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A novel metalloproteinase originally isolated from brain myelin membranes is present in many tissues

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A monoclonal antibody, CG4, was raised to a novel 60 kDa metalloproteinase purified from a bovine brain myelin glycoprotein fraction. Glycoproteins extracted from both myelin and nine different bovine tissues showed the 60 kDa CG4-immunoreactive band by immunoblotting in amounts that broadly paralleled enzymic activity of this metalloproteinase and varied relatively little among the tissues.

INTRODUCTION

Recent work in this laboratory has led to the identification and isolation of a metalloproteinase associated with brain myelin membranes. This activity was recognized by degradation of the major extrinsic membrane protein of myelin, i.e. myelin basic protein (MBP), when these membrane preparations were incubated at physiological ionic strength and neutral pH (Glynn *et al.*, 1987). The degradation of MBP in this system was substantially inhibited by phenanthroline and dithiothreitol and was thus attributed to a metalloproteinase (Chantry *et al.*, 1988).

The metalloproteinase activity was present in a lentil-lectin-binding glycoprotein fraction of proteins solubilized from bovine brain myelin membranes and was isolated from this fraction by ion-exchange and gel-filtration chromatography. The purified metalloproteinase had an apparent molecular mass of about 60 kDa by both gel-filtration chromatography and SDS/PAGE (Chantry *et al.*, 1989); its enzymic and molecular properties were distinct from those of three previously described membrane-bound metalloproteinases, isolated from mammalian kidney (Kerr & Kenny, 1974; Beynon *et al.*, 1981; Kenny & Ingram, 1987). As a step towards elucidating the physiological role of the myelin-associated proteinase, we asked whether this enzyme was restricted to brain, or present in a variety of tissues. The only substrates thus far identified for this proteinase are MBP and histones (Chantry *et al.*, 1989). MPB is degraded to a mixture of large fragments, among which a 10.3 kDa polypeptide, designated fragment C, is prominent. Fragment C has been identified as MBP-(74–170)-peptide, and an antiserum, anti-p28, specific for the neoepitope at its *N*-terminal has been raised (Groome *et al.*, 1988). MBP-fragment-C-generating activity can thus be detected by incubating tissue fractions with MBP, followed by immunoblotting with anti-p28 serum. However, formation of MBP fragment C alone is not proof that the same enzyme molecule exists in different tissues. To this end we raised a monoclonal antibody to the metalloproteinase purified from brain myelin and used this to screen various tissues immunochemically.

MATERIALS AND METHODS

Isolation of metalloproteinase from bovine brain myelin membranes

This was as described previously (Chantry *et al.*, 1989).

Production of a monoclonal antibody to the metalloproteinase

The method used was essentially that described by Galfre & Milstein (1981). Balb/c mice (8–12 weeks old) were injected (subcutaneously) with 10 μg of purified proteinase in Freund's complete adjuvant, followed by three booster immunizations (intraperitoneally) at 2-week intervals in Freund's incomplete adjuvant, and, finally, 3 days before cell fusion, received a further booster immunization (intraperitoneally) in phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4). Spleen cells from an immunized mouse were fused with NS1 mouse myeloma cells and cultured as described previously (Newcombe *et al.*, 1986). Tissue-culture supernatants were screened by e.l.i.s.a. with the use of plates coated with semi-pure proteinase (CM fraction; Chantry *et al.*, 1989). From four fusions, one rapidly growing hybridoma line gave a strong positive result in the e.l.i.s.a. This line was cloned twice by limiting dilution and the cloned hybridoma was designated CG4. Supernatant from CG4 cells was screened by e.l.i.s.a. with shown to comprise exclusively IgG1. CG4 cells were injected into pristane-primed mice for production of ascites fluid. IgG was isolated from the ascites fluid by hydroxyapatite chromatography.

