Isolation and Partial Characterization of a Glial Hyaluronate-binding Protein*

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A glial hyaluronate-binding protein (GHAP) with an isoelectric point of 4.3-4.4 was isolated from human brain white matter. The 60-kDa glycoprotein appeared to be quite resistant to proteolysis, and comparison with GHAP from a viable glioma removed at surgery showed that the protein isolated from autopsy material was not a degradation product resulting from postmortem autolysis. The protein was localized immunohistochemically with mouse monoclonal and rabbit polyclonal antibodies in cerebral white matter. Only small amounts could be found in the gray matter. After enzymatic deglycosylation, an immunoreactive 47-kDa polypeptide was obtained. Two amino acid sequences of GHAP showed a striking similarity (up to 89%) with a highly conserved region of cartilage proteins (bovine nasal cartilage proteoglycan and rat and chicken link protein). However, the amino acid composition and other amino acid sequences suggested that there are also differences between brain-specific GHAP and cartilage proteins.

Here, we report on the isolation and partial characterization of a protein related to but different from hyaluronectin, a glial hyaluronate-binding protein (GHAP). GHAP was originally identified by the immunoblotting and immunofluorescence procedures with monoclonal antibodies obtained from mice immunized with hyaluronectin (Bignami and Dahl, 1986a). The 60-kDa antigen was mainly found in brain and spinal cord white matter where it colocalized with the glial fibrillary acidic protein (Bignami and Dahl, 1986b), an astrocyte-specific intermediate filament protein (Dahl and Bignami, 1985).

No immunoreactive material was detected outside the brain and the spinal cord. Another difference with hyaluronectin was that the antigen first appeared relatively late in postnatal development (Bignami and Dahl, 1988a). It will be shown that GHAP is a new protein, showing structural similarities with cartilage proteoglycans and link proteins.

MATERIALS AND METHODS

Chondroitinase ABC, endoproteinase Arg-C, endoproteinase Lys-C, and staphylococcal V8 protease were purchased from Boehringer Mannheim. TPCK-treated trypsin was from Worthington. N-Glycanase and O-Glycanase were purchased from Genzyme Corporation. Neuraminidase, chondroitinase ABC, keratanase, concanavalin A, bovine serum albumin, and 3,3'-diaminobenzidine were obtained from Sigma. Goat anti-mouse rhodamine and peroxidase-conjugated antibodies were from Bio-Rad Laboratories. The 9/30/8-A-4 monoclonal antibody to link protein (Caterson et al., 1985) and the 12/21/1-C-6 monoclonal antibody to rat chondrosarcoma proteoglycan monomer (Caterson et al., 1986) were obtained as ascites fluid from Developmental Studies Hybridoma Bank. Amphotericin, molecular weight standards, and PI standards were from Pharmacia LKB Biotechnology Inc. Acetonitrile and trifluoroacetic acid were obtained from Pierce Chemical Co. Jackson was purchased from Vector Laboratories, Inc. and Na232P from Du Pont-New England Nuclear. All other chemicals were of analytical grade and were obtained either from Sigma or from J. T. Baker Chemical Co.

Isolation and Purification—Human brain white matter was dissected 5-24 h after death. Isolation of GHAP was done according to Delpech and Halavent (1981) as described previously (Bignami and Dahl, 1986a) with modifications. Briefly, 50 g of white matter were homogenized in 150 ml of 10 mM HCl by means of a Polytron. The pH was adjusted with 1 M HCl to 2.3. The homogenate was centrifuged for 15 min at 50,000 rpm in a Beckman Ti-60 rotor at 0°C for 20 min. The supernatant was adjusted to pH 5.6, incubated at 0°C for 30 min while stirring, and centrifuged in order to remove the glial fibrillary acidic protein (Dahl and Bignami, 1973). The supernatant was allowed to run through an HA-Sepharose column (2×15 cm) at a flow rate of 20 ml/cm²/h. Nonspecifically bound proteins were washed with 10 volumes of 1 M NaCl in 10 mM phosphate buffer, pH 7.2, and the HA-binding protein was eluted with either 10 mM HCl or 4 M guanidine HCl in 50 mM sodium acetate, pH 5.8.

Electrophoretic Procedures—SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (1970), and two-dimensional electrophoresis was done according to Celis and Bravo (1985). For the pH determination, the rod gels after the first dimension were...
sliced and equilibrated in 100 mM NaCl for 30 min. pH was measured with a Lazar PHR-146 micro-pH meter. For pl determination by two-dimensional gel electrophoresis, the pl calibration kit of Pharmacia (3.5-10) was used.

