Binding of the NG2 Proteoglycan to Kringle Domains Modulates the Functional Properties of Angiostatin and Plasmin(ogen)*

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Interactions of the developmentally regulated chondroitin sulfate proteoglycan NG2 with human plasminogen and kringle domain-containing plasminogen fragments have been analyzed by solid-phase immunoassays and by surface plasmon resonance. In immunoassays, the core protein of NG2 binds specifically and saturably to plasminogen, which consists of five kringle domains and a serine protease domain, and to angiostatin, which contains plasminogen kringle domains 1–3. Apparent dissociation constants for these interactions range from 12 to 75 nM. Additional evidence for NG2 interaction with kringle domains comes from its binding to plasminogen kringle domain 4 and to miniplasminogen (kringle domain 5 plus the protease domain) with apparent dissociation constants in the 18–71 nM range. Inhibition of plasminogen and angiostatin binding to NG2 by 6-aminohexanoic acid suggests that lysine binding sites are involved in kringle interaction with NG2. The interaction of NG2 with plasminogen and angiostatin has very interesting functional consequences. 1) Soluble NG2 significantly enhances the activation of plasminogen by urokinase type plasminogen activator. 2) The antagonistic effect of angiostatin on endothelial cell proliferation is inhibited by soluble NG2. Both of these effects of NG2 should make the proteoglycan a positive regulator of the cell migration and proliferation required for angiogenesis.

Several observations concerning the NG2 proteoglycan have suggested the possible involvement of this molecule in angiogenesis. NG2 is a prominent component of vasculature (1, 2), where its expression has been found to be developmentally regulated. The proteoglycan is expressed at high levels in immature vessels and then is down-regulated as the vessels mature (3–6). In mature animals NG2 is once again up-regulated in the neovasculature that develops during tumor formation and wound healing (4–7) further supporting a relationship between the proteoglycan and angiogenesis. Although endothelial cells have been proposed as one source of NG2 expression in neovascularature (6), a more likely possibility is that the proteoglycan is expressed by the mesenchymal cell component of the vasculature in question: i.e. cardiomyocytes in the heart, smooth muscle cells in arteries (8), and pericytes in microvessels (4, 5, 7).

Current evidence suggests that NG2 may be important for controlling both the motility of cells and their responsiveness to growth factors. NG2 has been shown to bind directly to PDGF-AA1 and bFGF (9) and thus may serve to modulate the effects of these growth factors on their cell surface signaling receptors. For example, we have shown that the PDGF-α receptor is unresponsive to PDGF-AA in aortic smooth muscle cells derived from NG2-null mice, suggesting a role for NG2 in sequestering the growth factor or in presenting it to the signaling receptor (10). PDGF and bFGF are both important angiogenic mitogens, so a role for NG2 in modulating the effects of the growth factors would be an important aspect of neovascularization.

Cell motility is also an important factor during angiogenesis, because both endothelial cells and pericytes migrate rapidly to sites where capillary beds are expanding. As a membrane-spanning molecule, NG2 interacts with both extracellular and intracellular components and may be able to trigger cytoskeleton-dependent changes in cell morphology and motility in response to the extracellular environment. An association of NG2 with the cytoskeleton has been suggested by several distinct lines of investigation (11–13), and evidence has been presented in support of the ability of NG2 to initiate changes in cell shape and migration that are dependent on the activation of Rho family GTPases (14, 15). On the exterior of the cell, an interaction of NG2 with the extracellular matrix component type VI collagen is well documented (1, 16–18). The ability of type VI collagen to promote cell migration in an NG2-dependent fashion (19) is further evidence of the capacity of the proteoglycan to mediate cellular responses to the environment.

NG2 is expressed by the majority of human melanoma cells and for this reason is also known as the human melanoma proteoglycan (15). Both the growth control and cell motility aspects of NG2 function can be seen in studies on melanoma progression. For example, we have shown that mouse melanomas expressing NG2 are both faster growing and more metastatic than their NG2-negative counterparts (20). The motility of tumor cells involves not only their ability to adhere to and migrate on extracellular matrix components but also their ability to degrade the extracellular matrix using a number of different proteases (21–23). Highly motile cells in normally developing tissues also utilize proteolysis as a tool for enhancing their migration. One proteolytic mechanism that is commonly used by both normal and neoplastic cells is the plasminogen system, wherein plasminogen is cleaved at the Arg561-Val562 peptide bond by the action of urokinase and tissue

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The abbreviations used are: PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; K, kringle; FCS, fetal calf serum; EC, extracellular; GAG, glycosaminoglycan; u-PA, urokinase plasminogen activator; BSA, bovine serum albumin; PBS, phosphate-buffered saline; 6-AHA, 6-aminohexanoic acid.
plasminogen activators to yield the proteolytically active two chain molecule plasmin (24–27). The action of plasmin is known to be an important factor in many developmental processes, including neovascularization and vascular remodeling (23, 28–30).

