The Membrane-spanning Proteoglycan NG2 Binds to Collagens V and VI through the Central Nonglobular Domain of Its Core Protein*

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NG2 is a membrane-spanning proteoglycan with a primary structure unique among cell surface or extracellular matrix proteins. To characterize the interaction between NG2 and extracellular matrix proteins, we have used a eukaryotic expression system to produce and purify several recombinant fragments covering not only the entire ectodomain of NG2 but also distinct subdomains of the molecule. Using a solid phase binding assay with various extracellular matrix proteins, we have identified two main ligands for NG2, namely, collagens V and VI. Consistent with previous models of glycosaminoglycan attachment, roughly 50% of the recombinant NG2 fragments containing the central domain have chondroitin sulfate chains attached to the protein core. These glycosaminoglycan chains are not directly involved in collagen binding, since chondroitinase-treated fragments exhibit an unimpaired ability to bind to both collagens. Using more restricted recombinant fragments of NG2, we mapped the binding site for both collagens to the central domain of NG2. Electron microscopy after rotary shadowing of native NG2 molecules indicates that this extended nonglobular domain provides a flexible connection joining the two N- and C-terminal globular regions of NG2. Rotary shadowing of mixtures of NG2 and collagen V or VI confirms a direct interaction between the molecules and indicates that the collagens align with the central region of NG2, giving the appearance of a rod between the N- and C-terminal globules.

The ability of cells to recognize and interact with the extracellular matrix is fundamentally important for normal development and for continued maintenance of tissue architecture and function throughout adulthood. The pathogenesis of a variety of diseases can be traced to defects in cellular recognition and function throughout adulthood. The pathogenesis of a variety of diseases can be traced to defects in cellular recognition and function throughout adulthood. The pathogenesis of a variety of diseases can be traced to defects in cellular recognition and function throughout adulthood. The pathogenesis of a variety of diseases can be traced to defects in cellular recognition and function throughout adulthood.

by cDNA cloning (2, 3). For example, syndecans, the best characterized of the transmembrane proteoglycans, can interact with a variety of matrix molecules, such as collagen I (4, 5), fibronectin (6), thrombospondin (7), and tenascin (8). Our laboratory has described another proteoglycan, NG2, with the potential to serve as a cell surface receptor for matrix components. This chondroitin sulfate-containing, membrane-spanning proteoglycan has a unique primary structure, which shares few obvious protein motifs with any other cell surface or matrix proteins (9). NG2 is expressed on immature progenitor cells in several types of developing tissues and is down-regulated when these precursors undergo terminal differentiation (10–13). However, high levels of expression are once again observed in some types of malignant cells, such as melanomas, glioblastomas, and chondrosarcomas (14, 15).

Integrin-mediated recognition of the extracellular matrix has been shown to initiate an intracellular cascade of second messenger-dependent signaling events that modulate cellular function such as cell adhesion, spreading, migration, and differentiation (16). In contrast, little information is available concerning intracellular events triggered by engagement of transmembrane proteoglycans. On the one hand, both NG2 and syndecan-1 have been shown to interact with the cytoskeleton (17–19). On the other hand, it has recently been shown that the engagement of syndecan-1 by its specific ligands is able to mediate cell spreading without the involvement of the syndecan cytoplasmic domain (20). This casts doubt on the idea that the proteoglycan itself can directly mediate signal transduction that leads to cell spreading. An alternative suggestion is that cell surface proteoglycans may function as co-receptors, acting in concert with other types of matrix recognition molecules.

This has been observed in the case of syndecans and the α5β1-integrin (21, 22). The co-receptor mechanism is thought to operate either by changing the conformation of the matrix ligand or by making it more available in some way. In addition, the human melanoma proteoglycan, sharing high homology with NG2 (23), has been shown to participate, along with the α4β1-integrin, in promoting spreading of melanoma cells on a fibronectin fragment (24, 25).

