A Segment of the Cartilage Proteoglycan Core Protein Has Lectin-like Activity*

Diane F. Halberg†, Gary Proulx‡, Kurt Dooeg‡, Yoshi Yamada‡, and Kurt Drickamer†‡

From the †Department of Biochemistry and Molecular Biophysics, Columbia University, New York, New York 10032 and the ‡Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892

A segment of 130 residues near the COOH terminus of the proteoglycan core protein derived from rat cartilage is highly homologous to the carbohydrate-recognition domain of the chicken hepatic lectin and other vertebrate carbohydrate-binding proteins. This portion of the protein has been expressed in an in vitro transcription and translation system and has been tested for its ability to interact with carbohydrates using affinity chromatography on immobilized sugars. A distinct specificity of the binding interaction is demonstrable, with fucose and galactose being the preferred ligands. However, the affinity of the expressed domain of the proteoglycan core protein is lower than that of the other known binding domains, since it elutes from the columns even in the presence of Ca2+. Proteoglycans are a prominent component of the extracellular matrix found in cartilage and other tissue. The proteoglycan found in cartilaginous tissues consists of a core protein (Mr = 300,000) and chondroitin sulfate and keratan sulfate side chains, which make up 80–90% of the mass of the molecule (1–3). Several functional domains have been localized along the core protein. These include a region near the NH2 terminus which interacts with hyaluronic acid and link protein, followed by a region of keratan sulfate attachment and a region of chondroitin sulfate attachment, which extends almost to the COOH terminus of the polypeptide. Recent sequence analysis of cDNAs corresponding to the 3′ portion of the rat proteoglycan core protein reveals an additional cysteine-containing segment of approximately 200 amino acids that lies beyond the glycosaminoglycan attachment region, at the COOH terminus of the protein (4). This sequence may correspond to a globular domain seen at the distal end of the molecule by electron microscopy (5).

A portion of the COOH-terminal segment of the rat proteoglycan shows striking sequence homology with the chicken hepatic lectin. Similar observations have been made for the chicken proteoglycan (6). The chicken hepatic lectin is one of a family of proteins that contains related carbohydrate-recognition domains associated either with membrane anchors (in the case of the chicken hepatic lectin and the mammalian asialoglycoprotein receptors (7–9)) or with collagen-like domains (in the case of the mannoside-binding proteins from liver and serum (10)). This suggested that the proteoglycan core protein might contain a domain which has the ability to interact with carbohydrates. The portion of the proteoglycan core that is homologous with the known carbohydrate-binding domains is roughly 130 amino acids in length and is followed by an additional cysteine-rich segment of approximately 90 amino acids found at the extreme COOH terminus of the protein.

Since it is impractical to prepare the COOH-terminal portion of the proteoglycan core protein directly from the intact proteoglycan, an alternative approach was necessary to test the possibility that this part of the protein forms a carbohydrate-recognition domain. An SP6 in vitro transcription system has previously been utilized to produce mRNAs for the major and minor forms of the asialoglycoprotein receptor (8, 11). When these mRNAs are translated in a reticulocyte lysate system, the protein products display carbohydrate-binding activity as judged by their ability to bind to galactose-containing affinity columns. Activity was observed only when the translation reaction was supplemented with dog pancreas microsomes, which allow correct folding of the polypeptide chain (12).

The in vitro transcription and translation system has been utilized to test for carbohydrate-recognition activity in the proteoglycan core protein. The results indicate that the proteoglycan core protein is capable of interacting selectively with monosaccharides, but the interaction is less stable than the binding of ligands to the asialoglycoprotein receptor tested in a similar way.

EXPERIMENTAL PROCEDURES

Recombinant DNA Procedures—All plasmids were grown in Escherichia coli strain HB101. Restriction enzymes were obtained from New England Biolabs, and T4 DNA ligase was from Boehringer Mannheim. Procedures for the construction of the template plasmids were as described previously (11).

In Vitro Transcription and Translation—Preparation of mRNA followed previously published procedures exactly (11). In vitro transcription was performed using rabbit reticulocyte lysate, dog pancreas microsomes, and radioactive methionine obtained from Amersham Corp. Reactions (30-μl total volume) contained 10 μl of lysate, 10 μl of microsomal membranes, 60 μCi of [35S]methionine, 1 mM oxidized glutathione, and approximately 1 μg of mRNA. Incubation was for 1 h at 30 °C, followed by centrifugation through sucrose and immunoprecipitation as described previously (11, 12). Precipitation with trichloroacetic acid was performed by addition of 1–2 μg of bovine serum albumin as carrier, followed by one-half volume of 30% (w/v) trichloroacetic acid. After incubation for 10 min on ice, the sample was collected by centrifugation for 5 min in an Eppendorf microcentrifuge. The pellet was washed twice with 0.5 ml of ethanol:ether (1:1), dried under vacuum, and dissolved directly in gel sample buffer. SDS–polyacrylamide gels were run in the buffer system of Laemmli

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† To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biophysics, Columbia University, 630 W. 168th St., New York, NY 10032.

