

Carbohydrate-Binding Protein 35: Identification of the Galactose-Specific Lectin in Various Tissues of Mice

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In previous studies, a lectin designated as carbohydrate-binding protein 35 (CBP35) has been isolated from cultured mouse 3T3 fibroblasts. In this study, antibodies directed against CBP35 were used to screen for cross-reactive proteins in various cultured cells and in various organs and tissues of mice. Cross-reactive proteins of the same molecular weight (M_r , 35,000) were found in human, mouse, and chicken fibroblasts and in a macrophage-like cell line, P388D1. Similarly, cross-reactive proteins were also found in the embryonic liver, lung, spleen, thymus, skin, and muscle tissue and in the lung, artery, thymus, and spleen of the adult mouse. Fractionation of extracts of mouse lung on affinity columns of asialofetuin-Sepharose yielded a protein whose molecular weight, carbohydrate-binding specificity, and immunological properties suggest that it is CBP35 derived from the lung, hereafter designated CBP35 (lung). The binding of ^{125}I -labeled CBP35 (lung) to rabbit erythrocytes was quantitated in the presence and absence of various carbohydrates. It was found that only carbohydrates containing galactose were inhibitors of the binding; the disaccharide lactose was 100-fold more potent as an inhibitor than was the monosaccharide galactose. When extracts of the adult mouse liver were fractionated by asialofetuin-Sepharose chromatography, only a protein corresponding to CBP16 was isolated; no CBP35 was found. These results corroborate the immunoblotting data, which indicated that CBP35 was not detectable in the adult mouse liver.

Although carbohydrate recognition has been implicated in a number of cellular processes, including the regulation of cell proliferation in cultured fibroblasts (6), very little detail concerning the molecular nature of the interacting components has been elucidated. In previous studies, we have purified three galactose-specific carbohydrate-binding proteins (CBPs) from cultured mouse 3T3 fibroblasts (19, 20). These CBPs were designated CBP35 (M_r , 35,000), CBP16 (M_r , 16,000), and CBP13.5 (M_r , 13,500). On the bases of molecular weights, isoelectric points, binding and agglutination properties, requirement for reducing agents, and immunological properties, it was suggested that CBP16 and CBP13.5 may be the murine analogs of lectins previously isolated from embryonic chicken (3, 16), electric eel (14), and bovine (1, 7, 17) tissues. In contrast, CBP35 represented a new lectin which was not a structural precursor to the other lectins of lower molecular weight.

For this reason, we wished to characterize the tissue distribution and molecular properties of CBP35 in some detail. We have screened for an analogous protein in various cultured cells and in various organs and tissues of both adult and embryonic mice. To corroborate the results of the screening assays, CBP35 has been isolated from mouse lung, an organ that showed an abundance of CBP35. In contrast, when the same isolation procedure was carried out with extracts of mouse liver, CBP16 instead of CBP35 was found.

MATERIALS AND METHODS

Materials. Mice (A/J) were obtained from Charles River Breeding Laboratories. Carrier-free Na^{125}I (100 mCi/ml) was purchased from Amersham Corp. Sepharose and Sephadex were from Pharmacia Fine Chemicals, Aquacide III was from Calbiochem-Behring, fetuin was from GIBCO Laboratories, nitrocellulose paper was from Schleicher & Schuell, Inc., and goat anti-rabbit immunoglobulin G (IgG) was from Sigma Chemical Co. The iodogen, 1,3,4,6-tetrachloro-3a,6a-

diphenylglycoluril (8), was a generous gift from J. C. Speck, Jr., Michigan State University. α_1 -Acid glycoprotein was a gift of the American Red Cross Blood Services Laboratories, Bethesda, Md.

The following fibroblasts were cultured by procedures described previously: (i) the murine fibroblast line 3T3 and 3T3 cells transformed by Kirsten murine sarcoma virus (24); (ii) human fibroblasts SL66 (21); and (iii) secondary chicken embryo fibroblasts and transformants obtained after infection with Rous sarcoma virus (22). These last two cell types were gifts of L. B. Crittenden. Cell lines murine P388D1 (CCL 46), Chinese hamster ovary cells (CCL 61), human intestine 407 (CCL 6), and human Chang liver (CCL 13) were from the American Type Culture Collection. Cultures of chicken myoblasts and myotubes were gifts of V. Bennett and C. Suelter; the procedures for establishing these cultures will be described elsewhere (V. D. Bonnett, H. D. Husic, J. H. Baxter, and C. H. Suelter, manuscript in preparation). Bovine endothelial cells, obtained by the method of Booyse et al. (4) and characterized by the presence of factor VIII antigen, were gifts of S. Huey and W. Smith.

