NG2 Proteoglycan-binding Peptides Target Tumor Neovasculature

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ABSTRACT
NG2 is the rat homologue of the human melanoma proteoglycan, also known as the high molecular weight melanoma-associated antigen. This developmentally regulated membrane-spanning chondroitin sulfate proteoglycan is expressed primarily by glial, muscle, and cartilage progenitor cells. Upon maturation, these cells type down-regulate NG2 expression. In adult animals, the expression of NG2 is restricted to tumor cells and angiogenic tumor vasculature, making this proteoglycan a potential target for directing therapeutic agents to relevant sites of action. To this end, we have identified specific NG2-binding peptides by screening a phage-displayed random peptide library on purified NG2. Several rounds of biopanning on NG2 resulted in the specific enrichment of two phage-displayed decapetides, TAASGVRSMH and LTLLRWVGLMS. The binding of these phages to NG2 was inhibitable both by soluble NG2 and by glutathione S-transferase. Interestingly, these NG2-binding fusion proteins cross-inhibited each other’s binding to NG2, suggesting that the two sequences bind to the same or overlapping sites on the proteoglycan. Upon injection into tumor-bearing mice, NG2-binding phages specifically homed to tumor vasculature in wild-type mice but did not localize to the tumor vasculature in NG2 knockout mice. The in vivo targeting capability of these sequences suggests that they can be used for tumor targeting.

INTRODUCTION
Current anticancer strategies have been directed at the identification and characterization of molecules that are preferentially expressed by either tumor cells or cells of the angiogenic blood vessels associated with the tumor. Once identified, these molecules serve as potential targets for directing chemotherapeutic or immunotherapeutic agents to tumor cells and/or their associated vasculature (1–7).

The rat proteoglycan NG2 (8) and its homologue, HMP (9), are possible targets for anticancer therapy. NG2/HMP is widely expressed by several different tumors, including glioblastomas, chondrosarcomas, melanomas, and some leukemias (10–13). Numerous reports have shown that NG2/HMP expression increases the proliferative capacity of melanoma cells (14–17). Moreover, antibodies against NG2/HMP inhibit melanoma cell growth both in vitro (15) and in vivo (14–16). Recently, we have shown that transfection of NG2 into NG2-negative B16F1 and B16F10 mouse melanoma cell lines increases both the proliferative capacity of these cells in vitro and tumor size in vivo (17). NG2 expression also increased lung colonization for both B16F1 and B16F10 cells in experimental metastasis studies.

Although the specific mechanism by which NG2 enhances the proliferative and metastatic properties of these cells is unclear, association of NG2 with known extracellular matrix ligands such as type VI collagen (18–21) or cellular ligands such as CD44 and αvβ3 integrin (17, 22) and its ability to enhance cellular responses to at least one growth factor, platelet-derived growth factor-AA (23, 24), appear to be important in these processes.

NG2/HMP is also widely expressed by angiogenic blood vessels. This is true not only for the expanding vasculature of normally developing tissues (23) but also for the neovasculature found in tumor stroma and in granulation tissue of healing wounds (12, 25, 26). In contrast, NG2/HMP is not detectable in normal quiescent vasculature. Immunohistochemical studies have suggested that NG2/HMP expression in neovasculature is limited to the neovascular pericytes (25, 26). However, NG2/HMP expression by endothelial cells in developing brain capillaries has also been reported (12, 23). Pericytes are intimately associated with endothelial cells in developing vasculature (27) and are thought to affect angiogenesis by regulating endothelial cell proliferation, directing microvessel outgrowth, and stabilizing capillary walls (27–30).

Because of the selective expression of NG2/HMP in tumor cells and tumor vasculature, several groups have chosen this molecule as a target for immunotherapy of cancer. An anti-NG2/HMP mAb-doxorubicin conjugate was shown to suppress malignant melanoma growth in a nude mouse model (31). Additionally, anti-NG2/HMP mAb-toxin and 131I-radiolabeled conjugates have been shown to have some therapeutic value for patients with malignant melanoma (32, 33). However, these trials have not been as successful as one might have hoped. In general, antibody-based therapies are often found to have limitations, mostly due to poor tissue penetration and unwanted immune responses (3, 6, 34–37). The alternative approach of using small peptides capable of targeting cells within tumor vasculature or stroma may alleviate many of the problems associated with antibody-based targeting strategies (2, 7, 37).

