Identification of carbohydrate-binding proteins from mouse and human fibroblasts

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Three carbohydrate-binding proteins (M_r 35 000, 16 000 and 13 500) were isolated from extracts of mouse 3T3 fibroblasts by affinity chromatography on polyacrylamide beads to which was covalently bound the ligand 6-aminoethyl 4- β -D-galactosyl-2-acetamido-2-deoxy- β -D-glucopyranoside. None of these proteins bind to polyacrylamide beads coupled with either 6-aminohexanol or 6-aminoethyl β -D-galactopyranoside. Therefore they appear to be carbohydrate-binding proteins specific for galactose-terminated glycoconjugates. A carbohydrate-binding protein was also purified from extracts of human foreskin fibroblasts. This protein (M_r 35 000) may represent the human counterpart of the mouse protein of similar M_r and binding properties.

Carbohydrate-binding proteins (CBPs), specific for recognizing galactose-terminated glycoconjugates, have been isolated from various mammalian and avian tissues [for reviews, see Ashwell & Harford (1982) and Barondes (1981)]. Of particular interest is a group of similar yet distinct lectins which have been postulated to play a role in intercellular interactions during tissue differentiation. These include the lectins from the eel electric organ (Levi & Teichberg, 1981), calf lung (De Waard *et al.*, 1976) and heart (Briles *et al.*, 1979), and chicken intestine (Beyer *et al.*, 1980) and muscle (Den & Malinzak, 1977; Nowalk *et al.*, 1977). In general, all of these proteins share the following properties: (a) lactose-binding specificity; (b) agglutination of similar erythrocytes; (c) subunits of low M_r (13 000–17 000); and (d) sensitivity to air oxidation.

We have undertaken a study of carbohydrate-specific proteins from cultured fibroblasts, presumably representing cells of a rather ubiquitous tissue distribution. Because many of the lactose-specific lectins have been isolated on Sepharose affinity columns covalently coupled to a glycoprotein such as asialofetuin, which contains terminal 4- β -D-

galactopyranosyl-2-acetamido-2-deoxy- β -D-glucopyranoside moieties, we have prepared affinity supports that mimic these structures in our search for fibroblast CBPs. In the present paper, we report the isolation, from extracts of mouse 3T3 fibroblasts, three distinct CBPs on the basis of their binding to polyacrylamide beads covalently coupled with the ligand 6-aminoethyl 4- β -D-galactopyranosyl-2-acetamido-2-deoxy- β -D-glucopyranoside (Gal-GlcNAc-HA).

Materials and methods

Culture and [35 S]methionine labelling of fibroblasts

Swiss 3T3 cells were obtained from the American Type Culture Collection (CCL92) and were grown to confluent monolayers in Dulbecco modified Eagle's medium containing 10% (v/v) calf serum (Steck *et al.*, 1979). The medium was replaced with fresh growth medium for 24 h. The cells were then cultured in serum-free Dulbecco modified Eagle's medium (10 ml/150 cm² growth area) containing 3 μ g of unlabelled methionine/ml and 20 μ Ci of [35 S]methionine/ml (Steck *et al.*, 1982). After 24 h, the medium was removed and the cells were washed before extraction and isolation of proteins.

Human foreskin fibroblasts (SL66) were gifts from Dr. V. Maher and Dr. J. McCormick (Michigan State University). They were cultured in Eagle's Minimum Essential Medium containing 20% foetal-bovine serum, 100 μ g of streptomycin/ml and 159 units of penicillin/ml (Roff *et al.*, 1983). They were labelled with [35 S]methionine (20 μ Ci/ml) and washed before the isolation of proteins.

Abbreviations used: CBP, carbohydrate-binding protein; PA, polyacrylamide; HA, 6-aminohexanol; Gal, galactose; GlcNAc, N-acetylglucosamine; SDS, sodium dodecyl sulphate.

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Synthesis of carbohydrate ligands containing 6-aminohexanol

The ligand 6-aminoethyl β -D-galactopyranoside (Gal-HA) was synthesized by the procedure of Chiang *et al.* (1979). The method of Barker *et al.* (1972) was used to synthesize the compound 6-aminoethyl 2-acetamido-2-deoxy- β -D-glucopyranoside (GlcNAc-HA). The aminohexanol-containing disaccharide Gal-GlcNAc-HA was synthesized by using UDPGal and GlcNAc-HA acceptor, catalysed by a partially purified preparation of bovine UDP-galactose:glucosaminide β -(1 $\rightarrow$ 4)-galactosyltransferase. The details of this preparation have been previously described (Rosevear *et al.*, 1982).