Isolation of tissue glycoprotein fractions

Bovine tissues were obtained from a local abattoir and were frozen in approx. 50 g portions. Thawed tissue was homogenized in 0.32 M-sucrose in medium A (10 mM-Tris/HCl buffer, pH 7.4, containing 0.02% NaN_3), and centrifuged (at 38000 *g* for 30 min). A portion of the soluble fraction was saved and the pellet was washed twice in 0.32 M-sucrose in medium A. Portions of the resuspended particulate fractions equivalent to 100 mg of protein [measured by the method of Lowry *et al.* (1951), with BSA as standard] were washed once in medium A containing 3 mM-*N*-ethylmaleimide and then resuspended in 35 ml of LL buffer (0.1 M-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl, 2 mM-$CaCl_2$ and 2 mM-$MgCl_2$) containing 8 μg of leupeptin (Sigma Chemical Co.)/ml and 2% (w/v) Triton X-100. The mixtures were rotated (at 20 °C for 1 h) in capped tubes and then centrifuged (at 38000 *g* for 30 min). Supernatants were loaded on to 1 ml columns of lentil lectin–Sepharose (Sigma Chemical Co.) equilibrated with LL buffer containing 0.25% Triton X-100. The columns were washed successively with 10 ml each of LL buffer containing 0.25% Triton X-100, LL buffer alone and LL buffer containing 0.4% CHAPS (Sigma Chemical Co.). Glycoproteins were then eluted from the columns with 0.5 M-

Abbreviation used: MBP, myelin basic protein.

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methyl α-mannoside (Sigma Chemical Co.) in LL buffer containing 0.4% CHAPS. Glycoproteins from heart and muscle were concentrated by vacuum dialysis, but all others were used directly for subsequent analyses.

Assay of MBP-fragment-C-generating activity

Glycoprotein fractions were diluted to 50 μg of protein/ml [determined by the method of Bradford (1976), with BSA as standard] with 0.5 M-methyl α-mannoside in LL buffer containing 0.4% CHAPS and incubated (at 37 °C) with 0.3 mg of purified human MBP (prepared by the method of Dunkley & Carnegie, 1974)/ml in a final volume of 75 μl. After 2 h reactions were stopped with 25 μl of SDS sample solution [containing 10% (w/v) SDS, 10% (w/v) sucrose and 0.02% Bromophenol Blue]. Mixtures were separated on SDS/15%-PAGE (Bio-Rad mini system) (Laemmli, 1970), blotted on to cellulose nitrate paper (Towbin *et al.*, 1979) and probed with a rabbit antiserum specific for MBP fragment C (anti-p28; Groome *et al.*, 1988) at a dilution of 1:250. Immunostaining used either peroxidase-conjugated anti-(rabbit IgG) antibody (ICN) diluted 1:1000 and diaminobenzidine or gold-conjugated anti-(rabbit IgG) antibody (1:150) and silver enhancement (reagents from Bio-Cell Laboratories, Cardiff, U.K.).

Detection of CG4-immunoreactivity by blotting

Glycoproteins isolated from myelin or from tissue particulate fractions were mixed (4:1) with SDS sample solution and electrophoresed on 10% polyacrylamide gels (Bio-Rad mini system) (Laemmli, 1970). Blots from these gels were probed with CG4 ascites fluid (1:100). Immunostaining was achieved with gold-conjugated anti-(mouse IgG) antibody (1:150) followed by silver enhancement (reagents from Bio-Cell Laboratories).

Immunoprecipitation of MBP-fragment-C-generating activity

Brain myelin glycoproteins (0.3 mg/ml) were incubated (for 18 h at 4 °C with either CG4 IgG1 or with control IgG1 (from MOPC 21 plasmacytoma; Sigma Chemical Co., catalogue no. M9269), both at 0.8 mg/ml. These mixtures were then transferred to tubes containing a pellet (equivalent to 25 mg dry/wt.) of Protein A–Sepharose coated with anti-(mouse IgG) antibody and were rotated for 2 h at 20 °C. [The coating procedure was as follows: Protein A–Sepharose (1 g dry wt.) was rotated (for 2 h at 20 °C) with 2 ml of rabbit antiserum to mouse IgG (Sigma Chemical Co., catalogue no. M6024); the antibody-coated Protein A–Sepharose was then washed and stored (at 4 °C) in phosphate-buffered saline containing 0.02% NaN_3.] After removal of antigen–antibody–Protein A–Sepharose complexes by centrifugation, samples of the supernatant were assayed for MBP-fragment-C-generating activity as described above.