A better understanding of the role played by cell surface proteoglycans in cellular function initially requires precise characterization of the interactions of these molecules with specific extracellular matrix ligands. We began to study matrix ligands for NG2 in a solid phase binding assay using NG2 purified from the B49 cell line (26). However, a major limitation in this work was the difficulty in purifying large amounts of the proteoglycan under native conditions. To overcome this difficulty and to achieve better in vitro characterization of the

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detailed mechanisms governing NG2-matrix interactions, we have produced recombinant fragments of NG2 covering not only the entire extracellular domain of NG2 but also distinct subdomains of the extracellular portion of the molecule. The fragments have been biochemically characterized and used in a solid phase binding assay along with a variety of potential extracellular matrix ligands. Our results identify two main ligands for NG2, namely, collagens V and VI. We show that both collagen bind to the central portion of NG2, which consists of a flexible rodlike domain separating two globular regions located at the N- and C-terminal ends of the NG2 ectodomain.

MATERIALS AND METHODS

Construction and Transfection of Expression Vectors—The full-length NG2 cDNA was excised from the pBluescript vector by digestion with XhoI and partial digestion with HindIII (27) and then subcloned into the EcoRV site of the eukaryotic expression vector pcDNA I/Amp (Invitrogen, La Jolla, CA). This construct (pcDNA Amp NG2) was used to generate several expression vectors coding for different domains of the extracellular part of NG2. cDNA fragments were generated by polymerase chain reaction (PCR)1 with the Pfu DNA polymerase (Stratagene) according to the supplier’s instructions.

The DNA in PC DNA NG2/c fragment coding for the entire extracellular part of NG2 (amino acid residues 1–2223) was generated by introducing a stop codon after nucleotide 6738. A fragment containing a stop codon and an XhoI site was obtained by PCR using 5’-primer 1 (nucleotide positions 5605–5622 of NG2 cDNA) and 3′-primer 2 (positions 6724–6739). This product was digested with XhoI and XbaI and ligated into the expression vector pcDNA Amp NG2 digested by the same enzymes, thus replacing the C-terminal end of the coding region with the PCR product and removing nucleotides 6739–7173 of the rat NG2 cDNA. A construct (pcDNA EC3) coding for domain 1 of NG2 and the major part of domain 2 (residues 1–1465) was made similarly by ligating a PCR fragment (5′-primer 3 (nucleotides 3494–3510) and 3′-primer 4 (nucleotides 4450–4464)) digested with Sphi-XbaI into the pcDNA Amp NG2 vector digested with the same enzymes, thus introducing a stop codon after nucleotide 4464.

For expression of internal domains of NG2, pcDNA Amp NG2 was used as a template for PCR to generate fragments for expression vectors. 5′-Primer 5 (nucleotides 1963–1979) and 3′-primer 6 (nucleotides 4420–4419) were used to construct the vector encoding the central domain of rat NG2 (CEP4/D2) (amino acid residues 632–1450). 5′-Primer 5 and 3′-primer 7 (nucleotides 4828–4846) and 3′-primer 8 (nucleotides 6679–6698) were used to generate the expression vector CEP4/D3 coding for the third domain of the extracellular part of NG2 (amino acid residues 1587–2218). Both fragments contain a unique 5′-NheI restriction site and introduce at the 3′-end a sequence coding for 6 histidine residues, followed by a stop codon and an XhoI site for cloning (28). The expression vector used in these two cases was the construct CEP4/D2 III 4 (29) based on the vector CEP4 (Invitrogen), kindly provided by Dr. Ernst Poschl (Max-Planck Connective Tissue Clinical Research Group for Rheumatology, Erlangen, Germany). The NG2 cDNA fragments were fused via their NheI site with the sequence coding for the signal peptide of human BM40 contained in this vector (30). Expression and processing result in secretion of proteins with four N-terminal amino acid residues (APLA) preceding the authentic NG2 domains. The coregion in-frame insertion of the inserts in all constructs was verified by restriction mapping and DNA sequencing.

In the case of the pcDNA NG2EC and pcDNA EC3 constructs, human embryonic kidney 293 cells (American Type Culture Collection) were co-transfected with plasmid psv2pac, which confers puromycin resistance. Stably transfected clones were selected by puromycin resistance following previously described procedures (31). Vectors CEP4/D2 and CEP4/D3 were used to transfect the 293 EBNA cell line (Invitrogen), which constitutively expresses the EBNA-1 protein from the Epstein-Barr virus, allowing epithelial replication of the vector. Transfected cells were selected by resistance to hygromycin B (300 µg/ml) (28).