Preparation of carbohydrate affinity columns

The method of Inman & Dintzis (1969) was used; this is described in a comprehensive treatise (Inman, 1974). Conditions were optimized so as to increase the coupling efficiency of our carbohydrate ligands, and only these details are described here. Bio-Gel P-150 (Bio-Rad Laboratories) (1 g dry wt.) was converted into the acyl hydrazide by incubation with 120 ml of 6.1 M-hydrazine at 50°C for 3 h. After extensive washing, the hydrazide derivative was treated with 100 ml of 0.3 M-HCl at 4°C for 10 min, after which 10 ml of 1 M-NaNO₂ was added. After 20 min of gentle stirring, the beads were washed with water and the resin was filtered on a Buchner funnel for 10 min to remove excess water.

The acyl azide resin was then made to react with the glycosides of 6-aminohexanol to couple the ligands covalently. For Gal-HA-PA, 8 ml of an aqueous solution of Gal-HA (462 μ mol) was adjusted to pH 11.5 and added immediately to 75% of the acyl azide resin made from 1 g of Bio-Gel P-150. The mixture was vortex-mixed at 30 min intervals, and after 1 h an additional 5 ml of water (pH 11.5) was added. The total reaction time was 4 h, after which an equal volume of 5 M-NH₃ (adjusted to pH 10 by addition of concentrated HCl) was added and the mixture was kept at 4°C for 20 h. The mixture was then washed extensively before use. The overall coupling yield was about 35%. The same procedure was used to couple Gal-GlcNAc-HA to Bio-Gel P-150 to make Gal-GlcNAc-HA-PA and to couple HA to the same beads to make HA-PA.

Isolation of carbohydrate-binding proteins

Extracts of 3T3 cells were prepared from [³⁵S]methionine-labelled cells in 150 cm² flasks. The cells were first washed with 15 ml of Tris/CaCl₂ buffer [50 mM-CaCl₂, 1 mM-Na₂N₃, 75 mM-Tris, pH 7.2]. They were then scraped with a rubber policeman into 2 ml of the same buffer containing 1% Triton X-100 and 1 mM-phenylmethanesulphonyl fluoride. The material was homogenized in

a Potter homogenizer (102–152 μ m clearance) by using five strokes for each 2 ml. Insoluble material was pelleted by centrifugation (3000 g for 15 min) and the supernatant was cooled on an ice bath.

The supernatant was applied to a column (0.8 cm $\times$ 10 cm) containing Gal-GlcNAc-HA-PA, equilibrated at 4°C with the same buffer. The column was washed extensively with Tris/CaCl₂ buffer containing 1% Triton X-100, 1 mM-phenylmethanesulphonyl fluoride and 2 mM- β -mercaptoethanol. The buffer was then changed to Tris/CaCl₂ buffer containing only 2 mM- β -mercaptoethanol. Proteins bound on the column were eluted with a linear 0–0.08 M-lactose gradient in Tris/CaCl₂ buffer containing 2 mM- β -mercaptoethanol (total volume 100 ml). Samples from the column effluent were assayed for radioactivity from [³⁵S]methionine-labelled proteins by using scintillation counting (Steck *et al.*, 1982).

The material eluted by lactose was pooled and dialysed against Tris/CaCl₂ buffer containing 2 mM- β -mercaptoethanol in tubing impermeable to molecules of *M_r* greater than 3500. The dialysis tubing also contained resin that was to be used for the next affinity column (Gal-HA-PA or Gal-GlcNAc-HA-PA). The dialysed contents were packed into a column for a second round of chromatography.