Phage display of random peptide libraries has proven to be successful in the isolation of peptides capable of binding to integrins (38–40), growth factor receptors (41), and other tumor cell-associated proteins (42–44). Moreover, in vivo phage targeting has allowed us to identify several peptides that home to vasculature of specific organs as well as to tumor neovasculature (45–47). Here, we have used phage display to isolate peptides that bind to the NG2 proteoglycan and home to NG2-expressing tumor neovasculature.

MATERIALS AND METHODS
Materials. B16F10 mouse melanoma cells were obtained from the Division of Cancer Treatment Tumor Bank, National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). Rat antimonouse CD31 antibodies were obtained from PharMingen (La Jolla, CA). Rabbit antibodies against NG2 have been described previously (23, 24).

Fus5 vector and K91 bacterial strain were a gift from G. Smith (University of Missouri-Columbia, Ref. 48). Construction of the random linear decapetide phage library has been described (39). The library titer was ~1013 TU/ml.

Isolation of NG2-binding Phages. A recombinant fragment of rat NG2 consisting of the NH2-terminal two-thirds of the extracellular domain (NG2EC3) was purified from transfected human embryonic kidney 293 cells as described (21). Recombinant NG2EC3 diluted in PBS (2 μg of NG2 per well) was coated onto microtiter wells overnight at 4°C. Wells were blocked with...
2% PBS-BSA for 1 h at room temperature. For biopanning, phages (1 × 10¹¹ TU) from a linear decapeptide phage library diluted in 2% BSA were added to proteoglycan-coated wells and incubated for 2 h at room temperature. Wells were washed with PBS containing 0.1% Tween 20 to remove unbound phages. Bound phages were recovered by direct infection of wells with exponentially growing K91kan bacteria, followed by phage amplification overnight at 37°C. Amplified phages were then subjected to four subsequent rounds of selection on NG2-coated wells. Phage binding was quantified by counting colonies from aliquots of phage-infected bacteria removed from NG2-coated wells. Phages were sequenced from randomly selected clones as described (39).

Binding of individual phage clones or an aliquot of unselected phage library control to NG2Δ3 or BSA-coated control wells was performed as described above using 1 × 10⁹ input phages per well. For competition studies, phage incubations were performed in the presence of increasing concentrations of soluble NG2Δ3 or GST fusion proteins. Soluble GST alone was used as a control in these competition experiments.

Solid-phase Binding Assays. GST fusion proteins containing the decapeptide inserts were constructed as described previously (47) and dissolved in PBS. Briefly, peptide inserts were PCR-amplified from the phages using specific M13 primers. PCR products were then digested with BamHI and EcoRI and inserted into the pGEX2TK vector. Fusion proteins were produced and purified according to manufacturer's instructions (Pharmacia, Buckinghamshire, England).

Solid-phase assays were performed as described previously (19). Briefly, GST fusion proteins or GST alone (2 μg/well) were coated onto microtiter

### Table 1 Selection of NG2-binding phage from a linear decapeptide phage library

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*An aliquot (2 × 10¹¹ TU) of a random linear decapeptide phage library was biopanned on immobilized NG2Δ3 as described in “Materials and Methods.” Five successive rounds of biopanning were performed. Shown are the peptide sequences from the inserts displayed from the last three rounds of selection. Numbers in parentheses are the numbers of clones displaying the same sequence.*

Fig. 1. Phage attachment assay. Purified TAASGVRSMH (TAA) and LTLRWVGLMS (LTL) phages or an unselected library mix (unamplified decapeptide library) were incubated in NG2Δ3-coated (●) or BSA-coated (□) microtiter wells and bound phages were quantified as described in “Materials and Methods.” Results are representative of three independent experiments. Columns, means from triplicate platings; bars, SE. All differences are statistically highly significant, as assessed by the Student’s t-test (P < 0.01).

Fig. 2. Inhibition of phage binding by soluble NG2. Purified TAASGVRSMH phage (○) and LTLRWVGLMS phage (■) were incubated in NG2Δ3-coated wells in the presence of the indicated concentrations of soluble NG2Δ3 (○ and ■) or soluble GST (□) and (△). Bound phages were quantified as described above. Results are representative of three independent experiments. Data points, means from triplicate platings of duplicate wells; bars, SE.