RESULTS

Monoclonal antibody CG4 detects a bovine proteinase with MBP-fragment-C-generating activity

Lentil-lectin-binding glycoproteins from brain myelin membranes degraded MBP and generated fragment C [MBP-(74–170)-peptide], which was recognized uniquely by anti-p28 serum. Production of anti-p28-immunoreactive material from MBP was proportional to the amount of myelin glycoprotein in the incubation (Fig. 1*a*). Incubation of the myelin glycoproteins with the new monoclonal antibody, CG4, followed by immunoprecipitation with anti-(mouse IgG) antibody and Protein A–Sepharose led to a marked decrease in the amount of fragment C formed in subsequent incubation with MBP (Fig. 1*b*).

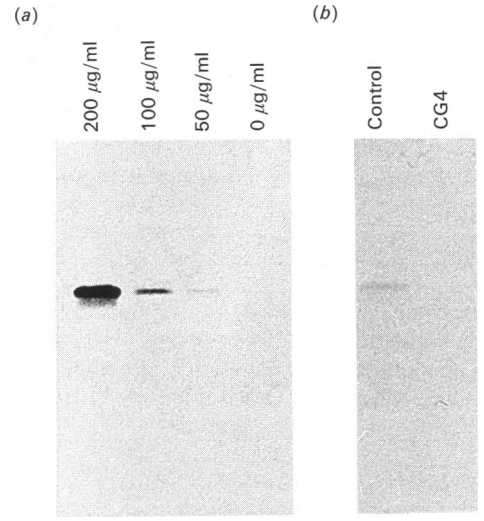

Fig. 1. MBP-fragment-C-generating activity in brain myelin glycoproteins: assay and immunoprecipitation

(*a*) Brain myelin glycoproteins at the indicated concentrations were incubated with MBP. Assay mixtures were separated by SDS/PAGE and blotted against anti-p28 serum, which binds uniquely to the 10.3 kDa MBP fragment C [MBP-(74–170)-peptide]. (*b*) Brain myelin glycoproteins (0.3 mg/ml) were incubated with either CG4 IgG1 or control IgG1 (0.8 mg/ml), followed by anti-(mouse IgG)-antibody-coated Protein A–Sepharose. After removal of antigen–antibody–Protein A–Sepharose complexes by centrifugation, samples of the supernatant were assayed for MBP-fragment-C-generating activity as above. Full details for (*a*) and (*b*) are given in the Materials and methods section.

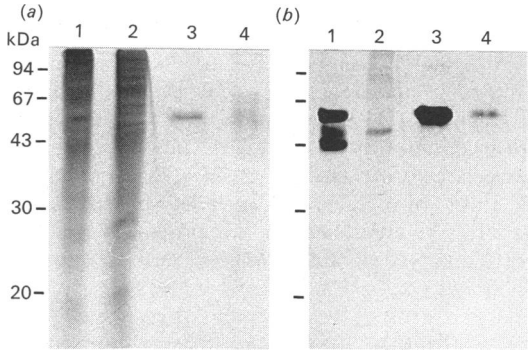

Fig. 2. Immunoblotting of glycoproteins and purified metalloproteinase from brain myelin against monoclonal antibody CG4

Brain myelin glycoproteins (3.2 μg) or purified metalloproteinase (0.2 μg) in SDS sample solution with or without 1% dithiothreitol were subjected to SDS/10%-PAGE and either (*a*) stained with Coomassie Blue or (*b*) blotted against CG4 monoclonal antibody as described in the Materials and methods section. Lane 1, glycoproteins; 2, glycoproteins + dithiothreitol; 3, metalloproteinase; 4, metalloproteinase + dithiothreitol. The positions of marker proteins are indicated at the left.