Gel-electrophoretic analysis

Polyacrylamide-gel electrophoresis in SDS was performed by the method of Laemmli (1970) in a 1 mm-thick, 9 cm-long, 5–16% linear gradient slab gel (0.67% bisacrylamide) with a 1 cm-long 4%-acrylamide stacking gel. Samples were dissolved in 1% SDS/4% β -mercaptoethanol and boiled for 1 min. After electrophoresis, the gels were fixed for 30 min in 10% trichloroacetic acid and stained with Coomassie Brilliant Blue. After destaining, the gel was subjected to fluorographic treatment (Bonner & Laskey, 1974) with Kodak X-Omat R (XR-5) film.

Results and discussion

Carbohydrate-binding proteins from 3T3 cells

Mouse 3T3 fibroblasts were cultured in the presence of [³⁵S]methionine to label the cellular proteins. After washing, these labelled cells were extracted with Triton X-100 and fractionated by affinity chromatography on a column of Gal-GlcNAc-HA-PA. Most of the radioactive material was not bound by the column (Fig. 1). After extensive washing in buffer containing Triton X-100, the column was developed with detergent-free buffer (arrow 1, Fig. 1). Finally, the column was eluted with a linear gradient of lactose (arrow 2, Fig. 1). This resulted in the appearance of a peak of radioactivity (Component A, Fig. 1), which accoun-

Fig. 1. Affinity chromatography of [^{35}S]methionine-labelled Triton X-100-solubilized extracts of 3T3 fibroblasts on a column (0.8 cm $\times$ 10 cm) of Gal-GlcNAc-HA-PA

The column was equilibrated with 50 mM-CaCl₂/1 mM-Na₂N₃/75 mM-Tris/1% Triton X-100/1 mM-phenylmethanesulphonyl fluoride, pH 7.2. The arrows mark the fractions at which the buffers were changed: 1, column buffer without Triton X-100; and 2, linear 0–0.08 M-lactose gradient (100 ml) in column buffer containing 2 mM- β -mercaptoethanol (----). Fractions (4 ml) were collected until the position of arrow 1; thereafter 1.5 ml fractions were collected. Samples (0.2 ml) were assayed for radioactivity. The horizontal bar labelled 'A' represents fractions pooled for analysis by SDS/polyacrylamide-gel electrophoresis.

ted for approx. 0.01% of the total radioactivity applied to the affinity column.

SDS/polyacrylamide-gel electrophoresis of Component A (Fig. 1) yielded three polypeptide bands, with M_r values of 35 000, 16 000 and 13 500 (lane a, Fig. 2). The relative proportions of the three bands were 3:1:1 respectively, as determined by densitometric tracing of the fluorograms. Similar results were obtained in both the presence and the absence of β -mercaptoethanol during the electrophoresis. Because the ligands used in the affinity support and in the elution of the polypeptides from the affinity column were saccharide moieties, the isolated proteins are presumably carbohydrate-binding proteins. Therefore the material corresponding to these three bands will be hereafter referred to as CBP 35, CBP 16 and CBP 13.5.

To explore the carbohydrate-specific requirements of the CBPs for the recognition, binding and elution from affinity supports, we have compared the

Fig. 2. SDS/polyacrylamide-gel electrophoresis of proteins derived from mouse (3T3) and human (SL66) fibroblasts after affinity chromatography on polyacrylamide beads covalently coupled with saccharide-containing ligands

(a) Component A (Fig. 1) (3200 c.p.m.); (b) Component A (Fig. 3a) (3200 c.p.m.); (c) Component B (Fig. 3a) (5000 c.p.m.); (d) Component A (Fig. 3b) (13 000 c.p.m.); (e) protein from SL66 human fibroblasts corresponding to Component A (Fig. 1) (3000 c.p.m.). For further details see the text.

chromatographic properties of the isolated polypeptides on columns of polyacrylamide beads coupled with various defined ligands (Fig. 3). Component A (Fig. 1) was dialysed against Tris/CaCl₂ buffer containing 2 mM- β -mercaptoethanol. During this dialysis, it was absolutely critical to include β -mercaptoethanol in the buffer; in its absence, all three polypeptides lost their binding activities. When the dialysed Component A (Fig. 1) was rechromatographed on a column of Gal-GlcNAc-HA-PA, approx. 80% of the radioactivity was bound by the column (Fig. 3a). The bound material could be completely eluted with galactose and yielded all three polypeptides (Fig. 2, lane c). No additional