Fig. 3. Inhibition of phage binding by cognate sequences. Purified phages were incubated in NG2Δ3-coated wells in the presence of the indicated concentrations of GST-TAASGVRSMH fusion protein (○), GST-LTLRWVGLMS fusion protein (■), or GST alone (△), and phage binding was quantified. Results are representative of three independent experiments. Data points, means from triplicate platings of duplicate wells; bars, SE. * considered highly significant by the Student’s t-test (P < 0.01). A, binding of TAASGVRSMH phage. B, binding of LTLRWVGLMS phage.
cells (1 x 10^6 cells in 0.2 ml of DMEM) were injected i.v. (lateral tail vein) into anesthetized (0.017 ml per g of Avertin) mice and allowed to circulate for 5 min. Mice were then perfused through the heart with 5 ml of DMEM. Tumors were removed and fresh-frozen, and 25-micron sections were cut on a cryostat. Tumor vascularization was visualized using a mixture of a rat antirat immunoglobulin and rhodamine isothiocyanate-conjugated antirat immunoglobulin antibodies (Biosource, Inc., Gaithersburg, MD). Cells (1 x 10^6) from these tissues were plated on microtiter wells overnight at 4°C. Wells were blocked with 2% BSA-PBS and incubated with soluble NG2Δ3 (1 μg/well) for 2 h at room temperature. After washing, wells were incubated with an anti-NG2 polyclonal antibody followed by washing and incubation with an 125I-labeled goat anti-rabbit IgG. After a final washing, bound radioactivity was determined using a gamma counter. For competition studies, soluble NG2Δ3 was preincubated for 15 min with increasing concentrations of soluble GST fusion proteins prior to incubation on GST fusion protein-coated wells. Preincubation with GST alone served as a control in these competition experiments.

In Vivo Phage Targeting. In vivo phage targeting was performed on 4–6-week-old NG2 knockout mice and control F1 wild-type mice. Generation and characterization of these mice has been previously described (49). For tumor generation, B16F10 mouse melanoma cells were harvested from subcutaneous cultures using nonenzymatic cell dissociation buffer (Life Technologies, Inc., Gaithersburg, MD). Cells (1 x 10^7 cells in 0.2 ml of DMEM) were injected s.c. into the mouse right flank. Tumors were monitored between 10 and 20 days postinjection, and animals bearing tumors of −1–2 cm in diameter were selected for target phage.

Tumor targeting using phages was performed as described previously (46). Briefly, phages (1 x 10^9–1 x 10^10) were injected i.v. (lateral tail vein) into anesthetized (0.017 ml per g of Avertin) mice and allowed to circulate for 5 min. Mice were then perfused through the heart with 5 ml of DMEM. Tumors and brains were removed and weighed. Tissues were homogenized in DMEM containing protease inhibitors (45), and phages were rescued and quantified from these tissues as described (45, 46).

Immunohistochemistry. For immunohistochemistry, B16F10 tumors were grown in NG2 knockout and F1 control mice as described above. Tumors were removed and fresh-frozen, and 25-μm sections were cut on a cryostat. Tumor vascularization was visualized using a mixture of a rat antimonious CD31 mAb (Pharmacia) and anti-NG2 polyclonal antibodies. Secondary staining was performed with FITC-conjugated anti-rabbit immunoglobulin and rhodamine isothiocyanate-conjugated antirat immunoglobulin antibodies (Biosource International, Camarillo, CA). Confocal images were obtained using a Zeiss LSM 410 laser scanning confocal microscope (Carl Zeiss, Thornwood, NY).

RESULTS

Isolation of NG2-binding Phages. To identify peptide motifs capable of interacting with NG2, we coated recombinant NG2 fragments consisting of the NH2-terminal two-thirds of the extracellular domain of the proteoglycan (NG2Δ3) onto microtiter wells and used them to select phage clones from a random decapeptide phage display library. Bound phages were isolated and used for successive rounds of panning on the proteoglycan. Random clones were sequenced from rounds II–V. Sequence analysis from the final three rounds of panning (Table 1) indicates the specific enrichment of two decapeptide sequences, TAAASGVRSMH and LTLRWVGLMS. These sequences first appeared in round II and III and became the exclusive motifs bound to NG2 in the subsequent rounds of selection.

Binding Characteristics of the TAAASGVRSMH and LTLRWVGLMS Phages. Phages displaying TAAASGVRSMH or LTLRWVGLMS were tested individually for their ability to bind to NG2Δ3-coated wells. The results showed that both phages specifically bind to the proteoglycan. An equivalent number of control phages from the unselected decapeptide phage library (Fig. 1) or phages containing no peptide inserts (data not shown) showed negligible binding to NG2. Moreover, binding of the TAAASGVRSMH and LTLRWVGLMS phages to BSA was minimal compared to their binding to the proteoglycan.