1 The abbreviation used is: SDS, sodium dodecyl sulfate.
Affinity Chromatography—Affinity resins were prepared by direct conjugation of sugars to Sepharose 6B with divinyl sulfone (14). All reagents for resin preparation were obtained from Sigma. Columns containing 3 ml of resin were prepared in dispo columns (Bio-Rad). Samples were dissolved as described previously (12) in loading buffer containing 125 mM NaCl, 20 mM imidazole-HCl, pH 7.8, 0.5% Triton X-100, and 25 mM CaCl2. In some cases, competing monosaccharides were dissolved in this buffer as well. Elution was performed with 20 ml of loading buffer, which was collected in 10 fractions of 2 ml. The column was then rinsed with 6 ml of buffer in which 2 mM EDTA was substituted for the CaCl2. Fractions were analyzed by precipitation with trichloroacetic acid and gel electrophoresis as described above. Individual lanes were scanned using a Bio-Rad Model 620 video densitometer. Peak elution positions were determined from plots of optical density in the band corresponding to the expressed fragment of the core protein as a function of elution volume.

RESULTS AND DISCUSSION

In previous work, a series of preproinsulin-asialoglycoprotein receptor hybrids were created to demonstrate that factors within the lumen of microsomes are necessary for the carbohydrate-binding domain of the receptor to achieve its native conformation (12). These constructions also demonstrated that the COOH-terminal carbohydrate-recognition domain of the receptor is capable of folding in the absence of the hydrophobic anchor which normally tethers it to the membrane. This suggested that synthesis of a preproinsulin-proteoglycan core protein hybrid could be used similarly to test for carbohydrate-recognition properties of this protein. As shown in Fig. 1, a template was prepared which was exactly analogous to those previously used to study the asialoglycoprotein receptor. The signal sequence of preproinsulin plus the first 256 residues of the B-chain are followed by the COOH-terminal 253 residues of the proteoglycan core protein.

Transcription of the pPG-2 mRNA with SP6 polymerase followed by translation in a reticulocyte lysate yielded the results shown in Fig. 2. The identity of the translation product was confirmed by the fact that it could be immunoprecipitated with polyclonal antiserum raised against the proteoglycan core protein. This immunoprecipitation could be blocked by inclusion of core protein in the immunoprecipitation reaction (data not shown). When dog pancreas microsomes were included in the translation reaction, the proteoglycan fragment became associated with the microsomes. The mobility of the translation product was increased in the presence of microsomes due to processing by the signal peptidase. Since the segment of the proteoglycan core being expressed does not contain any potential N-linked glycosylation sites, no upward shift in apparent molecular weight due to core glycosylation was observed.

The ability of the core protein fragment to interact with carbohydrate was assessed by passing the in vitro translation products over various carbohydrate-containing matrices (Fig. 3). Translations were performed in the presence of microsomes and oxidized glutathione under conditions which have been found to be optimal for obtaining active fragments from the major form of the asialoglycoprotein receptor. Under the translation and assay conditions employed, in excess of 90% of the receptor polypeptide would remain bound to galactose-Sepharose until released with EDTA. In contrast, two populations of proteoglycan core fragment are resolved on several of the affinity columns, most notably those containing galactose and fucose. Approximately half the material is only slightly delayed on the columns and appears in fractions 1 and 2. (Nonspecific translation products and the small amount of unprocessed core protein appear almost exclusively in the first fraction.) The remainder of the processed core protein is significantly retarded on the column, but is eventually eluted with Ca2+-containing buffer; no further material is released by treatment of the column with EDTA. Experiments in which the material in fractions 1 and 2 and that in fractions 6 through 10 from a fucose-Sepharose column were reapplied to identical columns revealed that each fraction elutes in exactly the position in which it was originally found (data not shown).

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2 Dawn Farrell, Diane Halberg, and Kurt Drickamer, unpublished observations.
We have previously observed differences in the efficiency with which various carbohydrate-binding domains expressed in vitro bind to immobilized ligand. For example, although essentially quantitative binding of the major form of the asialoglycoprotein receptor has been observed previously (6, 12), the in vitro translation system does not work as effectively for the minor form of this receptor, which has a distinct amino acid sequence (8). As in the case of the proteoglycan core protein investigated here, only about half of the minor form of asialoglycoprotein receptor is observed to bind to galactose-containing affinity columns under similar conditions (8). Likewise, differences in the efficiency of production of active mannose-binding protein have also been observed. A likely explanation for this behavior is that the rapidly eluting fraction is incorrectly folded, since in all cases the rapidly eluting (unbound) and retarded (bound) fractions run true when rechromatographed on the same columns.

The fact that a significant fraction of the translated material is retarded on several of the columns, and that this fraction runs true, suggests that the fragment interacts selectively with certain sugars, albeit with a lower affinity than does the asialoglycoprotein receptor. Elution of the column in the presence of EDTA (Fig. 3) indicates that the affinity for fucose-Sepharose is diminished when the concentration of free Ca++ is low. This is again similar to the behavior of the asialoglycoprotein receptor. Comparing the degree of retardation on the several columns tested, it appears that the fragment has a higher affinity for fucose- and galactose-Sepharose than for mannose- or N-acetylglucosamine-containing Sepharose.