Preparation of Antibodies—Monoclonal antibodies to GHAP were obtained from mice immunized with brain hyaluronate-binding fraction as reported (Bignami and Dahl, 1986a). Monoclonal RB50 was used for the present study. Polyclonal antibodies to GHAP were prepared in one female New Zealand White rabbit, 4 kg in weight. The rabbit was injected subcutaneously with 400 µg of enzymatically deglycosylated GHAP suspended in incomplete Freund’s adjuvant. The rabbit was bled from the ear 11 days later, and the serum was tested for immunoreactivity against GHAP by immunoblotting and immunofluorescence.

For absorption experiments, 10 µl of monoclonal supernatant RB50 or rabbit antisem were incubated overnight at 4°C with 5 µg of purified GHAP in 1 ml of blocking solution. The incubation mixtures were used on immunoblots of SDS and acid extracts of human white matter and purified GHAP. Furthermore, cryostat sections of human brain were stained by indirect immunofluorescence with the absorbed antibodies, respectively.

Immunoblotting—Electrophoretic transfer of proteins on nitrocellulose sheets was done according to Towbin et al. (1979). Nitrocellulose was blocked with 0.05% Tween 20 in PBS for 0.5 h and then incubated with monoclonal and polyclonal antibodies raised against GHAP diluted 1:250 and 1:500, respectively, in blocking solution. After washing three times for 5 min with blocking solution, nitrocellulose was incubated for 30 min with a goat anti-mouse peroxidase-conjugated antibody, washed with blocking solution as above, and stained with 3,3′-diaminobenzidine in 1 mg/ml imidazole and 0.3 µl/ml H2O2.

Immunofluorescence—Cryostat sections of human spinal cord, cerebellum, and cerebral cortex were briefly fixed in cold acetone and stained by indirect immunofluorescence with the absorbed antibodies, respectively.

Enzymatic Cleavage—Electrophoretic transfer of proteins on nitrocellulose sheets was done according to Ebert (1986). For amino acid sequencing, purified protein was reduced and carboxymethylated in the presence of 7 M guanidine HCl, 0.5 mM Tris-HCl, 2 mM EDTA, pH 8.2. Reduction was accomplished by making the solution 30 mM in dithiothreitol and incubating at 37°C for 2 h followed by alkylation with 70 mM iodoacetic acid for 30 min. The protein was then incubated in 1 M NH4HCO3, 0.1 mM CaCl2, 0.2% SDS, 0.1% Zwittergent 3-14, brought to 37°C, and sonicated. Incubation with TPCK-treated trypsin at an enzyme to substrate ratio of 1:100 (w/w) was carried out at 37°C for 12-24 h. After digestion, the samples were reduced to dryness on a Speed Vac (Savant Instruments, Inc.) in preparation for injection on HPLC. For V8 protease cleavage, the same buffer was used with the omission of CaCl2 and Zwittergent.

Peptides were separated by microbore reverse-phase HPLC on a Hewlett-Packard 1090 HPLC equipped with a 1040 Diode Array Detector, using a Brownless C8 column (2.1 × 100 mm). A linear gradient of 2-80% acetonitrile in 0.1% trifluoroacetic acid over 45 min was used at a flow rate of 200 µl/min. Chromatographic data at 220 and 280 nm and UV spectra from 214 to 320 nm of each fraction were obtained.

Automated Sequence Analysis—Automated Edman degradation was performed on an Applied Biosystems, Inc. 470A gas-phase sequenator equipped with an on-line 120A phenylthiohydantoin analyzer. All sequenator reagents and solvents were from Applied Biosystems, Inc.