The commonality of NG2 and plasminogen(ogen) to both the melanoma and angiogenesis systems has led us to speculate about a possible role for NG2 in mediating the function of this protease. Our curiosity has been heightened by the fact that plasminogen is also the precursor for another key player in angiogenic mechanisms, namely angiostatin. Full-length angiostatin, which comprises the first four kringle domains of plasminogen, is an endogenous inhibitor of angiogenesis and tumor growth (31–33). The angiostatin protein consisting of plasminogen kringle 1–3 (K1–3) retains the ability to inhibit endothelial cell proliferation and migration and thus is also an effective antagonist of angiogenesis and tumor growth (31–33).

In this study we have assessed the ability of NG2 to interact with and affect the function of plasminogen and angiostatin. Using solid phase binding assays and surface plasmon resonance technology, we demonstrate strong binding of NG2 to both molecules, interactions that appear to depend on the presence of the kringle domains. Moreover, these interactions with NG2 appear to modulate the functional properties of both molecules. Incubation with purified NG2 accelerates the urokinase-dependent activation of plasminogen and diminishes the ability of angiostatin to inhibit endothelial cell proliferation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Cell Proliferation Assays**

The Balb/c mouse aortic endothelial cell line 22106 (34) was provided by Drs. Yu Yamaguchi (The Burnham Institute, La Jolla, CA) and Robert Auerbach (University of Wisconsin, Madison, WI). Cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin sulfate. These cells do not express cell surface NG2 as determined by immunofluorescence microscopy.

For proliferation experiments, endothelial cells were harvested with trypsin-EDTA and seeded at 2000 cells/well in 96-well plates. Cells were allowed to recover for 8 h in Dulbecco’s modified Eagle’s medium plus 10% FCS and then incubated for 24 h in serum-free Dulbecco’s modified Eagle’s medium to allow synchronization of the cell cycle. Preliminary experiments established that optimal proliferation of these cells was achieved by a 72-h incubation period in Dulbecco’s modified Eagle’s medium containing 5% FCS and 10 ng/ml bFGF. For determining inhibition of proliferation, angiostatin or plasminogen (along with NG2 when indicated) was incubated with the starved cells for 1 h followed by the addition of the FCS/bFGF-containing medium for the 72-h period of stimulation. After the 72-h proliferation period, cell density was determined using the CellTiter 96 assay (Promega), in which the metabolic activity of viable cells converts tetrazolium dye into a formazan product that can be detected at 490 nm. The NG2/bFGF-containing medium for the 72-h period of stimulation. After the 72-h proliferation period, cell density was determined using the CellTiter 96 assay (Promega), in which the metabolic activity of viable cells converts tetrazolium dye into a formazan product that can be detected at 490 nm.

**Antibodies**

Rabbit antibody against human plasminogen and mouse monoclonal antibody specific for the kringle 1–3 region were purchased from Calbiochem. Affinity-purified rabbit antibodies against NG2 have been described (9). Peroxidase-labeled goat antibodies against rabbit and mouse immunoglobulins were obtained from Bio-Rad.

**Purified Proteins**

The production and characterization of recombinant NG2 fragments have been previously described (18). Four different NG2 fragments were used in our experiments: NG2/EC− (extracellular) without glycosaminoglycan (GAG) chains, comprising the entire extracellular domain (residues 1–2223 (35)); NG2/EC+ with GAG chains; central domain 2 (NG2/D2, residues 632–1450); and membrane-proximal domain 3 (NG2/D3, residues 1587–2218). The NG2/D2 preparation contains chondroitin sulfate chains, whereas NG2/D3 does not.

Human urokinase (u-PA) and plasminogen (glu-type) from human plasma were obtained from Calbiochem. Plasminogen fragments representing kringle 1–3 (K1–3), kringle 4 (K4), and kringle 5 plus the serine protease domain (miniplasminogen) were obtained from American Diagnostica Inc. (Greenwich, CT). These fragments were generated from human glu-type plasminogen by limited digestion with pancreatic elastase. The manufacturer's protocol specifies molecular masses for the fragments as 31.5 (K1–3), 14 (K4), and 38 kDa (miniplasminogen).

