differs from those of the MBPs because three of the five calcium-coordinating residues in MBP, i.e. Glu-185, Asn-187, and Asn-205, are replaced with TN residues Gln-143, Asp-145, and Phe-164, respectively. TN residues Gln-143 and Asp-145 coordinate calcium directly, whereas the side chain of Phe-164 is rotated away from the calcium ion, thereby allowing the accommodation of a fixed water molecule, which serves a role as calcium coordinator in place of the Asn-205 side chain of the MBPs (19).

Calcium binding site 2 is involved in carbohydrate binding in all C-type lectins known to exhibit calcium-dependent interaction with terminal sugar residues (reviewed in Ref. 20).

At the present stage, no carbohydrate ligand to the TN CRD has been identified. However, the presence of the calcium-coordinating residues Gln-143 and Asp-145 and the Pro-144, which is structurally conserved in the cis conformation, in addition to the presence of a surface-exposed aromatic residue (Phe-164) adjacent to the calcium binding site, may be taken as...
suggestive of a galactose-type specificity of the TN CRD.

Apart from calcium-dependent carbohydrate binding, CRDs of other C-type lectins have been reported to bind noncarbohydrate ligands, including proteins, lipids (21), and ice (22). Examples of protein ligands include the coagulation factors IX XI, binding protein (23), the low-affinity IgE-Fce receptor (CD23; Ref. 24), and lecticans (25).

In the present work, the Plg K4 binding site of TN is reported to be sensitive to calcium ions, to involve at least two of the calcium-coordinating residues in the CRD, and to be located at a site overlapping the putative carbohydrate binding site.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis, Expression, Refolding, and Processing of Recombinant Proteins—All single-residue mutations were performed using the Quickchange kit (Stratagene, La Jolla, CA). rTN(12) was used as a template for the mutagenesis. Mutagenesis primers were from DNA Technology (Aarhus, Denmark). The initial melting temperature of all primer pairs used for mutagenesis was designed to be at least 62 °C, and mutation sites were located in the central region of the primers. Mutations were verified by DNA sequencing using the Thermo Sequenase kit (U. S. Biochemical Corp.).

Expression in Escherichia coli, refolding, and purification of rTN, rTN3, and the single-amino acid mutations of rTN were performed as described previously (13). rPlg K4 was produced essentially as described previously (26).

Affinity Binding Analysis on rPlg K4 Immobilized on Sepharose CL-6B—rPlg K4 (12 mg) dissolved in 2.5 ml of 0.1 mM sodium phosphate, pH 7.5, was immobilized on 5 ml of Sepharose CL-6B (Pharmacia Biotech Inc.), which was activated as described previously (27) by the addition of 0.3 g of 1.1-carboxyl-dimethylamine in dry acetone. In preparation for binding analysis, the rPlg K4 column was washed with 1 column volume of 0.1 M NaCl, 50 mM Tris-HCl (pH 8.0), and 10 mM 6-aminohexanoic acid (6-AHA; Buffer B); 1 column volume of 0.5 M NaCl and 50 mM Tris-HCl (pH 8.0); and 4 column volumes of 0.1 M NaCl and 50 mM Tris-HCl (pH 8; Buffer B; Buffer A) at a flow rate of 0.5 ml/min. One ml of the protein sample at a concentration of 0.25 mg/ml (i.e. rTN, rTN single-residue mutants, or rTN3) was applied, and the column was washed with 3–4 column volumes of Buffer A before the elution of bound protein with Buffer B. Aliquots of the run-through, wash, and elution fractions were analyzed by nonreducing SDS-PAGE.

Calcium sensitivity of rTN binding was analyzed by equilibrating the rPlg K4 column with Buffer A containing either 1 mM CaCl2 or 1 mM calcium lactate before loading rTN, or by the application of rTN in Buffer A, without calcium, and applying a gradient from 0 to 1 mM of either CaCl2 or calcium lactate over 10 column volumes.