RESULTS

GHAP was isolated as described under “Materials and Methods.” The yield of the preparation was high. Approximately 4.1 mg of protein were prepared from 50 g of white matter, wet weight, as an average of six different preparations from four different brains. No significant differences between individual brains were observed. In some preparations, 1 mM phenylmethylsulfonyl fluoride dissolved in dimethyl sulfoxide was used as a protease inhibitor, but its omission did not have any effect, and the prepared protein by SDS-PAGE appeared to be intact. Although the method is simple and includes only one chromatographic step, the protein appeared well purified and free of major contaminants. The isolated polypeptide was more than 95% pure by SDS-PAGE. Reverse-phase HPLC confirmed this result. Only two peaks were observed at 56.3 and 59.7% acetonitrile which appeared to be identical by SDS-PAGE as well as by immunoblotting (data not shown). In some preparations and depending on the duration of the homogenization of the dissected material in the HCl solution, not only one but two bands, 62 and 60 kDa, were observed. However, both polypeptides were equally immunoreactive and appeared to have the same isoelectric point. In SDS, urea, or guanidine HCl extracts of white matter, only one polypeptide was detected on immunoblots corresponding to the 62-kDa band, but after extraction with 10 mM HCl, the molecular mass of the protein in most preparations was reduced to 60 kDa.

The polyclonal antibodies obtained from the rabbit immunized with purified GHAP selectively reacted with GHAP in extracts and purified preparations by the immunoblotting procedure. At 150 dilutions, the antiserum stained brain tissues with the pattern previously reported using GHAP monoclonal antibodies (Bignami and Dahl, 1986a, 1986b). The immunoreactivity was confined to the central nervous system. Connective tissues as well as mesencephalum and brain stem were stained with the pattern previously reported (Delpech and Delpech, 1984; Bignami and Delpech, 1985).

In order to investigate the specificity of the antibodies, absorption experiments were performed. SDS and HCl extracts were immunoblotted with the antibodies which were preincubated overnight with purified GHAP. Both the monoclonal and polyclonal antibodies lost their ability to recognize
Brain-specific Hyaluronate-binding Protein

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FIG. 1. GHAP specificity of the antibodies. 10 μl of cell culture supernatant RR50 and rabbit antisem were incubated overnight at 4 °C with 5 μg of purified GHAP in 1 ml of blocking solution. The incubation mixtures were used for immunoblot on HCl extract of human white matter (lane 1) and purified GHAP (lane 2) as described under "Materials and Methods." a, immunoblot with RR50; b, immunoblot with RR50 preincubated with GHAP; c, immunoblot with the rabbit antisem; d, immunoblot with the rabbit antisem preincubated with GHAP.

Fig. 1. GHAP specificity of the antibodies. 10 μl of cell culture supernatant RR50 and rabbit antiserum were incubated overnight at 4 °C with 5 μg of purified GHAP in 1 ml of blocking solution. The incubation mixtures were used for immunoblot on HCl extract of human white matter (lane 1) and purified GHAP (lane 2) as described under “Materials and Methods.” a, immunoblot with RR50; b, immunoblot with RR50 preincubated with GHAP; c, immunoblot with the rabbit antiserum; d, immunoblot with the rabbit antiserum preincubated with GHAP.

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the antigen on nitrocellulose after the incubation, indicating that they contained antibodies which bind only to GHAP (Fig. 1). The same results were obtained by indirect immunofluorescence on cryostat section of human brain (data not shown).

Fig. 2. GHAP in human glioma removed at surgery and in human white matter removed at autopsy. Biopitic and autopic tissues were extracted with 10 mM HCl and run on a 10–20% SDS-PAGE. From the same extract, proteins corresponding to 250 μg of starting material, wet weight, were used for Coomassie Brilliant Blue staining (panel a), and proteins corresponding to 500 μg of starting material, wet weight, were used for Western blot (panel b). Lane 1, tumor biopitic material. Lane 2, autopic white matter. M, molecular weight standards. From top to bottom: phosphorylase b, 97,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase 30,000; trypsin inhibitor, 20,100; α-lactalbumin, 14,400. Arrow points to the glial fibrillary acidic protein, a major protein in the glioma.
were incubated in 0.2 M phosphate buffer, pH 8.6, 100 mM β-mercaptoethanol at 37 °C for 16 h with 2.5 units of N-Glycanase (lane 2). pH was adjusted to 6.1 with H3PO₄. GHAP was incubated for 1 h at 37 °C with 0.2 units of neuraminidase (lane 3) and then for 6 h at 37 °C with 0.03 units of O-Glycanase (lane 4). After each step, 5 μg of protein were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue (panel a), transferred to nitrocellulose, and tested for ConA-binding activity (panel b) or transferred to nitrocellulose and tested for their immunoreactivity with monoclonal antibody RR50 (panel c). Lane 1, GHAP control. M, molecular weight standards (as in Fig. 2). Note in panel b the staining of ovalbumin which contains mannose residues.