**Reagents**

Research grade CM5 SensorChips (carboxymethylated dextran matrix), Pioneer B1 chips with reduced negative charge, amine-coupling kit [N-ethyl-N ’-dimethylaminopropyl] carbodi-imide/N-hydroxysuccinimide], and HBS buffer (10 mM HEPES with 0.15M NaCl, 3.4 mM EDTA, and 0.005% surfactant P20 at pH 7.4) were all obtained from BIAcore AB (Uppsala, Sweden). TurboTMB (3,3’,5,5’-tetramethylbenzidine) was obtained from Pierce, 6-aminohexanoic acid was purchased from Sigma, and the plasin substrate S-2251 (H-b-valyl-l-leucyl-l-lysine-p-nitroanilide dihydrochloride) was purchased from Chromogenix AB (Molndal, Sweden).

**Kinetic Measurements Using Surface Plasmon Resonance**

A BIAcore 3000 surface plasmon resonance-based biosensor (BIAcore AB) was used to measure binding parameters for the interaction between soluble NG2 fragments (analytes) and various immobilized plasminogen species (ligands). We did not attempt to measure the binding of soluble plasminogen species to immobilized NG2, because the extremely basic nature of plasminogen kringle domains leads to high levels of nonspecific interaction with the negatively charged sensor chips. Instead, plasminogen and plasminogen fragments were immobilized on the sensor chip surface by the amine-coupling method according to the manufacturer’s instructions. Low levels of ligand (<500 resonance units) were immobilized to minimize mass transfer effects. For all kinetic measurements we used a flow path involving all four cells of the BIAcore 3000. In one set of experiments, the first flow cell contained a chip coated with glu-plasminogen, the second flow cell contained a chip coated with angiostatin (plasminogen fragment K1–3), and a third flow cell contained a blank chip to serve as a reference surface. The fourth flow cell was not monitored. In a second set of experiments, cell one contained a K4-coated chip, cell two contained a miniplasminogen-coated chip, and cell three again served as a reference. Between binding cycles these coated chips were regenerated by injection of 1 M NaCl. Binding specificity was checked by injecting BSA (1.25 and 2.5 μg) over each of the coated chips.

Purified recombinant NG2 species at the indicated concentrations were injected over the coated surfaces in HBS, pH 7.4, at a flow rate of 20 μl/min. A wide range of analyte concentrations (~1–10 × Kd) were applied to assure recovery of data suitable for curve fitting. Typically, sensorsgrams were obtained at five different analyte concentrations for each of the analyte/ligand pairs. The set of sensorsgrams was analyzed using BIAevaluation software version 3.0. This software corrects for
the systematic upward drift that occurs during many measurements and allows for subtraction of background sensorgrams from experimental sensorgrams. These adjustments yield corrected sensorgrams.

The association and dissociation phases of corrected sensorgrams were fit simultaneously, assuming a simple bimolecular reaction model: \( A + B \rightarrow AB \). Both an association rate constant \( k_a \) (m\(^{-1}\) s\(^{-1}\)) and a dissociation rate constant \( k_d \) (s\(^{-1}\)) were obtained for the entire data set (global fit), and the apparent equilibrium dissociation constant \( K_D \) was obtained from the ratio of the off and on rate constants \( k_d/k_a \). All our kinetic data were fit most adequately by assuming a simple bimolecular model for interaction between soluble analyte and immobilized ligand, equivalent to the Langmuir isotherm for adsorption to a surface. Fits were not improved by using a mass transport model or a two-state (conformational change) model that assumes cooperativity between multiple binding sites.

In addition to the kinetic analysis of binding using association and dissociation rates, we also used Pioneer B1 chips to obtain steady binding data for the interaction of NG2/EC− with plasminogen, angiostatin, and miniplasminogen. The surfaces of these chips have a low degree of carbamoylmethylation and are therefore less negatively charged than the CM5 chips, resulting in lower levels of nonspecific binding. Very low ligand immobilization levels (−50 resonance units) were also used in these experiments. The ligand-coated chips were analyzed in flow cells one through three with flow cell four serving as the reference surface. To derive apparent dissociation constants under equilibrium binding conditions, peak response levels achieved in the steady state region of the sensorgrams were plotted against analyte concentration (C). This plot was fit to a single site binding equation (Langmuir isotherm) to determine \( K_D \) values.