1 The abbreviations used are: PCR, polymerase chain reaction; EC, extracellular; PAGE, polyacrylamide gel electrophoresis; P, peak; TBS, Tris-buffered saline; GAG, glycosaminoglycan.
RESULTS

Expression and Purification of Recombinant NG2 Fragments

Several stable human 293 cell clones were obtained, which expressed different fragments of the NG2 proteoglycan. These fragments are summarized in Fig. 1. The construct NGOEC codes for the entire extracellular part of NG2. This region can be roughly divided into three domains (9): domain 1, the N-terminal region containing 8 cysteine residues; the central domain 2 lacking cysteine residues but containing the putative attachment sites for chondroitin sulfate chains; and domain 3, the membrane proximal region containing two cysteine clusters. A construct coding for domain 1 and the main part of domain 2 is designated EC3A3. Two smaller constructs correspond to the internal domains of NG2, D2 and D3. For production of these four polypeptides, serum-free medium from 293 cell clones or 293 EBNA-transfected cells was screened by SDS-PAGE for the presence of proteins of the expected sizes, which were absent in medium from nontransfected 293 cells (36). Expression of the correct protein was confirmed by Western blotting with a polyclonal antiserum against NG2 (data not shown). Positive cells showed significant production and secretion of recombinant NG2 fragments, ranging from 0.3 μg/ml for D2 to 8 μg/ml for NGOEC. For both fragments NGOEC and EC3A3, ~50% of the core proteins were substituted by chondroitin sulfate chains and migrated on SDS-PAGE as broad bands of high molecular mass (~350–450 kDa). The other portion of these two recombinant proteins is represented by the protein core free of GAG chains and migrates in SDS-PAGE as a broad band of 250–300 kDa. However, the apparent molecular masses of the two recombinant proteins are higher than the calculated masses based on the amino acid sequence.

Purified NGOEC appeared on electrophoresis as two bands of 110 and 125 kDa. Edman degradation of both bands showed the same N-terminal sequence, APLARGGPAQD, which corresponds to the expected sequence from the construct PCEP4/D2 (starting at position 632 in the NG2 sequence). Since both the 110- and 125-kDa proteins have been affinity-purified by means of the 6 histidine residues at their C-terminal ends, it seems likely that the difference in electrophoretic migration of the two polypeptides results from posttranslational modifications other than proteolytic processing. These modifications did not involve N-glycosylation, as judged by the electrophoretic pattern seen after N-glycanase treatment; the increase in electrophoretic mobility of both species indicates that they are equally N-glycosylated. In addition, a portion of the recombinant NGOEC molecules contain some GAG chains and migrate in SDS-PAGE as a broad band of 250–300 kDa. However, the proteoglycan form of D2 does not exceed 20% (Fig. 2).
Binding Properties of Recombinant NG2 Fragments

Binding to the Large Fragments NG2EC and EC Δ3—We have previously shown that the NG2 proteoglycan interacts with collagen VI both in vitro in solid phase assays (26) and in situ, in which it anchors the collagen at the cell surface (27, 32). In addition, NG2 seemed to be able to interact with other collagen types, particularly the fibrillar collagens II and V. The recombinant NG2 fragments give us the ability to study in more detail these interactions between NG2 and collagens. When NG2EC and EC Δ3 fragments were used as soluble ligands for different immobilized collagens, strong and saturable binding of both fragments was obtained to pepsin-solubilized human collagens V and VI (Fig. 3, A and B). In contrast, no binding of either fragment to collagens I and II was detected. Using radiolabeled ligand, apparent dissociation constants of 140 and 70 nM were derived for binding of EC Δ3 to type V and VI collagens, respectively (data not shown).