Fig. 5. Sequential deglycosylation of GHAP. 500 μg of GHAP were incubated in 0.2 M phosphate buffer, pH 8.6, 100 mM β-mercaptoethanol at 37 °C for 16 h with 2.5 units of N-Glycanase (lane 2). pH was adjusted to 6.1 with H3PO₄. GHAP was incubated for 1 h at 37 °C with 0.2 units of neuraminidase (lane 3) and then for 6 h at 37 °C with 0.03 units of O-Glycanase (lane 4). After each step, 5 μg of protein were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue (panel a), transferred to nitrocellulose, and tested for ConA-binding activity (panel b) or transferred to nitrocellulose and tested for their immunoreactivity with monoclonal antibody RR50 (panel c). Lane 1, GHAP control. M, molecular weight standards (as in Fig. 2). Note in panel b the staining of ovalbumin which contains mannose residues.

Fig. 6. Two-dimensional gel electrophoresis of deglycosylated GHAP. GHAP was sequentially deglycosylated as described in Fig. 5, and 2 μg of protein were subjected to two-dimensional gel electrophoresis with pI standards 3.5–9.7. a, GHAP control; b, GHAP after incubation with N-Glycanase; c, GHAP after incubation with N-Glycanase and neuraminidase; d, GHAP after incubation with N-Glycanase, neuraminidase, and O-Glycanase. Arrows in a–d point the position of GHAP.

positive reaction with the mouse monoclonal antibody.

In order to investigate whether GHAP is a glycoprotein, the isolated protein was allowed to bind on a ConA-Sepharose column. The protein was eluted at 0.2 M D-mannoside by applying a gradient of 0–0.5 M D-mannoside.

Sequential cleavage of the sugar residues was performed with N-Glycanase, neuraminidase, and O-Glycanase. N-Glycanase cleaves the N-glycosidic bonds, neuraminidase the sialic acid residues, and O-Glycanase the O-glycosidic bonds. After incubation with the N-Glycanase, a reduction of the apparent molecular mass (60 kDa) and the appearance of two bands at 54 and 52 kDa were observed. After treatment with O-Glycanase, the molecular mass was further reduced to 47 kDa, while neuraminidase digestion did not have any significant effect (Fig. 5a). Binding of ConA on the protein on nitrocellulose sheets was abolished after incubation with N-Glycanase, indicating a complete removal of the mannose residues (Fig. 5b, lane 2). Staining with jacalin, which recognizes O-glycosidic bonds, showed that it was not possible to remove these sugar residues completely. Indeed, chemical
were run in 10-20% SDS-PAGE following proteolytic cleavage at 37°C with 0.07 units of endoproteinase Lys-C (panel a), 6 units of endoproteinase Arg-C (panel b), 50 μg of V8 protease (panel c), and 20 μg of trypsin (panel d). Aliquots were taken after 0 min (lane 1), 10 min (lane 2), 20 min (lane 3), 30 min (lane 4), 1 h (lane 5), 3 h (lane 6), 5 h (lane 7), and 24 h (lane 8). For trypsin cleavage, aliquots were taken after 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 20 min (lane 4), 30 min (lane 5), 1 h (lane 6), 2 h (lane 7), 4 h (lane 8), 8 h (lane 9), 24 h (lane 10). M, molecular weight standards (as in Fig. 2).

deglycosylation with trifluoromethanesulfonic acid (Edge et al., 1981) caused a reduction of the molecular mass to 41 kDa, but this could be because of chemical cleavage of the protein and not only because of removal of all sugar residues. Deglycosylation had no effect on the immunoreactivity of GHAP. All deglycosylated forms of the protein bound equally well to monoclonal and polyclonal antibodies, thus indicating that the antibodies are directed against specific amino acid sequences and not the sugar residues (Fig. 5c). The same results were obtained when these reactions were allowed to proceed in the presence of SDS, Nonidet P-40, and 1,10-phenanthroline hydrate as a protease inhibitor.

With regard to the isoelectric point of GHAP, after incubation with neuraminidase, there was a shifting from 4.3 and 4.4 to one isoelectric point of 4.8, while after incubation with N-Glycanase and O-Glycanase, no change was noted (Fig. 6).

Incubation of the protein with chondroitinase ABC and keratanase did not have any effect on the electrophoretic mobility of the protein.