Preparation of tissue samples. Tissues were removed from a freshly sacrificed adult male mouse or mouse embryos on day 17 of gestation and washed with buffer containing 0.9% NaCl, 0.1% (ethylenedinitrilo)tetraacetic acid, 0.05% iodoacetamide, 2 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM citrate, and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 6.1) at 4°C. The tissues were minced and homogenized (10 strokes per ml) with a Potter homogenizer (clearance, 102 to 152 μm) in 0.9% NaCl-1% sodium deoxycholate-1% Triton X-100-0.1% sodium dodecyl sulfate (SDS)-0.05% iodoacetamide-2 mM PMSF-10 mM Tris (pH 7.2) (3 to 4 volumes per tissue volume). Bone extracts were prepared by removing the femurs, breaking them into small pieces, and extracting with the detergent-containing buffer for 30 min. Skin extract was prepared from minced hide which had been trimmed of excess hair. Erythrocytes were obtained by the method of

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Lis and Sharon (15). The homogenates were centrifuged at $1,500 \times g$ for 10 min, and the resulting supernatants were diluted with an equal volume of buffer containing 1% SDS, 4% β -mercaptoethanol, and 60 mM Tris (pH 6.8). After incubation at 95°C for 20 min, insoluble material was removed by centrifugation at $1,500 \times g$ for 10 min. The protein content of the resulting supernatants was determined (5). Samples were diluted with electrophoresis sample buffer, and samples containing 100 μ g of protein were electrophoresed.

Preparation of samples from cultured cells. Cells were scraped into phosphate-buffered saline (1.5 ml per 100-mm dish) and centrifuged at $1,500 \times g$ for 5 min. The resulting cell pellet was frozen at -70°C until used. The pellets were resuspended in 1.5 ml of 0.5% Triton X-100-2 mM PMSF-5 mM phosphate buffer (pH 8.0) and centrifuged for 5 min at $1,500 \times g$. The resulting supernatants were boiled for 2 min, dialyzed overnight against water, and lyophilized. For polyacrylamide gel electrophoresis, the samples were suspended in water, and the protein content was determined (5). The samples were then adjusted to 1% SDS-4% β -mercaptoethanol-10% glycerol-60 mM Tris (pH 6.8). Samples containing 100 μ g of protein were electrophoresed.

Polyacrylamide gel electrophoresis and analysis by immunoblotting. Polyacrylamide gel electrophoresis in SDS was performed by the method of Laemmli (13) on 5 to 16% acrylamide gradient slabs (0.21 to 0.67% bisacrylamide) (thickness, 1 mm; length, 9 cm) with 4% stacking gels (length, 1 cm). Samples were dissolved in 1% SDS-2% β -mercaptoethanol-60 mM Tris (pH 6.8) 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 gels were dried, and radioactive bands were detected by autoradiography, using Kodak X-Omat RP (XRP-5) film (23).

The procedures for transferring proteins from polyacrylamide gels to nitrocellulose paper, for staining with amido black, and for blotting with antisera were those described by Tobin et al. (26) with minor modifications. After electrophoresis, the proteins were transferred at 400 mA for 4 h. The nitrocellulose paper was then placed in saturating buffer overnight, followed by incubation with anti-CBP35 (3T3) (antiserum raised against CBP35 derived from 3T3 cells [20]) at a dilution of 1:250 for 5 h. After washing, the paper was incubated with 1.6×10^6 cpm/10 ml of ^{125} I-goat anti-rabbit IgG (1.9×10^8 cpm/mg) for 5 h. The papers were washed, dried, and subjected to autoradiography.

Isolation of CBPs from murine lung and liver. Lungs from female A/J mice were excised and washed with 0.9% NaCl-0.1% EDTA-0.1% glycerol-2 mM PMSF-20 mM citrate-25 mM HEPES (pH 6.5), followed by 50 mM CaCl_2 -2 mM PMSF-2 mM NaN_3 -75 mM Tris (pH 7.1) (buffer A). The washed lungs were minced in buffer A containing 1% Triton X-100. The final volume of buffer and minced lung was ca. 1 ml per lung. The slurry was homogenized with one stroke in a Potter-Elvehjem homogenizer at 1,200 rpm, and the resulting homogenate was stirred for 30 min at 4°C. The homogenate was centrifuged at $12,000 \times g$ for 20 min, and the layer of fat was carefully aspirated and discarded. The remaining supernatant was loaded onto a column of asialofetuin-derivatized Sepharose and eluted as previously described for the purification of CBPs from 3T3 cells (20). The CBPs were further purified by a second round of affinity chromatography. Identical procedures were used for lectin purification from mouse liver. For the isolation of CBP35, the affinity-purified proteins were fractionated by gel filtration as previ-

ously described (20). All procedures were performed at 4°C. Column eluates were assayed for protein by the method of Bradford (5).