### Solid Phase Binding Assays

**Plasminogen and Kringle Domain Binding to Immobilized NG2**—Assays were performed as described previously (9). Briefly, 96-well microtiter plates (Greiner, Nuertingen, Germany) were coated with purified NG2 fragments at 4 μg/ml at 4 °C, washed three times with PBS containing 0.05% Tween 20 (PBST), and then blocked for 1 h with 1% BSA in PBS. Plasminogen or plasminogen fragments dissolved in 100 μl of PBS containing 1% BSA were incubated in the wells for 2 h at 20 °C and then washed three more times as described above. For inhibition experiments, binding of plasminogen or angiostatin to immobilized NG2 was measured in the presence of different concentrations (0.1−50 mM) of the lysine analog 6-aminohexanoic acid (6-AHA).

Bound plasminogen, miniplasminogen, and K4 were detected with rabbit anti-plasminogen, whereas bound angiostatin was detected with monoclonal antibody against the plasminogen K1–3 fragment. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG, respectively, were used as second antibodies. Quantitation of antibody binding was accomplished using 3,3′,5,5′-tetramethylbenzidine as the peroxidase substrate. Plates were read at 450 nm with a Titertek microtiter instrument measures analyte/ligand interactions directly in

**Results**

### Plasminogen Binding to Immobilized NG2—Interaction of the NG2 ectodomain and the subdomains NG2/D2 and NG2/D3 with plasminogen and plasminogen fragments could be effectively measured using a solid phase immunoassay. As shown in Fig. 1, significant binding to BSA-coated wells was not observed for plasminogen or any of its fragments. However, specific saturable binding of soluble plasminogen, angiostatin (K1–3), K4, and miniplasminogen was observed to several species of immobilized NG2, including NG2/EC−, NG2/EC+, NG2/D2, and NG2/D3. These results indicate that binding sites for plasminogen may be present in more than one location on the NG2 polypeptide and suggest that chondroitin sulfate chains are unlikely to be important for the NG2/plasminogen interaction. The apparent dissociation constants (\( K_D \)) calculated from the binding data by nonlinear regression analysis range from 16 to 71 nM. These values for each pair of ligands are listed in Table I. Scatter in the values obtained for binding of the K4 fragment made this data inadequate for the determination of reliable \( K_D \) values.

The strong binding of NG2 to the K1–3 and K4 fragments, which lack the C-terminal serine protease domain of plasminogen, suggests that NG2 could be interacting with kringle domains, which comprise the N-terminal two-thirds of the plasminogen molecule. As a further test of this possibility, we incubated plasminogen and angiostatin with NG2 in the presence of increasing concentrations of 6-AHA, a known inhibitor of the lysine binding sites present in the kringle domains. In the presence of 10 μM 6-AHA, we observed greater than 90% inhibition of plasminogen and angiostatin binding to all four NG2 species, indicating the involvement of the kringle-associated lysine binding sites in plasminogen binding to NG2. Fig. 2 presents the inhibition data obtained with 6-AHA for interaction of plasminogen and angiostatin with the NG2/EC− and NG2/D2 fragments.

**NG2 Binding to Immobilized Plasminogen**—As an additional assessment of the specificity of plasminogen binding to NG2, we also performed solid phase assays with NG2 as the soluble component and various plasminogen species as the immobilized components. As expected, NG2 fragments bound effectively to wells coated with plasminogen, miniplasminogen, K1–3, and K4. Low background binding of NG2 to BSA-coated wells was observed in all cases. Fig. 3 presents the data obtained for the NG2/EC− and NG2/D2 fragments. Nonlinear regression analysis yielded apparent \( K_D \) values between 21 and 41 nM for these interactions (see Table I), which is in good agreement with the values obtained in binding studies done in the reverse format (Table I).