**Binding of GHAP to Hyaluronic Acid**—The ability of the protein to bind to HA in a saturable manner was investigated by a binding assay of GHAP onto HA immobilized on AH-Sepharose. By increasing the amount of GHAP offered in the mixture, there was an absolute increase in the binding to HA, but the percentage of binding was decreasing starting from 75% of the offered GHAP to only 20% (Fig. 7a). The binding of the protein to immobilized HA could be drastically decreased when increasing amounts of either unlabeled GHAP were taken after 0 min (lane 1), 10 min (lane 2), 20 min (lane 3), 30 min (lane 4), 1 h (lane 5), 3 h (lane 6), 5 h (lane 7), and 24 h (lane 8). For trypsin cleavage, aliquots were taken after 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 20 min (lane 4), 30 min (lane 5), 1 h (lane 6), 2 h (lane 7), 4 h (lane 8), 8 h (lane 9), 24 h (lane 10). M, molecular weight standards (as in Fig. 2).
Peptides, particularly one at 29 kDa in panels a, b, were observed with bovine nasal cartilage link protein (BNCPG) (Neame et al., 1986; Perin et al., 1986), rat link protein 2 (RCLP2) (Neame et al., 1986), and chicken link protein (CLP) (Deák et al., 1986). b, comparison of T22 sequence obtained after cleavage with trypsin with BNCPG, RCLP2, and CLP. Identical amino acids are connected by a solid line. Amino acids which represent common changes based upon the Dayhoff tables of evolutionary similarity (Dayhoff et al., 1983) are connected by a dotted line.

Proteolytic Degradation—Protein samples were cleaved with specific endoproteinases Arg-C and Lys-C, with trypsin, and with V8 protease (Fig. 8). GHAP was quite resistant to proteolytic degradation, and some very stable peptides, particularly one at 29 kDa in panels a, b, and c, were observed. Cleavages with the Arg-C and Lys-C endoproteinases were more effective after deglycosylation. Efforts to isolate specific peptides that bind to the HA-Sepharose column have not been successful yet. However, all peptides derived after 24-h incubation with the Lys-C-specific endoproteinase were allowed to bind to the HA-Sepharose and were eluted only after application of 4 M guanidinium HCl, suggesting a nonspecificity of the binding, whereas peptides from complete cleavage with the Arg-C-specific endoproteinase did not show any affinity for HA.

Amino Acid Analysis and Sequencing—Purified protein was subjected to amino acid analysis according to the procedure of Ebert (1986). The results were compared with those reported for other HA-binding proteins (Table I). Similarities could be observed with bovine nasal cartilage link protein (Baker and Caterson, 1979), rat chondrosarcoma link protein 2 (Neame et al., 1986), and chicken cartilage link protein (Deák et al., 1986), suggesting that GHAP belongs to the same family of proteins. Comparison with the amino acid composition of hyaluronectin (Delpech et al., 1986) showed that the proteins are similar but also appear to have significant differences.

Amino acid sequence data were obtained after cleavage with V8 protease and trypsin. The following seven amino acid sequences have been determined after degradation with V8 protease

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Description</th>
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<tr>
<td>S4</td>
<td>VKVGSFPRGSLSGKVS</td>
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<tr>
<td>S6a</td>
<td>EQPFAAYDFEQCDAGWLAQTYVIPVAPRQVPC</td>
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<tr>
<td>S6b</td>
<td>YRCDVMYGIE</td>
</tr>
<tr>
<td>S7a</td>
<td>QLFAAYEDGFQCD</td>
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<tr>
<td>S7b</td>
<td>AAGLCSLGDVGAIAT</td>
</tr>
<tr>
<td>S8</td>
<td>VTLVAQDQ</td>
</tr>
<tr>
<td>S9</td>
<td>EETTVLVAQDGNI</td>
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<tr>
<td></td>
<td>KIGQDYKGRVSVPT</td>
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<td>HPEAVGDASLT</td>
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With three exceptions (S6a, T22, T37), the sequences were unique, i.e. no significantly similar amino acid sequences were found when a computer-assisted search of the National Biomedical Research Foundation protein database was performed (Devereux et al., 1984). The sequences of peptides S6a, T22, and T37 have been compared with those of BNCPG (Neame et al., 1986; Perin et al., 1986), RCLP2 (Neame et al., 1986), and CLP (Deák et al., 1986) (Fig. 9). Indeed, these GHAP sequences show a striking similarity to certain regions of these proteins. A 48-amino acid long sequence derived from the combination of two overlapping sequences, S6a and T37, shows 83% identity and 89% similarity to a region of BNCPG which is probably part of the binding site to HA. The same sequence shows 53% identity and 87% similarity and 49% identity and 79% similarity to the same regions of RCLP2 and CLP, respectively (Fig. 9). Another sequence (T22) shows 65% identity and 76% similarity to another region of BNCPG, RCLP2, and CLP (Fig. 9). It should be noted that the 48-amino acid long sequence contains 3 cysteine residues at the same position as in BNCPG, RCLP2, and CLP. Through disulfide bonds, these cysteine residues allow the formation of one of two loops which contain the binding sites of the proteins to HA according to Goetinck et al. (1987) and Neame et al. (1986). (For an opposing opinion, see Neame et al., 1987; Doege et al., 1987.) Although this is one of the regions reacting with monoclonal antibody 9/30/8-A-4 (Goetinck et al., 1987), binding of the antibody to GHAP occurred only at very low dilutions. Also, no reactivity was observed with an antibody to rat chondrosarcoma proteoglycan (monoclonal 12/21/1-C-6, Caterson et al., 1986).
In this paper, we report on the isolation and partial characterization of GHAP, a 60-kDa glycoprotein derived from human brain white matter. The same molecular mass was determined by immunoblotting with a monoclonal antibody to GHAP in extracts of a surgically removed glioma thus indicating that the protein isolated from autopsy material is not a degradation product.