The isolated lectins, CBP35 (lung) and CBP16 (liver), were electrophoresed on SDS gels. Gel slices with bands corresponding to M_r s of 35,000 and 16,000 were incubated overnight in 1 ml of water containing 1% SDS. After centrifugation to remove the acrylamide gel, the solution was lyophilized. The lyophilized powder was extracted twice with 1 ml of methanol, and the residue was then suspended in water, dialyzed, and lyophilized. The amino acid composition was determined by reverse-phase chromatography of phenylthiocarbamoyl amino acids (12); this analysis was carried out by the University of Michigan Protein Sequencing Facility.

Iodination procedures. CBP35 was labeled with ^{125} I by the method of Fraker and Speck (8). Extracts of lung tissue were subjected to two rounds of affinity chromatography, and the material eluted by lactose was concentrated to 2.5 ml by reverse dialysis against Aquacide III. This concentrate was added to a test tube which had been precoated with 50 μ g of chloroglycoluril. To this was added 800 μ Ci of Na^{125}I , and the solution was incubated on ice for 20 min with intermittent swirling. The labeled material was chromatographed over Sephadex G-15 (1.4 by 27 cm). Material eluted in the void volume was pooled, concentrated by reverse dialysis, and chromatographed on a Sephadex G-150 column (0.8 by 115 cm). Material chromatographing to a region corresponding to an M_r of 35,000 was pooled. Iodination and subsequent steps were done in buffer A containing 2 mM β -mercaptoethanol and 50 mM lactose, except the final chromatography over Sephadex G-150 in which the lactose was omitted.

Iodination of immunoglobulins was performed as described by Hunter and Greenwood (10). Unincorporated ^{125} I was removed by passage of the reaction mixture over Dowex 1-X8 (chloride form).

Binding of lectin to erythrocytes. Trypsinized, glutaraldehyde-fixed rabbit erythrocytes were prepared by the method of Nowak et al. (16). To 175 μ l of a 20% (vol/vol) suspension of erythrocytes was added 100 μ l of ^{125} I-labeled CBP35 (1.1×10^4 cpm/ μ g; 1.6×10^3 cpm), 100 μ l of a solution containing a saccharide, and 25 μ l of buffer A. All reagents were in buffer A containing 2 mM β -mercaptoethanol. The suspension was incubated for 20 min at 4°C and centrifuged at $1,000 \times g$, and the supernatant was discarded. The erythrocyte pellet was resuspended in 0.4 ml of buffer A containing 2 mM β -mercaptoethanol and repelleted at $1,000 \times g$, and the supernatant was discarded. Radioactive CBP35 bound to the erythrocytes was determined by counting the washed pellet on an LKB 1271 Riagamma counter.

RESULTS

Analysis of the presence of CBP35 in cultured cells. To test for the presence of CBP35 in different cells, extracts of various cells were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose paper, and blotted with antisera directed against CBP35 (3T3). The results (Fig. 1) showed: (i) cross-reactive proteins of the same molecular weight (M_r , 35,000) were found in all fibroblasts, irrespective of the species of origin (mouse 3T3, human SL66, and normal and Rous sarcoma virus-transformed secondary chicken embryo fibroblasts); (ii) 3T3 fibroblasts transformed with Kirsten murine sarcoma virus showed cross-reactive proteins of molecular weight 35,000 and 34,000; (iii) P388D1, a cell line with macrophage-like