On blots of myelin glycoproteins, CG4 bound to a band with the same mobility (apparent molecular mass about 60 kDa) as the purified metalloproteinase (Fig. 2*b*, cf. lanes 1 and 3). In addition, when, as in this experiment, relatively large amounts of glycoprotein were blotted, a second band of about 43 kDa was evident, together with a fainter band of about 48 kDa (Fig. 2*b*, lane 1). Titrations showed that the minimum amount of metalloproteinase detectable reproducibly by CG4 on blots was about 20 ng (results not shown). When dithiothreitol was

included in the SDS sample solution, the metalloproteinase migrated as a more diffuse band but still centred around 60 kDa. After dithiothreitol treatment of the samples CG4-immunoreactivity with the 60 kDa band (and the 43 kDa band) was almost abolished, whereas the faint 48 kDa band persisted.

MBP-fragment-C-generating activity is present in human and rat myelin membrane preparations (Glynn *et al.*, 1987; Chantry *et al.*, 1988) and in glycoprotein fractions from these sources. However, CG4 did not react with any antigens on blots of human and rat myelin glycoproteins (results not shown).

Tissue distribution of CG4-immunoreactivity and MBP-fragment-C-generating activity

Lentil-lectin-binding glycoproteins were prepared from brain myelin membranes and from particulate fractions of nine bovine tissues. In most of the tissues recoveries of glycoproteins amounted to 0.2–0.3% of the total particulate protein. From peripheral-nerve particulate fraction and brain myelin membranes glycoprotein yields were half this amount, and heart and muscle yielded only about 50 μg of glycoprotein from 100 mg of particulate protein (results not shown).

Equal amounts of glycoprotein from the different tissues were assayed for CG4-immunoreactivity and MBP-fragment-C-generating activity. To provide a semi-quantitative index of variation between samples, brain myelin glycoproteins were tested at both 50 and 16.6 μg/ml; generation of the anti-p28-immunoreactive MBP fragment (Fig. 3*a*) and CG4-immunoreactivity (Fig. 3*b*) were only barely detectable in the 3-fold-diluted myelin glycoprotein sample (Figs. 3*a* and 3*b*, lanes 1). CG4-immunoreactivity was detected in glycoprotein fractions from all tissues examined. In fact, with the exception of liver, which had only trace amounts, CG4-immunoreactivities among glycoproteins from different tissues varied by less than the range of the two dilutions of myelin glycoproteins in lanes 1 and 2 of Fig. 3(*b*). MBP-fragment-C-generating activity generally paralleled the distribution of CG4-immunoreactivity among the various tissue glycoproteins (Fig. 3*a*). Enzyme activities in lung and peripheral nerve were perhaps rather low compared with their CG4-immunoreactivities, but again the overall variations among tissues came within the range of the two dilutions of the myelin glycoprotein sample.

Neither CG4-immunoreactivity nor MBP-fragment-C-generating activity was detected in cytosolic fractions from these tissues. In addition, the 60 kDa CG4-immunoreactive band was not detected on blots of total particulate fractions (results not shown).