Additional studies were carried out to compare the binding ability of the two different pools of the NG2EC and EC Δ3 recombinant proteins obtained from the DEAE purification, P2 containing GAG chains and P1 representing the protein core. Fig. 3, A and B, shows that only the recombinant fragments possessing chondroitin sulfate chains (P2) are able to bind to immobilized collagens V and VI. Although these results initially indicated to us that NG2 binding to type V and VI collagens was mediated by the chondroitin sulfate chains, additional experiments showed that this was not the case. First, the P2 pools of the fragments NG2 EC and EC Δ3 were digested by chondroitinase ABC prior to use in binding assays. Although digestion appeared complete when analyzed by SDS gel electrophoresis (see Fig. 2), both undigested and digested P2 samples bound more strongly to collagen V or VI than the corresponding P1 samples (Fig. 3C). Second, we could show that increasing concentrations of free chondroitin sulfate in the binding assay do not inhibit the binding of either P2 recombinant fragment to collagen VI (Fig. 4). Finally, when collagens were used as the soluble ligands, no difference was seen between the ability of immobilized P1 and P2 forms of EC Δ3 to bind both collagens V and VI (Fig. 5). Similar results were obtained with the NG2EC fragment (not shown). Taken altogether, these results suggest that the chondroitin sulfate chains of NG2 do not participate directly in the binding of NG2 to collagens. The inability of P1 pools of NG2 EC and EC Δ3 to bind effectively to immobilized collagens V and VI remains an anomaly. Although this result may represent a quirk of the solid phase binding assay (involving hidden versus exposed epitopes on the immobilized molecules), it could also indicate that addition of GAG chains to the NG2 core polypeptide induces a conformational state that facilitates binding to the collagens. Adsorption of P1 polypeptides to plastic might produce a conformation that mimics that of the P2 polypeptide.

Binding to the Restricted Fragments D2 and D3—In an attempt to localize more precisely the collagen binding site of NG2, binding assays with the two smaller fragments, D2 and D3, were performed using collagens V and VI as soluble ligands (Fig. 6). For the binding to collagen VI, decorin served as a positive control (38). Negative controls included the basement membrane proteins laminin-1 and collagen IV. Significant binding could be obtained with the central D2 fragment of NG2 to collagen VI (Fig. 6A) or V (Fig. 6B). Binding was similar to or higher than that obtained with the larger fragments NG2EC and EC Δ3. Binding of the third domain of NG2, D3, to collagen VI was as low as that seen with the controls laminin and collagen IV. Binding of D3 to collagen V occurred at the background level seen for bovine serum albumin, confirming that this domain is not involved in the binding of NG2 to collagens.