Although the method used for the isolation of GHAP is similar to that used for the preparation of hyaluronectin (Delpech and Halavent, 1981), the results shown here indicate that in accordance with previous immunohistochemical studies conducted with monoclonal antibodies and now confirmed with polyclonal antibodies (as discussed in the Introduction), GHAP and hyaluronectin are different proteins. First, there is only one 60-kDa polypeptide isolated, and reverse-phase HPLC gave rise to only one major and one minor peak consisting of the same protein, not several polypeptides with a major band at 68 kDa (Delpech and Halavent, 1981). Second, the amino acid composition of GHAP is different from that reported for hyaluronectin (Delpech et al., 1986). It is possible that differences in the starting material, i.e. dissected white matter (GHAP) versus whole brain (hyaluronectin), may explain why similar purification procedures yielded different products.

The binding of the protein to ConA indicates that GHAP is a glycoprotein. After treatment with N-Glycanase which specifically cleaves sugars bound through N-glycosidic bonds, a reduction of the molecular mass of about 8000 alcalins and the appearance of two bands at 52 and 54 kDa were observed. No change in the isoelectric point was seen, indicating that the cleaved sugar residues were not charged. Binding of ConA was completely abolished, suggesting that all mannoside residues were removed. After cleavage of the sialic acids with neuraminidase, there was almost no change in the apparent molecular weight but a shifting of the isoelectric point of the polypeptides from 4.3 and 4.4 to a more basic one (4.8). The change from two isoelectric variants to only one after this incubation indicates that the two variants are present not because of different degrees of phosphorylation or any other modification but very likely because of different and specific degrees of glycosylation. After treatment with O-Glycanase, there was another change observed in the molecular mass of the protein (reduced to 47 kDa) but not in regard to the isoelectric point.

After deglycosylation of the protein, both chemical and enzymatic, we could not detect any difference in its immunoreactivity, and we conclude that the monoclonal and polyclonal antibodies are directed against specific amino acid sequences and not the sugar residues.

The amino acid composition of GHAP was similar but different from those obtained for cartilage proteoglycans and link proteins (Baker and Caterson, 1979; Neame et al., 1986). However, two amino acid sequences showed marked similarity with the tandem repeated, evolutionarily conserved sequences of cartilage proteoglycans and link proteins (Fig. 9). According to Neame et al. (1986) and Goetinck et al. (1987), these contain the site for interaction with hyaluronic acid. However, in more recent publications (Neame et al., 1987; Doege et al., 1987), the site has been changed in the amino-terminal loop, the tandem repeated sequences apparently being involved in link protein-proteoglycan binding (see Fig. 4 in Neame et al., 1987).

As noted previously, cartilage and brain white matter are among the few tissues in the body where axons do not grow. Although the phenomenon led to experiments resulting in the identification of a brain protein with structural similarity to cartilage proteins, the evidence as to an inhibitory role for axonal growth is still missing except for a preliminary report showing that axons in tissue culture do not readily grow on GHAP-coated coverslips (Bignami et al., 1988).

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