**Fig. 3.** Affinity chromatography of proteoglycan (COOH-terminal domain: interaction of pPG-2 translation products with immobilized carbohydrates. Following translation of pPG-2 mRNA in the presence of dog pancreas microsomes, the microsomes were isolated by centrifugation through a sucrose cushion, dissolved in column loading buffer, and applied to various resins. After elution with loading buffer (fractions 1-10), the columns were further rinsed with EDTA-containing buffer (fractions 11-13). Following precipitation, fractions were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Competing sugars were present at the indicated concentration in the sample and in the loading buffer used to elute the column. In the panel marked +EDTA, buffer containing 2 mM EDTA in place of CaCl₂ was used to dissolve the sample and elute the column.

**Fig. 4.** Effect of competing monosaccharides on elution of pPG-2 translation product from fucose-Sepharose. Chromatography on fucose-Sepharose was performed as described in Fig. 3, but employing several different competing monosaccharides. Peak elution positions of the retarded fraction were estimated by scanning densitometry. Data were modeled with an equation describing simple competition between free and immobilized monosaccharide; the lines were generated by fitting to this equation using a nonlinear least squares algorithm (17). Because the degree of substitution of the resin is not known, only relative dissociation constants can be derived from these data. Relative to the dissociation constant for fucose, the constant for galactose is 0.6, for glucose is 2.5, for mannose is 3.0, and for N-acetylglucosamine is 7.1.
Because it is impossible to ensure that the columns used for the experiments in Fig. 3 have identical degrees of substitution with monosaccharides, competition experiments were performed to determine the relative affinities of the core protein fragment for various monosaccharides. Since the greatest retardation was observed with fucose-Sepharose, this resin was used for the competition studies. Examples of the types of results obtained are shown in Fig. 3, using fucose as the competing ligand. These results were quantified and are summarized in Fig. 4, along with the results of similar experiments employing a range of other monosaccharides as competitors.

Using a simple kinetic model to describe approximately the competition observed, it is possible to estimate relative dissociation constants for the ligands tested. From this analysis, it is concluded that the fragment has highest affinity for fucose and galactose among the sugars tested. The affinities for glucose and mannose are intermediate, and the affinity for N-acetylgalactosamine is considerably lower. The fact that the core protein domain is less retarded on the galactose column than on the fucose column in Fig. 3 presumably reflects a difference in the concentration of bound sugar on the two resins.

The selectivity of the interaction observed is similar to that observed for the rat asialoglycoprotein receptor, which has also been shown to bind to fucose as well as galactose (18). However, the receptor also binds to N-acetylgalactosamine, while the core protein fragment shows no interaction with this sugar (data not shown). The specificity observed is also quite distinct from that of the chicken hepatic lectin (which binds primarily N-acetylgalactosamine) and the soluble mannos-binding proteins, which bind N-acetylgalactosamine and mannose in addition to fucose (19, 20). These results suggest that the homologies between these proteins are probably reflected in a common overall structure which forms a carbohydrate-binding site, while some of the sequence differences are related to the generation of unique binding specificities.
than a single sugar residue. Also, the conditions used in the binding assay, while optimal for several members of this class of animal lectins, may not approach those in the unique, charge-dense environment of the cartilage matrix. While our data suggest that the COOH-terminal segment of the core protein has a carbohydrate-binding function in the cartilage matrix, the target for this binding is unknown. Type II collagen is a predominant component of cartilage and carries numerous hydroxylysine-linked galactose substituents which might serve as ligands (22, 23). However, we were not able to show any interaction between the domain expressed in vitro and type I or II collagen immobilized on agarose under the conditions used to demonstrate binding to monosaccharide columns (data not shown). Of course, a specific fibrillar structure of the collagen may be crucial for interaction to occur.

Other investigators have studied interactions between isolated cartilage proteoglycan and type II or other minor cartilage proteins (24–29). These studies give a complex picture in which the results depend on the method of assaying interactions and on the physical state (denatured, native, or aggregated) of the collagens. While these studies disagree on the ability of glycosaminoglycan-free core protein to interact with collagen (26, 27, 29), in all cases the chondroitin-sulfate chains at least modulate collagen binding. It is unlikely, however, that the proteoglycan preparations in these studies contained significant amounts of the COOH-terminal lectin-like domain, since it is apparently quite labile (30). It is possible that the domain is involved in an early event in secretion or deposition of the proteoglycan, such as binding to the chondrocyte surface, and is then removed by proteolytic processing. Other cartilage matrix components have also been reported to bind proteoglycan (31), and it is likely that a multiplicity of such interactions contribute to the overall properties of the cartilage. Finally, it should also be noted that very recently an otherwise unrelated proteoglycan core protein from human fibroblasts has also been found to contain a domain homologous with the carbohydrate-recognition domain discussed here (32).

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