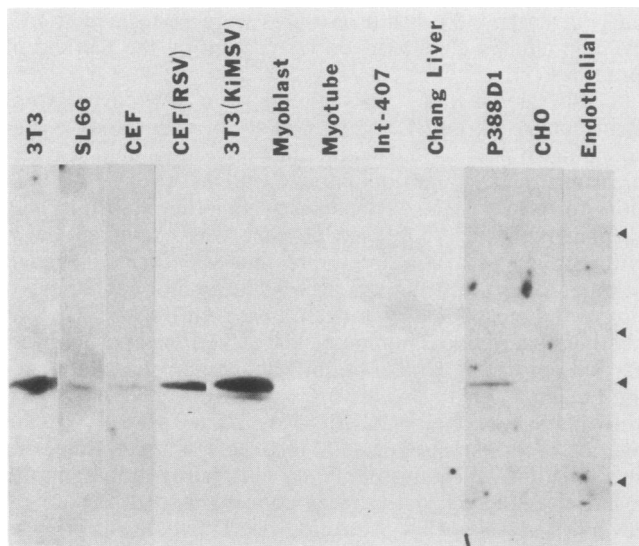


FIG. 1. Survey of the presence of CBP35 in various cultured cells. Extracts of cells (100 μ g) were subjected to polyacrylamide gel electrophoresis, transferred to nitrocellulose paper, and blotted with anti-CBP35 (3T3), followed by ^{125} I-goat anti-rabbit IgG as described in the text. The cells used were: (i) Swiss 3T3 murine fibroblasts; (ii) SL66 human fibroblasts; (iii) secondary chicken embryo fibroblasts (CEF); (iv) chicken embryo fibroblasts transformed by Rous sarcoma virus [CEF(RSV)]; (v) 3T3 fibroblasts transformed by Kirsten murine sarcoma virus [3T3(KiMSV)]; (vi) chicken myoblasts; (vii) chicken myotubes; (viii) cells derived from a human intestine (Int-407); (ix) cells derived from a human liver (Chang liver); (x) cell line derived from cultured murine lymphoblastoid cells (P388D1); (xi) Chinese hamster ovary (CHO) cells; and (xii) bovine endothelial cells. Molecular weight markers (▲) from top to bottom: myosin (M_r , 200,000), bovine serum albumin (M_r , 68,000), aldolase (M_r , 40,000), and cytochrome *c* (M_r , 12,500).

properties (11), also showed a protein (M_r , 35,000) cross-reactive with anti-CBP35 (3T3); and (iv) none of the other cells showed detectable amounts of a protein analogous to CBP35. These last cell types included cells derived from

human liver and intestine, bovine endothelial cells, Chinese hamster ovary cells, and chicken myoblasts and myotubes.

Survey of the distribution of CBP35 in mouse tissues. Using a similar protocol, we have searched for the presence of CBP35 in different mouse tissues. Autoradiography of the blotted nitrocellulose paper revealed relatively large amounts of reactive material with M_r s of 35,000 in the lung, artery, thymus, and spleen (Fig. 2A and B). The reactive material is also present to a lesser extent in the small intestine, stomach, bone, eye, cartilage, and skin. Finally, it was not detected in the erythrocytes, heart, liver, kidney, brain, muscle, and testes. An additional band was observed in the lung and brain. This material migrated to a position with an M_r of 42,000. There was no reactive material corresponding to CBP16 and CBP13.5 in any of the tissues tested with anti-CBP35.

When a similar analysis was carried out with embryonic tissues of the mouse (day 17 of gestation) and compared with the results obtained with adult tissues, several observations were evident (Fig. 2 and 3). (i) There was an abundance of a cross-reactive protein (M_r , 35,000) in the embryonic lung, thymus, and spleen, as was found in corresponding tissues of the adult. (ii) In contrast, there were drastic differences between embryonic and adult tissues when the presence of CBP35 was compared in the liver, muscle, and skin. Whereas a cross-reactive protein was abundant in the embryonic tissues, a similar protein was not found or was hardly detectable in the adult. (iii) Conversely, the adult stomach and intestine showed the presence of a cross-reactive protein, which was absent or barely detectable in the embryonic counterparts. (iv) Finally, no CBP35 analogs were found in either adult or embryonic tissues of the heart, kidney, and brain.

Purification of CBP35 from mouse lung. Because the adult lung tissue showed a relatively large amount of material reactive with anti-CBP35 (Fig. 2A, lane 2), we tested whether this tissue could serve as a source for large-scale preparation of CBP35. Homogenates of the lung were fractionated by two cycles of affinity chromatography on asialofetuin-Sepharose. Upon the addition of lactose, a peak of protein