Surface Plasmon Resonance (SPR) Assay—The SPR binding assays were performed on a BIAcore 2000 instrument (BIAcore). rPlg K4 or Plg (human Glu-plasminogen was a gift from Dr. Lars Sottrup-Jensen, University of Aarhus, Aarhus, Denmark [28]) was immobilized onto CM5 BIAcore sensor chips using the Amine Coupling Kit as described previously (26). After chip activation, rPlg K4 dissolved in 0.1 M sodium phosphate (pH 4.5) at a concentration of 0.32 mg/ml or 0.005% Surfactant P20. Binding of rTN or immobilized rPlg was analyzed with various concentrations of dissociation were allowed before the chip was regenerated with 0.5 M NaCl and 50 mM Tris-HCl (pH 8.0; Buffer B; Buffer A) at a flow rate of 0.5 ml/min. One ml of the protein sample at a concentration of 0.25 mg/ml (i.e. rTN, rTN single-residue mutants, or rTN3) was applied, and the column was washed with 3–4 column volumes of Buffer A before the elution of bound protein with Buffer B. Aliquots of the run-through, wash, and elution fractions were analyzed by nonreducing SDS-PAGE.

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Binding analysis was performed at a flow rate of 5 µl/min. Before loading of the protein sample, the chip was equilibrated in 0.1 mM NaCl, 50 mM Tris-HCl (pH 8.0), and 0.005% Surfactant P20. Aliquots (25 µl) of rTN or single-residue mutants at a concentration of the trimeric calcium-sensitive Plasminogen Binding of Tetranectin CRD

![Image](354x552 to 508x729)

**Fig. 1.** SPR analysis of the calcium sensitivity of rTN binding to rPlg K4 and Plg. The R<sub>A</sub> levels, which were normalized to the R<sub>4</sub> level in a calcium-depleted buffer, are plotted versus calcium concentration. ●, rTN binding to rPlg K4; ○, binding to Plg. Data points are the means of four binding experiments, and vertical bars indicate the S.D.

**RESULTS**

Initial mapping of the Plg K4 binding site on TN was carried out using the rPlg K4 affinity column for the detection of lysine-sensitive binding of rTN deletion variants. Results from these experiments showed that rTN deletion constructs containing the α-helical trimerization region as well as the CRD bound to immobilized rPlg K4, whereas free CRD (rTN3) exhibited no apparent binding (data not shown). However, the observation that bound rTN could be eluted from the rPlg K4 column by either CaCl2 or calcium lactate (at approximately 0.2 mM calcium) prompted us to focus the single-residue mutational analysis on the CRD domain. The calcium sensitivity of the interaction was further analyzed in a series of SPR experiments in which the binding of rTN to either immobilized rPlg K4 or immobilized Plg was analyzed with various concentrations of calcium in the buffer (Fig. 1). The shapes and positions of the two binding curves are virtually identical, substantiating that TN binds exclusively to the Plg K4 domain in Plg. Half-maximal binding was found to occur at approximately 0.2 mM calcium.

Binding affinities of rTN and rTN3 to rPlg K4 in solution were determined by ITC. The data fitted well to a one-binding site model for rTN3 and a one-binding site/subunit model for rTN (Fig. 2), yielding dissociation constants of 53 and 52 µM, respectively, for this strongly enthalpy-driven association (Table I).

To determine the location of the Plg K4 binding site on the CRD in greater detail, a series of single-residue substitutions in rTN was generated on the basis of inspection of the pub-
Calcium-sensitive Plasminogen Binding of Tetranectin CRD

TABLE I
Binding parameters obtained from the microcalorimetric titration of rTN and rTN3 with rPlg K4

|       | rTN  | rTN3 |
|-------|------|------|
| Stoichiometry | 0.95 ± 0.08 | 3.2 ± 0.1 |
| $K_d$ (μM) | 51.8 ± 5.6 | 52.9 ± 3.1 |
| $\Delta G$ (kJ/mol) | −24.5 ± 0.3 | −24.4 ± 0.1 |
| $\Delta H$ (kJ/mol) | −137 ± 14 | −113 ± 4 |
| $-\Delta S$ (kJ/mol) | 113 ± 14 | 89 ± 4 |