### BIACore Analysis of the NG2/Plasminogen Interaction

Kinetic information obtained by classical immunoassay methods is influenced by several complex phenomena, such as steric hindrance because of antibody binding and lengthy incubation times. Surface plasmon resonance technology can circumvent or minimize some of these problems, most notably the issue of antibody reactivity and incubation time, because the BIACore instrument measures analyte/ligand interactions directly in...
Fig. 1. Binding of plasminogen and plasminogen fragments to immobilized NG2 species. Increasing concentrations of plasminogen (A), K1–3 (B), miniplasminogen (C), or K4 (D) were incubated in microtiter wells coated with BSA (●) or with the NG2 fragments NG2/EC– (●), NG2/EC+ (○), NG2/D2 (□), and NG2/D3 (▲). Bound plasminogen species were quantified immunochemically, as described under “Experimental Procedures.” These experiments were performed three times with comparable results in each case. For the data shown, each point represents the mean of values obtained from duplicate wells. Binding curves represent the best fit determined by nonlinear regression analysis. Apparent dissociation constants derived from this analysis are presented in Table I.

Table I

|     |     |     |     |
|-----|-----|-----|-----|
|     |     |     |     |
| D2  | D3  | EC–GAGs | EC+GAGs |
| Plg | 22.3 ± 1.5 | 15.8 ± 1.5 | 47.5 ± 8.8 |
| K1–3 | 24.5 ± 3.7 | 26.9 ± 5.8 | 26.3 ± 5.8 |
| m-Plg | 64.9 ± 8.7 | 57.4 ± 9.3 | 62.4 ± 9.1 |

TABLE I

Solid-phase binding data

Dissociation constants (nM) were calculated by nonlinear regression analysis for the interaction between soluble plasminogen species and adsorbed NG2 fragments (A) and for the interaction between soluble NG2/EC– or NG2/D2 and adsorbed plasminogen species (B).

real time. We therefore used the BIAcore 3000 to determine rate constants for the association and dissociation of soluble NG2 fragments with plasminogen species immobilized on sensor chips.

Representative sensorgrams are shown in Fig. 4 for binding of NG2/D2 to plasminogen, angiostatin, miniplasminogen, and K4. The binding curves are all relatively steep in the association phase and shallow in the dissociation phase, both of which are indicative of the high affinity of NG2 for the kringle structures of plasminogen. The sensorgrams obtained from injection of soluble NG2/D3, NG2/EC–, and NG2/EC+ were similar to sensorgrams for the NG2/D2 (sensorgrams not shown). All sensorgrams have good signal to noise ratios and exhibit concentration-dependent increases in both the rate and extent of binding. Bound NG2 could be eluted by injection of 1 M NaCl, suggesting that interactions between charged residues are important for binding of NG2 to plasminogen and its fragments. Injection of 1.25 and 2.5 μM BSA over immobilized plasminogen and plasminogen fragments resulted in curves that reveal little or no specific interaction between BSA and the various plasminogen species (data not shown).

The association and dissociation portions of all sensorgrams were analyzed by curve fitting and proved to be adequately fit by a simple bimolecular reaction model. More complex binding models either did not improve the goodness of fit or else were inferior in fitting the experimental data. Equilibrium dissociation constants calculated from the forward and reverse rate constants for the various bimolecular reactions are presented in Table II. To confirm that the use of this bimolecular model provides KD values that reflect the actual strength of the NG2/plasminogen interactions, we also performed steady state affinity measurements for the binding of NG2/EC– to plasminogen, angiostatin, and miniplasminogen. To determine affinity constants from the steady state binding levels (Req) observed at various concentrations of analyte (C), plots of Req versus C were created for all three sets of interactions (Fig. 5). These plots were fit to a general model of equilibrium binding to obtain another set of KD values, which are presented in Table III. These values are in good agreement with the values obtained by kinetic analysis (Table II). In addition, both sets of values agree well with the apparent equilibrium dissociation constants derived from the solid phase assays (Table I).

NG2 Inhibition of the Antiproliferative Effect of Angiostatin on Endothelial Cells—Angiostatin is known to exert a pronounced inhibitory effect on endothelial cell proliferation (32, 36). To determine whether NG2 can influence this capability of angiostatin, we utilized an in vitro assay of mouse aortic endothelial cell proliferation. In preliminary experiments, these cells were exposed to various concentrations of bFGF, with or without FCS, for 24, 48, 72, or 96 h. From these tests it was
determined that the most satisfactory induction of endothelial cell proliferation occurred after 72 h in the presence of 10 ng/ml bFGF and 5% FCS (data not shown). The effect of angiostatin and plasminogen on this bFGF/FCS-induced proliferation was examined by preincubation of the endothelial cells with these agents prior to induction (Fig. 6). Plasminogen at a concentration of 1 μM had no effect on endothelial cell proliferation. In contrast, angiostatin at 0.25 μM produced a 65% level of inhibition, and 0.5 μM angiostatin yielded an 85% inhibition of FCS/bFGF-induced proliferation. In the presence of 1 μM NG2/EC or 5 μM NG2/D3, this inhibitory effect of angiostatin is essentially abolished, suggesting that NG2 can interfere with the antagonistic effect of angiostatin on endothelial cell proliferation. NG2 by itself has no discernible effect on the endothelial cells.