Thus, the central portion of NG2 appears sufficient to achieve binding of the proteoglycan to collagens V and VI. To confirm the specificity in binding of recombinant fragments to collagen VI, inhibition experiments with soluble fragments were performed. Preincubation of soluble collagen VI (30 nM) with an excess of the different fragments NG2EC, EC Δ3, and D2 (150 nM) inhibits from 65 to 90% of the collagen binding to wells coated with the same fragments (Fig. 7). These results provide evidence of specific interaction of the NG2 fragments with collagen VI.
Rotary shadowing of native NG2 purified from the B49 cell line revealed abundant amounts of large globular domains (Fig. 8A). Two globules were often found in apposition, separated by a distance ranging from 30 to 110 nm ($n = 60$). In rare cases, a threadlike connection was observed between the globules (Fig. 8A, inset 1). Moreover, this central region could be labeled by a monoclonal antibody, the epitope of which has been localized to the recombinant D2 domain by enzyme-linked immunosorbent assay and dot blot experiments (Fig. 8A, inset 2). These observations indicate first that the structure of the central domain cannot be easily resolved by the rotary-shadowing technique due to the absence of extensive folding, and second, that the central portion of NG2 is flexible. These data are consistent with the putative folding pattern of NG2 deduced from its primary structure (9), which indicates the presence of N- and C-terminal cysteine-rich domains (respectively, D1 and D3) separated by a 978-amino acid segment free of cysteine residues (D2). Rotary shadowing of mixtures of native NG2 and collagen V or VI were performed to visualize directly the interaction between the molecules. Pepsin-extracted colla-
Several putative extracellular matrix ligands have been previously proposed to interact with the NG2 proteoglycan, *i.e.* collagens II, V, and VI as well as the glycoproteins laminin-1 and tenascin-C (26). However, the difficulty of purifying large amounts of NG2 precluded a complete analysis of the binding interactions between purified molecules. In the present study, we have successfully produced and purified several recombinant fragments of NG2. High yields of these fragments have allowed us to confirm and characterize in more detail the interactions of NG2 with collagens V and VI, which in our assays represent the extracellular matrix ligands with the strongest affinities for the proteoglycan. Electron microscopy of rotary-shadowed molecules confirms not only the collagen binding properties of NG2 but also several aspects of NG2 biochemistry. Rotary-shadowed preparations of native NG2 indicate that this molecule comprises two globular domains joined by a rodlike central domain that is difficult to visualize. This structure is compatible with the model deduced from the cDNA sequencing of NG2 in which domains 1 and 3, with globular conformations stabilized by intrachain disulfide bonds, lie at either end of the extended, cysteine-free second domain (9). The high levels of expression obtained in our recombinant system with the D3 region are consistent with the idea that this domain represents a stable, independent unit, capable of folding efficiently in the absence of the rest of the molecule. The D2 domain was expressed at a lower level and was partially modified posttranslationally, although the nature of the modification remains to be determined. The largest discrepancy in expression was seen with a construct encoding the 650 N-terminal amino acid residues of domain 1. Extremely low levels of a 90-kDa protein were obtained with this construct, even though it contained all 8 cysteine residues that might be involved in disulfide-mediated stabilization of its globular structure (not shown). Preliminary results suggest that additional residues C-terminal to this 650-amino acid segment (*i.e.* in the initial portion of domain 2) may be necessary for proper folding of the N-terminal globular domain. The case of the larger NG2EC fragment also seems complex, since several distinct proteolytic events appear to occur at the C-terminal end of this polypeptide. It has been shown that recombinant proteins expressed in 293 cells can undergo proteolytic trimming, most frequently after a BXBB site (B for basic amino acid) (28, 36). Such sequences are absent in NG2, but two sites in the ectodomain (Arg1774-Arg and Arg2095-Arg) may represent cleavage sites for dibasic endoproteases (40). Truncated NG2 species of 275 and 290 kDa have been characterized in various NG2-positive cell lines (37), indicating a sensitivity to proteolysis in this part of the molecule. Although this process could represent a pathway for regulation of NG2 as a cell surface receptor, no information is available at present concerning in vivo occurrence of cleaved forms of the proteoglycan.

Biochemical studies with the recombinant NG2 fragments confirm that the D2 region is the site for attachment of chondroitin sulfate chains to the NG2 core polypeptide. This raises the question of whether the chondroitin sulfate chains are involved in the collagen binding mechanism. We previously suggested that binding of native NG2 to collagen VI was independent of the presence of the chondroitin sulfate chains (26, 32). Our current data confirm that chondroitin sulfate is not directly involved in the binding mechanism and that the pro-
tein core of NG2 derived by chondroitinase digestion can bind effectively to collagens V and VI. However, we have also demonstrated that soluble NG2 fragments synthesized without GAG chains fail to interact with immobilized collagens V and VI. One possible explanation for this result is that addition of the GAG chains may modulate folding of the protein core to produce a conformation that facilitates recognition of the collagen ligands. Thus the process of chondroitin sulfate chain attachment could represent a means of regulating the receptor-ligand interaction. In this respect, it is of interest that NG2 is expressed both as a proteoglycan and as a protein core free of GAG chains. Although the GAG content of the NG2 core polypeptide in tissues has not been characterized in detail, different cell lines exhibit different ratios of mature proteoglycan and core protein (41), with one or two extreme cases expressing the core protein almost exclusively. 2 This phenomenon seems to be rather common, since both proteoglycan and core protein forms of other cell surface molecules such as CD44 and β-glycan have also been described (42, 43). Interestingly, although CD44 also serves as a cell surface receptor for extracellular matrix effectors such as fibronectin, in contrast to NG2, its chondroitin sulfate chains are directly involved in the binding mechanism (44, 45). The NG2 extracellular domain binds collagens V and VI with moderately high affinities, i.e., apparent dissociation constants of 140 and 70 nM, respectively, as determined from solid phase assays. In comparison, another cell surface proteoglycan, syndecan, has been shown to have dissociation constants of 5 and 320 nM, respectively, for these two collagen species (46).