Fig. 2. Thermograms and binding isotherms from the isothermal calorimetric titration of rTN and rTN3 with rPlg K4. A, titration of rTN with rPlg K4 at 25 °C. B, titration of rTN3 with rPlg K4 at 25 °C. The thermodynamic parameters from the binding analysis are given in Table I.

lished CRD structure (19). Firstly, each of the surface-exposed lysine residues in the CRD region of rTN was replaced by alanine residues (K134A, K148A, K162A, and K166A). Secondly, several of the calcium-coordinating residues, in particular those involved in coordination at calcium site 2, were substituted by alanine residues (D116A, E120A, Q143A, D145A, E150A, N151A, and D165A). Finally, additional surface-exposed residues in the vicinity of calcium site 2 were substituted by alanine or other residues (I140A, F164L, R167A, and R169A). Each mutant protein discussed here is represented by an entry in Table II.

Protein Expression and Refolding of rTN and Mutants—Expression levels of the single-residue mutants of rTN were similar to the wild-type level. However, refolding efficiency differed by up to 50% relative to that of the wild type. One mutant, rTN F164A, failed to re-fold (data not shown). Purified protein preparations appeared to be homogeneous, correctly refolded, and free from contaminating disulfide-linked oligomer species as judged by nonreducing SDS-PAGE (Fig. 3).

On the basis of the light absorption at 280 nm and amino acid analysis, A(1%)280 values for rTN and rTN3 were determined to be 2.0 and 2.8 g L⁻¹ cm⁻¹, respectively. SDS-PAGE staining intensities for rTN and the single residue mutants confirmed that the use of the rTN A(1%)280 Value for the mutant proteins was justifiable, and that the mutants exhibited a hypochrome effect similar to that of rTN.

rPlg K4-Sepharose Affinity Chromatographic Analysis—The mutants were grouped into three classes with respect to their affinity for rPlg K4-Sepharose (Table II), namely: (i) mutants binding essentially as strongly as the wild-type protein rTN (E120A, K134A, Q143A, D145A, N151A, K162A, F164L, and R169A); (ii) mutants that were retarded on the column and eluted during the washing step (D116A, I140A, K166A, and R167A); and (iii) mutants that appeared not to bind to immobilized rPlg K4 at all and hence eluted in the flow-through fractions (K148A, E150A, and D165A).

SPR Binding Analysis—Representative sensograms from the SPR analysis of rPlg K4 and Plg binding corresponding to the same concentration of each TN derivative are shown in Fig. 4. A and B, respectively. All experiments consistently showed the occurrence of a fast association and a biphasic dissociation phase with a fast and a slow component, an observation reminiscent of observations for similar protein systems (30, 31). For the group of poorly binding mutants, the binding component with slow dissociation was found to be especially pronounced relative to the fast component of dissociation. Both components of the dissociation phase reflect specific interactions, because they can be efficiently competed by 5 mM 6-AHA (data not shown).

rTN and each of the rTN mutants are all homotrimeric proteins with three potential binding sites. Therefore, a simple kinetic analysis may not be applied within this system. We have therefore chosen to evaluate the SPR binding data using a semiquantitative analytical method similar to that of MacKenzie et al. (30). In this approach, binding is assumed to be defined by a simple Langmuir relationship described by the following equation.

$$R_{pl} = R_{max} \left( \frac{[L]}{[L] + K_p} \right)$$

(Eq. 1)

$R_{pl}$ denotes the measured response at the plateau, $R_{max}$ is the maximum response at binding site saturation, $[L]$ is the ligand concentration, and $K_p$ is the dissociation constant.

Firstly, estimates of $R_{max}$ and the apparent dissociation constant $K_p$ were determined by measuring the $R_{pl}$ at different concentrations of rTN ligand. The data from both the rPlg K4 chip and the Plg chip were then plotted versus ligand concentration according to the rearranged equation, which is as follows.

$$\frac{R_{max}}{R_{pl}} = 1 + \frac{K_p}{[L]}$$

(Eq. 2)

$R_{max}$ and $K_p$ were estimated by linear regression analysis (Fig. 5). $R_{max}$ for the rPlg K4 chip was found to be 598 ± 52 RU, and $K_p$ was 0.50 ± 0.05 μM. For the Plg chip, $R_{max}$ was found to be 1159 ± 34 RU, and $K_p$ was 0.17 ± 0.01 μM (Table III).