NG2 Stimulation of the Urokinase-dependent Activation of Plasminogen—It is well established that binding to cell surface receptors and other macromolecules induces conformational changes in plasminogen, which render the proenzyme much more susceptible to activation by both u-PA and tissue-type plasminogen activators (25, 37). To determine whether NG2 binding has such an effect on plasminogen, we monitored plasmin production by u-PA in the presence of NG2/EC or NG2/D3. As shown in Fig. 7, the nonspecific ligand BSA induces only a slight increase in plasmin production, a fact that has been reported by other investigators (38). In contrast, all three of the NG2 species were extremely effective in stimulating u-PA-dependent plasminogen activation. The interaction of NG2 with plasminogen therefore has a dramatic physiological consequence.

DISCUSSION

The plasminogen/plasmin system is involved in the regulation of development, remodeling, and repair in both normal and pathological tissues (39–42). Angiostatin, a proteolytic fragment of plasminogen, is thought to play a key role in regulating endothelial cell proliferation and migration during the process of angiogenesis (43–45). Macromolecules that control the localization and/or regulate the activity of plasminogen and angiostatin are therefore of interest in terms of their involvement in these same biological processes. These molecules might be integral membrane components, anchored in the extracellular matrix, or free in solution.

In this study we show that the integral membrane proteoglycan NG2 contains high affinity binding sites for human plasminogen. NG2 also binds tightly to plasminogen fragments such as angiostatin, which contain kringle domains, the characteristic disulfide-bonded modules that occupy the N-terminal two-thirds of the plasminogen polypeptide (46–48). Very similar specificities and binding affinities for NG2/plasminogen interactions are observed using both solid phase immunoassays and BIAcore measurements. The apparent dissociation constants derived from this analysis are presented in Table IB.
strong inhibition of NG2/plasminogen binding is obtained with 6-AHA, which is known to block the lysine binding sites present in kringle domains 1, 2, 4, and 5 (54–56). K3 apparently lacks a functional binding site for zwitterionic ligands such as 6-AHA (57). Kringle domains are thought to be important for the binding of plasminogen to cell surface receptors, and some studies have concluded that plasminogen can assume several different orientations on the endothelial cell surface because of this idea, both the central NG2/D2 subdomain and the membrane-proximal NG2/D3 subdomain exhibit strong binding to each of the plasminogen species tested. Even though two or more plasminogen binding sites may exist on the NG2 polypeptide, they appear to function independently of each other, because our kinetic data can be adequately fit by a simple bimolecular binding model that does not include cooperativity between interactive binding sites. We have obtained similar evidence for multiple, noninteractive binding sites on the NG2 core polypeptide for the growth factors bFGF and PDGF-AA (9). These growth factors also have positively charged sequences that may interact with the clusters of acidic residues (9). These growth factors also have positively charged sequences that may interact with the clusters of acidic residues (9). These growth factors also have positively charged sequences that may interact with the clusters of acidic residues (9).

The sensitivity of the NG2/plasminogen interaction to treatment with 1 M NaCl, which we observed in the BIAcore experiments, is also consistent with the involvement of charged residues in the binding mechanism. The isolated kringle domains are basic polypeptides with isoelectric points between 8 and 10, whereas all of the NG2 species we used are acidic with pI values between 5 and 6. Although the similarity in behavior between the NG2/EC– and NG2/EC+ species provides no evidence for the involvement of NG2 chondroitin sulfate chains in plasminogen binding, the NG2 core protein itself contains numerous clusters of acidic residues (35), which might participate in binding to properly positioned positively charged residues in the kringle modules. The fact that acidic clusters are distributed throughout the NG2 polypeptide might suggest the existence of multiple binding sites for plasminogen. In support of this idea, both the central NG2/D2 subdomain and the membrane-proximal NG2/D3 subdomain exhibit strong binding to each of the plasminogen species tested. Even though two or more plasminogen binding sites may exist on the NG2 polypeptide, they appear to function independently of each other, because our kinetic data can be adequately fit by a simple bimolecular binding model that does not include cooperativity between interactive binding sites. We have obtained similar evidence for multiple, noninteractive binding sites on the NG2 core polypeptide for the growth factors bFGF and PDGF-AA (9). These growth factors also have positively charged sequences that may interact with the clusters of acidic residues found in NG2.