To confirm the specificity of these interactions, we incubated both species of NG2-binding phages with increasing concentrations of soluble recombinant NG2Δ3 prior to incubation with NG2Δ3-coated wells. The results show a dose-dependent inhibition of binding of both phage populations to the NG2-coated substratum (Fig. 2). As a control, preincubation of the two phage species with GST did not inhibit binding to NG2Δ3. In addition, the low level of binding of unselected phages to NG2Δ3 (as illustrated in Fig. 1) was not affected by incubation with either soluble NG2Δ3 or GST, confirming the nonspecific nature of this binding (data not shown).

Inhibition of Phage Binding with Cognate Sequences. GST fusion proteins containing the two NG2-binding motifs were constructed and tested for their ability to inhibit binding of phages to NG2Δ3-coated wells. When TAAASGVRSMH phages were allowed to bind to NG2Δ3 coated wells in the presence of increasing concentra-

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**Figure 4. Binding of NG2 to immobilized fusion proteins.** Purified NG2Δ3 was incubated in GST or GST fusion protein-coated wells in the absence of inhibitor (4) or after preincubation with 50 μg of soluble GST (3), GST-TAAASGVRSMH (3), or GST-LTLRWVGLMS (3). Binding of NG2Δ3 was determined as described in "Materials and Methods." Results are representative of three independent experiments. Columns, means from triplicate wells; bars, SE. *, considered significant by Student’s t test (P < 0.05); **, considered highly significant by Student’s t test (P < 0.01), A, binding to GST-coated wells (GST). B, binding to GST-TAAASGVRSMH-coated wells (GST-TAA). C, binding to GST-LTLRWVGLMS-coated wells (GST-LTL).
A similar result was obtained when the binding of LTLRWVGLMS phage was tested in the presence of increasing concentrations of GST-LTLRWVGLMS. In contrast, incubation of the phages with a control GST protein without a peptide insert did not significantly inhibit binding.

A similar result was obtained when the binding of LTLRWVGLMS phage was tested in the presence of increasing concentrations of GST fusion proteins. Both fusion proteins inhibited the binding of this phage species to NG2Δ3, whereas the control GST protein had no significant effect on the binding (Fig. 3B).

**Solid-phase Binding of GST Fusion Proteins to NG2.** The ability of soluble NG2Δ3 to bind to GST, GST-TAASGVRSMH, and GST-LTLRWVGLMS was tested by using a solid-phase assay. The results indicate that the soluble proteoglycan binds much more effectively to the immobilized fusion proteins than to GST alone (Fig. 4). In addition, preincubation of NG2Δ3 with increasing concentrations of GST-TAASGVRSMH resulted in a dose-dependent decrease in binding of the proteoglycan to wells coated with this same fusion protein (Fig. 4B). Preincubation of NG2Δ3 with increasing concentrations of GST-LTLRWVGLMS also inhibited binding of the proteoglycan to wells coated with GST-TAASGVRSMH. These results reinforce the notion that the two peptides bind to similar sites on NG2. Both of the soluble fusion proteins also inhibited the binding of NG2Δ3 to wells coated with GST-LTLRWVGLMS (Fig. 4C). In both cases, preincubation of the proteoglycan with soluble GST failed to give significant inhibition of binding to the GST fusion proteins (Fig. 4, B and C). In addition, the level of nonspecific binding of NG2Δ3 to GST alone was not further reduced by preincubation with GST or GST fusion proteins (Fig. 4A).

**Tumor Targeting in Vivo Using NG2-binding Phages.** We were interested in determining whether NG2-binding phages were capable of targeting NG2 within tumor vasculature. We compared the ability of NG2-binding phages to home to the vasculature of B16 melanoma xenografts growing in either wild-type or NG2-null mice. When an equivalent number of NG2-binding phages were injected i.v. into the two lines of tumor-bearing mice, both TAASGVRSMH and LTLRWVGLMS phages were found to home specifically to tumors of NG2-expressing wild-type mice. In contrast, there was much less homing to tumors established in NG2 null mice (Fig. 5A). In addition, control phages did not show selective accumulation to the tumors established in either wild-type or NG2 knockout mice. In a separate experiment, tumor targeting phage previously shown to bind to α5 integrins (46) exhibited equivalent abilities to target tumors in wild-type and NG2-null mice (Fig. 5B). This result indicates that the homing of NG2-binding phages to tumors in wild-type mice is mediated by NG2 expression and is not due to other differences between tumor vasculature of wild-type and NG2-null mice.