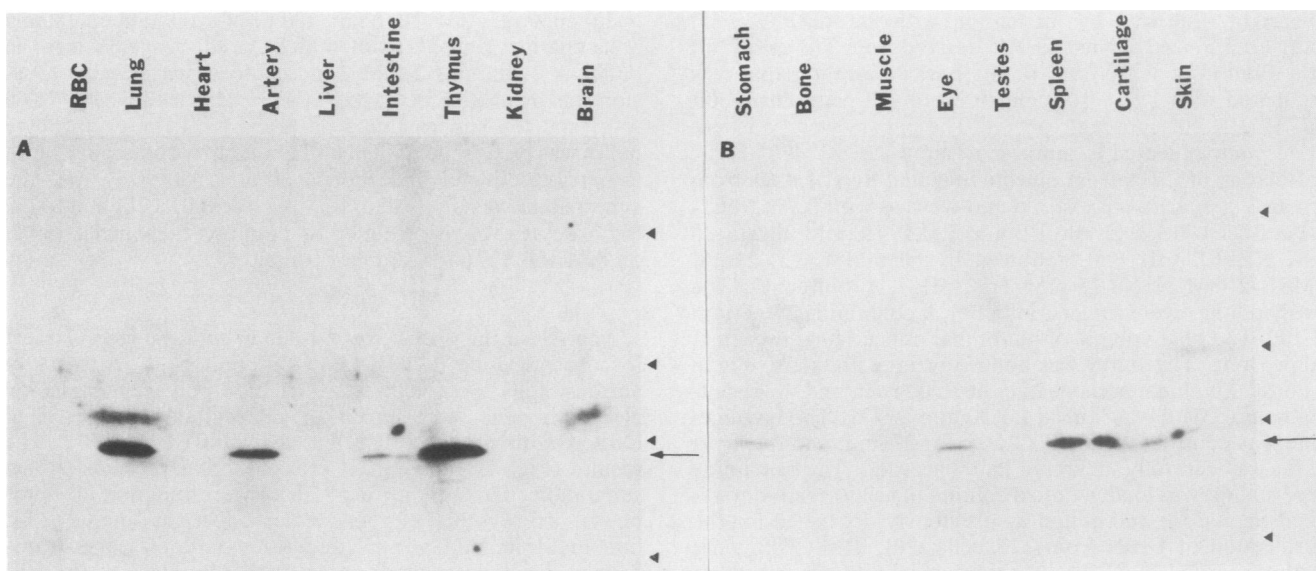


FIG. 2. Survey of the tissue distribution of CBP35 in adult male mouse. Tissue extracts (100 μ g) were subjected to polyacrylamide gel electrophoresis, transferred to nitrocellulose paper, and blotted with anti-CBP35 (3T3), followed by ^{125} I-goat anti-rabbit IgG as described in the text. The arrow marks the position of migration of CBP35 from 3T3 cells. Molecular weight markers (▲) are as described for Fig. 1.

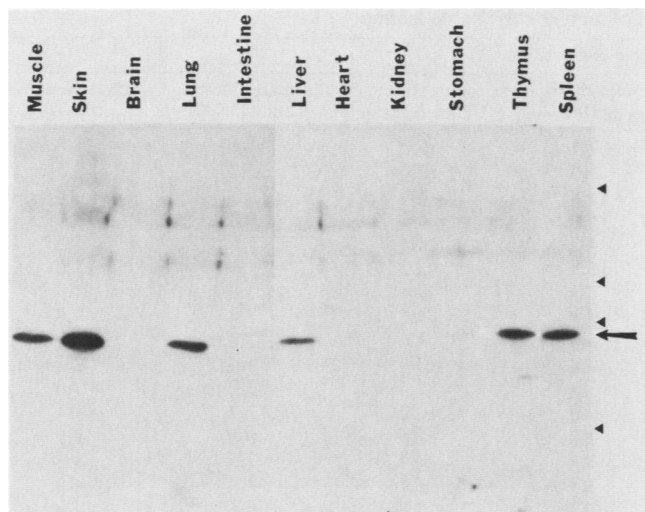


FIG. 3. Survey of the tissue distribution of CBP35 in embryonic mice (day 17 of gestation). Tissue extracts (100 μ g) were subjected to polyacrylamide gel electrophoresis, transferred to nitrocellulose paper, and blotted with anti-CBP35 (3T3), followed by ^{125} I-goat anti-rabbit IgG as described in the text. The arrow marks the position of migration of CBP35 from 3T3 cells. Molecular weight markers (▲) are as described for Fig. 1.

was eluted from the columns (Fig. 4). A polypeptide (M_r , 35,000) was present in this peak as determined by gel electrophoresis and Coomassie brilliant blue staining.