We have been able to address the functional implications of NG2 binding to plasminogen and angiostatin by examining the effect of purified NG2 on the in vitro activity of the two mole-

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**TABLE II**

**BIAcore kinetic data**

Association and dissociation rate constants and apparent dissociation constants for the binding of immobilized plasminogen species to soluble NG2 fragments are shown. These values represent the mean of triplicate measurements made on the same sensor chip.

| Immobilized ligand | Soluble analyte | Association rate constant | Dissociation rate constant | Dissociation constant |
|--------------------|----------------|--------------------------|---------------------------|----------------------|
| Plg                | D2             | $1.7 \times 10^5$ s$^{-1}$ | $5.7 \times 10^{-3}$ s$^{-1}$ | 37.6 nM              |
| K1–3              | D2             | $1.6 \times 10^5$ s$^{-1}$ | $5.1 \times 10^{-3}$ s$^{-1}$ | 32.8 nM              |
| m-Plg             | D2             | $3.2 \times 10^5$ s$^{-1}$ | $9.8 \times 10^{-3}$ s$^{-1}$ | 31.0 nM              |
| K4                 | D2             | $4.2 \times 10^5$ s$^{-1}$ | $1.1 \times 10^{-2}$ s$^{-1}$ | 26.4 nM              |
| Plg                | D3             | $6.4 \times 10^4$ s$^{-1}$ | $1.5 \times 10^{-3}$ s$^{-1}$ | 23.4 nM              |
| K1–3              | D3             | $1.4 \times 10^5$ s$^{-1}$ | $1.7 \times 10^{-3}$ s$^{-1}$ | 11.7 nM              |
| m-Plg             | D3             | $2.8 \times 10^5$ s$^{-1}$ | $7.5 \times 10^{-3}$ s$^{-1}$ | 26.8 nM              |
| K4                 | D3             | $3.5 \times 10^5$ s$^{-1}$ | $9.0 \times 10^{-3}$ s$^{-1}$ | 25.4 nM              |
| Plg                | EC–            | $4.0 \times 10^4$ s$^{-1}$ | $1.9 \times 10^{-3}$ s$^{-1}$ | 47.6 nM              |
| K1–3              | EC–            | $1.1 \times 10^5$ s$^{-1}$ | $2.2 \times 10^{-3}$ s$^{-1}$ | 20.3 nM              |
| m-Plg             | EC–            | $1.9 \times 10^5$ s$^{-1}$ | $4.0 \times 10^{-4}$ s$^{-1}$ | 21.0 nM              |
| K4                 | EC–            | $3.5 \times 10^5$ s$^{-1}$ | $1.1 \times 10^{-3}$ s$^{-1}$ | 31.4 nM              |
| Plg                | EC+            | $5.1 \times 10^4$ s$^{-1}$ | $1.7 \times 10^{-2}$ s$^{-1}$ | 34.6 nM              |
| K1–3              | EC+            | $3.4 \times 10^4$ s$^{-1}$ | $6.5 \times 10^{-4}$ s$^{-1}$ | 19.3 nM              |
| m-Plg             | EC+            | $2.3 \times 10^4$ s$^{-1}$ | $4.3 \times 10^{-4}$ s$^{-1}$ | 18.5 nM              |
| K4                 | EC+            | $1.2 \times 10^5$ s$^{-1}$ | $2.6 \times 10^{-3}$ s$^{-1}$ | 21.6 nM              |
cules. In the case of plasminogen, binding of NG2 fragments to the proenzyme greatly accelerates u-PA-dependent activation of plasminogen to plasmin. This is consistent with previous observations suggesting that binding of plasminogen to cell surface or extracellular matrix receptors induces conformational changes in the proenzyme that make it more susceptible to proteolytic activation (25, 58). It is of interest to note that although NG2 is an integral membrane component, it can also be shed from the cell surface by proteolytic clipping (59). Further experiments may help determine whether NG2 is more effective in promoting plasminogen activation in its membrane-bound or shed form.