Immunohistochemical examination of the tumor vasculature in wild-type mice showed NG2 expression was limited to vascular pericytes, which were abuminally associated with CD31-positive endothelial cells (Fig. 6, A–C). NG2-knockout mice exhibited no NG2 immunoreactivity but showed normal CD31-positive endothelial cell staining (Fig. 6, D–F). No other major difference between vasculature of B16 tumors grown in wild-type and NG2 knockout mice was observed.

**DISCUSSION**

Here, we have described the use of phage display to identify two novel peptide ligands for the NG2 proteoglycan. These decapeptides compete with one another for binding to NG2, suggesting that they recognize the same or overlapping sites on the proteoglycan. The two sequences are clearly different but may act as mimotopes of each other on the basis of small areas of similarity (VR versus LR, SM versus MS, and ASG versus LTL). Database searches revealed no significant similarities between the two decapeptide sequences and known ligands for the proteoglycan (17, 19, 21). They, therefore, represent either novel ligands or mimotopes of sequences present in previously characterized ligands.

Both of the NG2-binding sequences are able to direct the homing of peptide-bearing phages to the NG2-positive neovasculature of melanoma xenografts in mice. Significantly, this tumor homing is greatly reduced in NG2-null mice, illustrating the importance of NG2 as the target molecule. In wild-type mice, we were able to localize NG2 expression to pericytes in the angiogenic vasculature of the xenografts. This agrees with earlier reports of NG2 localization in tumor vasculature (25, 26). Because the phages are relatively large particles and not likely to be able to penetrate an intact endothelial layer in the short time we used for the homing, our results show that pericytes in tumor vessels are accessible to circulating probes. The reason for this may be that tumor vessels are “leaky” (50–52). The localization and accessibility of NG2 on pericytes suggest the potential use of NG2-homing sequences for targeting delivery of therapeutic agents to
tumors. Several reports have suggested that pericytes play an important role in controlling endothelial cell proliferation and stabilization during angiogenesis (27–30). Thus, anticancer strategies based on the targeting of pericytes in angiogenic vasculature may complement approaches based on endothelial cell targeting. Because NG2 is also expressed by the tumor cells themselves in many types of tumors (10–13), the NG2-binding peptides could deliver therapeutics to the tumor cells themselves in addition to targeting tumor vasculature. The small peptides may prove superior to antibodies in terms of penetration into tumors. Future studies will evaluate the relative merits of peptides and antibodies as targeting vectors for NG2.

Specific targeting of tumor vasculature or combined targeting of vasculature and tumor cells offers several advantages over therapies that are strictly tumor-directed. Probes that target tumor cells themselves are limited by both the heterogeneous expression of tumor antigens within the tumor, as well as by the high rate of tumor cell mutation (1, 2, 6, 53). In contrast, cells that comprise tumor vasculature are nonmalignant, relatively homogenous cell populations. The development of resistance to chemotherapy resulting from the high rate of tumor cell mutations is also circumvented by targeting normal cells of the tumor vasculature (54–56).

Finally, the NG2-binding peptides may allow us to identify additional physiological ligands for NG2. Such insights may help elucidate the role of NG2 during development and in pathological conditions. NG2 expression has been found to affect cellular responses to platelet-derived growth factor-AA and cellular interactions with extracellular matrix components (18, 20, 23, 24). In addition, the expression of NG2 increases the malignant potential of tumor cells (17). These findings suggest that NG2 may play a functional role in angiogenesis and tumor development. The peptides we have isolated in this work may prove to be useful probes for analyzing these functions.

REFERENCES


Fig. 6. Immunohistochemical analysis of tumor vasculature in NG2-knockout and wild-type mice. Cryostat sections prepared from B16F10 tumors isolated from NG2-knockout (D–F) or F1 wild-type (A–C) mice were double labeled with a polyclonal antiserum directed against NG2 (A and D) and a rat mAb against CD31 (B and E). Sections were then stained with fluorescein-conjugated goat antirabbit and rhodamine-conjugated goat antirat secondary antibodies, and confocal images through a single section were obtained. Superimposition of confocal images reveals NG2 expression on pericytic cells abluminally apposed to CD31-positive endothelial cells in wild-type mice (C). No NG2 expression was observed in the tumor vasculature of the NG2 knockout mouse (D and F). Note that the B16 cells themselves are NG2 and CD31 negative and do not contribute to the staining pattern. Scale bar (D), 10 μm.


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