Secondly, the binding of each single-residue mutant was analyzed in a series of experiments in which ligand (rTN) was injected over the rPlg K4 and Plg chips at equal conditions (i.e. concentration, flow, and buffer), and the $K_p$ was estimated from the $R_{pl}$ level (Table II).

Three mutants (rTN K148A, rTN E150A, and rTN D165A) showed very low binding to both rPlg K4 and Plg. Each of these mutants was also identified as a nonbinder on rPlg K4-Sepharose. Both rTN E150A and rTN D165A represent mutations of calcium-coordinating residues at calcium site 2. The rest of the single-residue mutations only exhibited little effect on binding. Three single-residue substitutions, rTN K166A, rTN R167A, and I140A, all showed a lower affinity for rPlg K4 and Plg. These three mutants were also retarded on the rPlg K4 column.

The calcium sensitivity of binding was further studied by characterization of mutants rTN D116A, rTN E120A, rTN Q143A, rTN D145A, and rTN N151A, which are all known calcium-coordinating residues. The SPR responses at the plateau levels were measured using both the rPlg K4 chip (Fig. 6) and the Plg chip (data not shown) at different calcium concentrations. Not only did these mutations affect the calcium sensitivity of rPlg K4 and Plg binding, but the transition from binding to nonbinding was found to be less sharp for this
钙敏感受激纤蛋白原介导的四正性CRD的钙敏感结合

表 II
钙敏感受激纤蛋白原对重组四正性TN衍生物的结合特性

| rTN类型 | 绑定到K4-琼脂糖 | rPlg K4 | Plg |
|--------|----------------|--------|-----|
| 野生型 | + 286 ± 13     | 0.55 ± 0.10 | 850 ± 22 |
| rTN3   | - NA           | NA     | NA  |
| D116A  | (+) 251 ± 7    | 0.69 ± 0.11 | 497 ± 50 |
| E120A  | + 334 ± 3      | 0.40 ± 0.08 | 974 ± 75 |
| K134A  | + 292 ± 13     | 0.53 ± 0.09 | 824 ± 57 |
| I140A  | (+) 221 ± 3    | 0.85 ± 0.12 | 554 ± 30 |
| Q143A  | + 305 ± 27     | 0.48 ± 0.11 | 918 ± 31 |
| D145A  | + 372 ± 4      | 0.31 ± 0.07 | 1042 ± 62 |
| K148A  | - 71 ± 7       | 3.7 ± 0.5  | NA  |
| K206A  | - 119 ± 12     | 2.0 ± 0.3  | 50 ± 46 |
| D232A  | - 323 ± 4      | 0.40 ± 0.08 | 1102 ± 40 |
| K162A  | - 319 ± 10     | 0.44 ± 0.08 | 1042 ± 49 |
| R163A  | - 283 ± 5      | 0.56 ± 0.09 | 775 ± 18 |
| D165A  | - 94 ± 10      | 2.7 ± 0.4  | 36 ± 24 |
| K166A  | (+) 206 ± 6    | 1.0 ± 0.1  | 685 ± 12 |
| R167A  | (+) 166 ± 21   | 1.3 ± 0.2  | 310 ± 19 |
| R169A  | + 296 ± 9      | 0.51 ± 0.09 | 811 ± 28 |

a +, ligand binds to K4-琼脂糖; (+), ligand elutes during washing; and −, no binding.

b NA (not applicable), the ligand binding is weak and out of range.

图 3. SDS-PAGE分析的rTN和突变蛋白产物。用非降解SDS-PAGE分析制备的rTN蛋白产品在等摩尔浓度下。图。Lanes 1–7, rTN K166A, rTN F164L, rTN K162A, rTN K148A, rTN D116A, rTN, and rTN3, respectively. Lane M, molecular mass values for marker proteins are 94, 67, 43, 30, 20.1, and 14.4 kDa (from top to bottom).