In vivo, NG2 modulation of plasminogen activation by u-PA and tissue-type plasminogen activators may lead to accelerated plasmin degradation of extracellular matrices and basement membranes, a process which is required for cell migration, tissue remodeling, and wound healing, and which also plays an important role in pathological processes such as inflammation and neoplasia (22–24, 28, 29).

The inhibition of endothelial cell proliferation and migration...
by angiostatin is currently a subject of intense interest, because it has important implications for understanding not only the regulation of normal angiogenesis, but also prevention of the neovascularization which is necessary for tumor growth (60, 61). Neither the mechanism of angiostatin inhibition nor the receptors that mediate the effect of the molecule are well defined at present. Our results suggest that NG2 might function, not as a mediator of the antagonistic effect of angiostatin on endothelial cells, but as a regulator that blocks this negative effect of angiostatin. In this context it will be important for us to define more clearly which vascular cell types express NG2. Although there have been reports of NG2 expression by brain capillary endothelial cells (6, 8), the proteoglycan is not found on the mouse aortic endothelial cells used in our current study, and a more consistent alternative is that NG2 is always expressed by the particular mesenchymal cell population, which is associated with endothelial cells in the vasculature in question. Thus, in the heart NG2 is expressed by cardiac myocytes, in large vessels it is expressed by smooth muscle cells (8, 10), and in capillaries it is expressed by pericytes (4, 5, 7). The intimate relationship between endothelial cells and their mural cell companions (62–64) allows for extensive cross-talk, and evidence exists for signaling in both directions between the two cell types (65–70). As a cell surface component of mural cells, NG2 is in position to sequester angiostatin, which otherwise would be available to inhibit proliferation and migration of endothelial cells. This type of mechanism runs counter to suggestions that pericyte interaction with endothelial cells occurs secondarily to the initial events of endothelial tube formation and that investment by pericytes serves to inhibit endothelial cell growth (71–73). However, other reports indicate that pericytes are present at very early stages of capillary formation and could serve to stimulate and guide the formation of endothelial tubes (4, 5, 74, 75). This model would appear more likely to allow the type of anti-inhibitory activity we have observed for NG2. Both in vitro and in vivo studies with tissues from the NG2 knockout mouse (10) may be useful in determining what role NG2 plays in pericyte modulation of endothelial tube development.

In the case of both plasminogen activation and angiostatin scavenging, NG2 would appear to function as a positive regulator of angiogenesis. Because plasmin-mediated proteolysis is required for the endothelial cell migration/invasion that occurs during the formation of new vasculature, the ability of NG2 to potentiate plasmin activation would be expected to promote neovascularization. NG2 binding of angiostatin should also promote neovascularization by neutralizing the inhibitory effects of angiostatin on endothelial cell proliferation and migration. One implication of these results is that binding of NG2 to angiostatin might interfere with the ability of the proteoglycan to potentiate the urokinase-mediated activation of plasminogen, thereby nullifying to some extent the positive effect of the proteoglycan on angiogenesis. However, it is important to remember that angiogenesis and tumor progression each are multistep processes that involve cell proliferation, cell migration, cell-cell interactions, cell-matrix interactions, and both the degradation and synthesis of basement membranes. The contributions of plasmin and angiostatin to neovascularization and tumor progression may therefore occur at different stages of the overall processes. It may be possible, for example, that NG2 is free to potentiate plasminogen activation during early stages of vessel formation/tumor growth and may only be affected by the accumulation of angiostatin during later stages of these processes. More sophisticated in vitro and in vivo assays will be required to fully understand the importance of NG2 interaction with plasminogen and angiostatin and to assess the respective contributions of these molecules to different aspects of vessel and tumor development.

Our results with NG2 and angiostatin are also consistent with a report that smooth muscle cells are the principal mediators of angiostatin binding in atherosclerotic coronary artery, another site of extensive neovascularization (76). This study showed that angiostatin can inhibit hepatocyte growth factor-induced smooth muscle cell proliferation and migration. It would be of great interest to investigate the effect of angiostatin on smooth muscle cell proliferation and migration in more detail and to assess whether NG2 can block the effects of angiostatin as it does in the case of endothelial cell growth. It seems possible that the proteoglycan could have a different function in smooth muscle cells because of its expression by the smooth muscle cells themselves rather than by an adjacent cell type. Perhaps in this case NG2 actually mediates the inhibitory effect of angiostatin on the smooth muscle cells. Once again, further work will be required to expand our binding and preliminary cell behavior data into a better understanding of vascular cell regulation by NG2 during angiogenesis.

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