Fig. 3. Chromatography of [^{35}S]methionine-labelled CBP 35, CBP 16 and CBP 13.5 on columns of (a) Gal-GlcNAc-HA-PA and (b) Gal-HA-PA

The radioactive CBPs were isolated by fractionating extracts of 3T3 fibroblasts on a column of Gal-GlcNAc-HA-PA (Fig. 1). The CBP-containing fractions were eluted with lactose, dialysed (in tubing impermeable to molecules of M_r greater than 3500) against 50 mM- CaCl_2 /1 mM- NaN_3 /75 mM-Tris/2 mM-mercaptoethanol, pH 7.2. The material was then chromatographed on affinity columns (0.8 cm $\times$ 10 cm) equilibrated with the same buffer. The arrows mark the addition of (1) galactose (0.15 M) and (2) lactose (0.15 M) in the developing buffer. Fractions (2 ml) were collected and 0.4 ml samples were analysed for radioactivity. The horizontal bars designated 'A' and 'B' represent fractions pooled for SDS/polyacrylamide-gel electrophoresis.

radioactivity was eluted with lactose. In contrast with these results, no binding was observed with any of the polypeptides when a column containing only the monosaccharide galactose (Gal-HA-PA) was used (Fig. 3b and Fig. 2, lane d). Finally, there was also no binding to HA-PA or PA columns. Inasmuch as these affinity supports differ only in the saccharide portion of their chemical structures, these results demonstrate unequivocally that the three polypeptides are carbohydrate-specific proteins.

We have also found that none of the CBPs bound on the Gal-GlcNAc-HA-PA column could be eluted by the addition of Ca^{2+} -free buffer containing

10 mM-EDTA. Moreover, the extent of the initial binding of CBP 35, CBP 16 and CBP 13.5 to the affinity column was similar in the presence and absence of Ca^{2+} ions. These results suggest that the interactions of the CBPs with the carbohydrate ligands do not require the bivalent cation.

Molecular identities of the carbohydrate-binding proteins

Several lines of evidence indicate that CBP 16 and CBP 13.5 are mouse fibroblast counterparts of lactose-specific lectins isolated from eel electric organ (Levi & Teichberg, 1981), calf heart (Briles *et al.*, 1979), calf lung (De Waard *et al.*, 1976), chicken intestine (Beyer *et al.*, 1980) and muscle (Den & Malinzak, 1977; Nowalk *et al.*, 1977). Their similarities include: (a) molecular weights of the polypeptide chains; (b) galactose/lactose specificity; and (c) sensitivity to air oxidation and requirement for the presence of reducing agents (β -mercaptoethanol). Moreover, preliminary immunoprecipitation experiments lend further support to this conclusion. An antiserum raised against the chicken lactose-specific lectins causes immune precipitation of CBP 16, but not of CBP 35 (C. Roff & S. Ullrich, unpublished work).

Gel-filtration studies of Component A (Fig. 1) on Sephadex G-50 in Tris/ CaCl_2 buffer containing 2 mM- β -mercaptoethanol have shown that CBP 35 can be separated from CBP 16 and CBP 13.5. The isolated CBP 35 retained carbohydrate-binding activity, assayed by binding to the same affinity column, independent of the presence of the other two polypeptides. Conversely, CBP 16 and CBP 13.5 also re-bind to the same affinity column after separation from CBP 35. A protein analogous to CBP 35 has not been previously identified and isolated in other species and from other cell types. Therefore it represents a new carbohydrate-binding protein.

Isolation of carbohydrate-binding protein from human fibroblasts

In a similar series of experiments, normal human foreskin fibroblasts (SL66) were labelled with [^{35}S]methionine, extracted with Triton X-100, and fractionated by direct affinity chromatography on a column of Gal-GlcNAc-HA-PA. A small amount of radioactive material bound to the column, and this could be eluted with lactose in a fashion similar to that observed with 3T3 cells. Polyacrylamide-gel electrophoresis and fluorography of this material yielded one predominant band, migrating at the position corresponding to CBP 35 (Fig. 2, lane e). Thus it appears that in a single step we have been able to obtain a highly purified protein that probably represents the human counterpart to CBP 35 of mouse 3T3 cells. These results strongly suggest that

polyacrylamide beads covalently coupled with defined carbohydrate structures provide a highly efficient method for the isolation of CBPs.

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