We have been able to localize the binding site for collagens V and VI to the central, nonglobular domain of NG2. All three recombinant NG2 fragments (NG2EC, ECΔ3, and D2) containing the central domain are capable of binding to both collagens. The D2 fragment appears to have a binding capacity equal to or greater than those of the two larger fragments, suggesting that this segment is largely responsible for the collagen binding properties of NG2. In contrast, the isolated D3 domain exhibits no ability to bind collagens.

Rotary-shadowed mixtures of NG2 and collagen V or VI contained examples of NG2 molecules in which the collagenous triple helical domain spanned the space between the N- and C-terminal globules, apparently in alignment with the central D2 domain. This observation suggests that the collagenous domain may have multiple points of contact with the central nonglobular domain of NG2. Thus the collagen binding domain might not be localized to an extremely restricted segment of NG2 but may comprise an extended portion of the central segment. The binding mechanisms of collagens V and VI present some similarities: 1) they interact with the same domain of the NG2 core protein; and 2) the triple helical domain of the collagen is involved in the interaction. Despite the presence of the Gly-X-Y motif in the collagenous domain of both molecules, these two collagens do not share a high degree of homology in their sequence (47). However, binding of NG2 does not occur to all types of collagenous sequences. We did not obtain binding of our recombinant fragments to collagens I or II, regardless of whether these collagens were in a monomeric or fibrillar state (data not shown). These results differ from those of Burg et al. (26), which showed that native NG2 could bind to collagen II. This discrepancy could indicate that NG2 has a weaker affinity for collagen II and/or a different mechanism for binding to collagen II than to collagens V and VI.

Previous suggestions have been made concerning the biological significance of the collagen VI-NG2 interaction (26, 27, 32). Collagen VI is considered to be a key component in extracellular matrix assembly due to its ability to interact with other extracellular matrix proteins such as collagen I, collagen XIV, von Willebrand factor, and proteoglycans, either with the protein core of the molecule or with the glycosaminoglycan side chains (48). The fibrillar collagen V is widely distributed as a minor component of the extracellular matrix in various tissues. It is thought to be a crucial component for connective tissue architecture, as collagen V generally co-polymerizes with the more abundant collagen I to form heterotypic fibers (49). Mice producing a structurally abnormal subunit, α2(V), present some major disorders in the organization of the extracellular matrix in different tissues (50). Collagen V has been shown to bind to many extracellular matrix components through its triple helical domain, although it is thought to be rapidly masked in situ by collagen I molecules. However, accumulation of collagen V has been observed during tissue remodeling and in neoplasia. In such cases, collagen V could become transiently available as a potential ligand that fulfills a very specialized function (51). Collagens V and VI also promote adhesion and spreading of numerous tumor cell lines, of smooth muscle cells, and of corneal fibroblasts in primary culture (48, 51). The integrins α1β1 and α2β1 are generally considered the dominant receptors for cell adhesion to collagens V and VI (52, 53). However, collagen V has also been shown to be a substrate for glycosaminoglycan-mediated cell attachment (54). No data are available at present concerning the possible involvement of NG2 as a receptor in mediating cell adhesion to collagen V or VI. However, there is evidence that transmembrane proteoglycans and other cell surface receptors such as integrins can exert a coordinate effect during interactions with extracellular matrix components. In particular, it has been shown that the melanoma-associated proteoglycan, which shares 81% homology with rat NG2 (23), participates in concert with the α4β1 integrin in the spreading of melanoma cells on a fibronectin fragment (24, 25). It is not known whether a similar mechanism may be involved in cell spreading on collagen V or VI, but this type of interaction could be particularly relevant during normal development or tumor metastasis when levels of NG2 expression are maximal.

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The Membrane-spanning Proteoglycan NG2 Binds to Collagens V and VI through the Central Nonglobular Domain of Its Core Protein
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