图 4. 代表性传感器gram。代表性传感器gram的从SPR结合分析来rTN, rTN K148A, rTN K166A,和rTN R167A在浓度为0.5 μM和rTN3在浓度为1.5 μM到（A）固定的rPlg K4和（B）固定的rPlg。

讨论

该研究证明了Plg K4与TN的结合是钙敏的。ITC分析显示，这种结合并非发生在TN的CRDs上。TN的CRD-Ca2+复合物的存在NS, as measured in solution, which probably explains why binding of the free monomeric CRD (rTN3) to Plg K4 was only detectable by ITC. As would be anticipated (30, 31) from the presence of three independent K4 binding sites in each TN homotrimer, the interaction between TN and Plg K4 was found to be significantly stronger and allowed the demonstration of binding to immobilized Plg K4 or Plg and the estimation of binding constants by SPR (K'p, 0.5 and 0.2 μM, respectively). Thus, our detailed understanding of the interaction warrants the use of SPR analysis for characterization of mutant TN homotrimers as representative of the interaction between the mutant CRDs and Plg K4.

The mutational analysis maps the Plg K4 binding site to a localized region defined by two of the calcium-coordinating residues in CRD calcium site 2 (Glu-150 and Asp-165) and to a surface-exposed lysine residue located close to (Lys-148). Other surface-exposed residues in the region appear to contribute little to the affinity. The observation that Plg K4 binding can be titrated by calcium and the decreased sensitivity exhibited by the calcium-coordinating mutants indicate that binding of Plg K4 only occurs when calcium site 2 is not occupied by a calcium ion. The calcium level corresponding to half-maximal binding, which was found to be 0.2 mM for both rPlg K4 and Plg, is in agreement with this hypothesis and also corroborates the original observation that only the Plg K4 domain in Plg interacts with TN.

Notably, one of the mutants, TN D116A, involving one of the calcium-coordinating residues of site 1 that is located on the distal side of calcium site 1 in relation to site 2 exhibits slightly decreased affinity toward Plg K4 and a significantly decreased sensitivity to calcium. This finding, together with the observation that Glu-150, like Glu-193 in rat MBP, coordinates both to the calcium ion in site 1 with the backbone carbonyl oxygen and to the calcium ion in site 2 with the side chain (19), may be taken as indication of a general cooperativity in calcium binding of the C-type lectin CRDs with two bound calcium ions.
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The dissociation constant in the binding of 6-AHA to the binding pocket of Plg K4 in solution, which has been reported to lie within the range of 20–80 μM at 0–0.5 M NaCl, respectively (26). Substantial additional interactions between the Plg K4 and the CRD would be expected to contribute to a significantly lower dissociation constant.

Calcium-dependent binding of the TN CRD to ligands other than Plg K4 has not been demonstrated. The binding of complex sulfated oligosaccharides (i.e., fucoidan and heparin) to TN is calcium-independent, does not involve the CRD, and has recently been shown to involve residues within the NH2-terminal domain of approximately 20 amino acid residues of TN. 4

The present finding may guide more specific and clarifying biological investigations of the physiological role of TN. In particular, the sensitivity to calcium ions of the weak interaction between TN and Plg may provide a means for a tissue to recruit Plg from plasma, thus requiring only the initiation of a process, which may provide its own regulation by calcium-modulated binding and release of Plg or Plg-derived products at TN-rich regions in the tissue.

To our knowledge, the interaction between TN and Plg represents the first example of a calcium-sensitive protein-protein interaction directly involving the putative carbohydrate binding site of a C-type lectin CRD. Other C-type lectin CRDs have been reported to bind proteins (23–25). These interactions all appear to be calcium-dependent and to involve residues located outside the calcium binding sites(s).

The number of proteins containing C-type lectin CRDs is large and is still growing. C-type CRDs have been reported to bind such diverse compounds as carbohydrates, proteins, lipids, and even ice. We anticipate that the Plg K4 binding site on TN, together with the mannose-binding site of MBP and possibly the ice-binding site of herring antifreeze protein, offers unique protein engineering-based opportunities to study the requirements of surface topology and chemistry for grafting new binding sites for very diverse compounds on an essentially conserved single-chain structural unit.

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