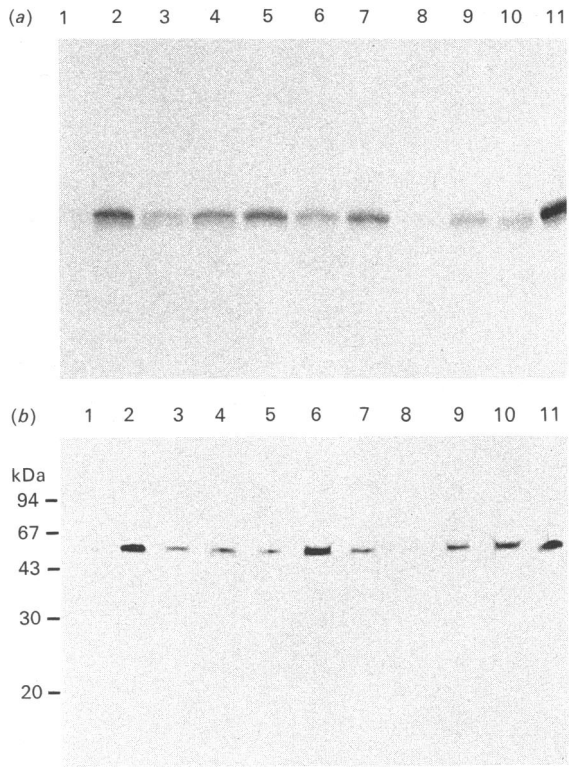

Fig. 3. Tissue distribution of MBP-fragment-C-generating activity and CG4-immunoreactivity

Tissue glycoproteins, at 50 μg/ml (except myelin ×0.3 in lane 1), were (*a*) assayed for MBP-fragment-C-generating activity or (*b*) subjected to SDS/PAGE without reducing agent and blotted against CG4 monoclonal antibody, both as detailed in the Materials and methods section. Lane 1, brain myelin ×0.3; 2, brain myelin; 3, heart; 4, muscle; 5, adrenal; 6, lung; 7, kidney; 8, liver; 9, spleen; 10, peripheral nerve; 11, brain. The positions of marker proteins are indicated at the left of part (*b*).

DISCUSSION

The present data indicate that a novel metalloproteinase originally isolated from bovine brain myelin membranes is widespread in bovine tissues. Among glycoprotein fractions from the tissues the concentrations of the enzyme appeared to vary relatively little. This rather uniform tissue distribution can be compared with that of the most fully studied of the membrane-bound metalloproteinases, endopeptidase 24.11. The latter proteinase is highly enriched in kidney compared with all other tissues examined, including central nervous system (1% or less relative to kidney), adrenal (0.4%), lung (0.1%), liver, spleen and muscle (< 0.1%) (Gee *et al.*, 1985; Kenny *et al.*, 1985).

On the basis of specific enzyme activity we calculated previously that the metalloproteinase comprised 5% of the lentil-lectin-binding glycoprotein fraction of brain myelin membranes (Chantry *et al.*, 1989). Since the yields of glycoprotein from particulate fractions are around 0.1–0.2%, the enzyme constitutes only about 0.005–0.01% of the total protein of myelin membranes, and, from the present results, would comprise a similar minute fraction of the total protein of the particulate fractions of the various tissues. The detection limit for the metalloproteinase on blots by the monoclonal antibody CG4 was 20 ng. Thus to detect a CG4-immunoreactive band on blots of total particulate fraction would require loading 0.2–0.4 mg, well above the capacity of the gels.

The monoclonal antibody CG4 bound the proteinase responsible for generating fragment C from MBP, as shown in the immunoprecipitation experiment. The major band at 60 kDa with the same electrophoretic mobility as the purified metalloproteinase was always present in glycoprotein samples in which CG4-immunoreactivity was detectable (cf. Fig. 3*b*), whereas the 43 kDa band was evident only with large amounts of glycoprotein. The metalloproteinase is quite labile at all stages of its purification, and the 43 kDa band may well be a breakdown fragment. The CG4-epitope on both the 60 kDa and 43 kDa bands was sensitive to dithiothreitol, and this may reflect a requirement for an internal disulphide linkage for its integrity. By contrast, CG4 binding to the fainter 48 kDa band was not dithiothreitol-sensitive, and may represent a non-specific reaction.

The relative ubiquity of the MBP-fragment-C-generating proteinase suggests that its function is not restricted to catabolism of myelin proteins. However, it does not degrade azocasein, a large protein that has been used as a substrate for meprin and

endopeptidase-2 (Beynon *et al.*, 1981; Kenny & Ingram, 1987). The demonstration that the new metalloproteinase is present in a wide range of cell types may allow use of various cultured cell lines to pursue further studies of its functions.

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