Although CBP16 and CBP13.5 were not detected by staining with Coomassie brilliant blue, they were present in the material purified by two cycles of affinity chromatography (Fig. 4). When this material was labeled with ^{125} I (see below) and subjected to gel electrophoresis and autoradiography, three major bands were seen (data not shown). These corresponded to CBP35 and to proteins with M_r s of 16,000 and 13,500. These two low-molecular-weight proteins may be the mouse lung counterparts to CBP16 and CBP13.5 isolated from 3T3 fibroblasts.

The material isolated by two cycles of affinity chromatography was further fractionated by gel filtration on Sephadex G-150 (20). Fractions chromatographing at a position corresponding to CBP35 (3T3) were pooled and analyzed by SDS gels. The results showed one predominant polypeptide (M_r , 35,000) stained with Coomassie brilliant blue (Fig. 5a). To ascertain that this protein was CBP35, its reactivity with antibodies raised against CBP35 derived from 3T3 cells (anti-CBP35 [3T3]) was tested. The protein was transferred onto nitrocellulose paper and blotted with anti-CBP35 (3T3), followed by ^{125} I-labeled goat anti-rabbit IgG. Autoradiography revealed a reactive polypeptide with an M_r of 35,000 (Fig. 5b). Relative to molecular weight standards, this polypeptide migrated to a position identical to that of the Coomassie brilliant blue-stained polypeptide. The CBP35 isolated and identified from lung tissue will be hereafter designated CBP35 (lung).

In both the Coomassie brilliant blue-stained and immunoblotted samples (Fig. 5a and b), there was also a minor polypeptide band (M_r , 60,000). Preliminary evidence indicates that this band may be a higher-molecular-weight precursor of CBP35. This polypeptide also has the capacity to bind to carbohydrate.

The amino acid composition of CBP35 (lung) is listed in Table 1. The amino acid compositions of other lactose-inhibitable lectins from various sources are also tabulated.

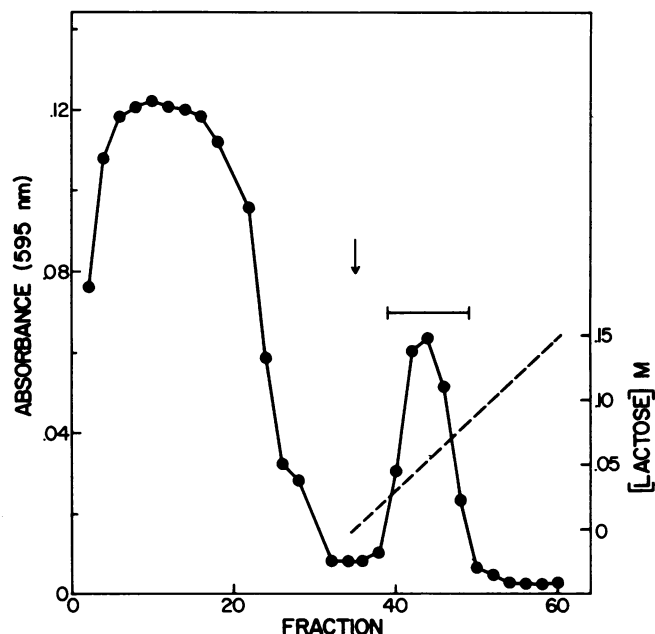


FIG. 4. Representative column profile showing the affinity chromatography of mouse lung extracts on an asialofetuin-Sepharose column (1.2 by 15 cm). Lung extracts from 100 mice were subjected to affinity chromatography on asialofetuin-Sepharose as described in the text. The lactose-eluted material from the first cycle of affinity chromatography was dialyzed in the presence of asialofetuin-Sepharose and repacked into a second column. Fractions (4 ml) were collected, and 0.4-ml samples were assayed for protein by the method of Bradford (5). The arrow marks the beginning of the lactose gradient (0 to 150 mM lactose; total volume, 100 ml) (---). The horizontal bar indicates fractions which were pooled for gel electrophoretic analysis, for gel filtration on Sephadex G-150, and for iodination.

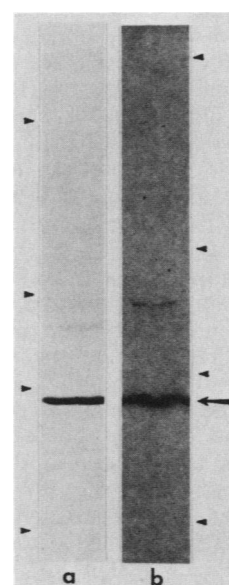


FIG. 5. Polyacrylamide gel electrophoresis of CBP35 purified from the mouse lung. (a) Coomassie brilliant blue-stained gel; (b) immunoblot of nitrocellulose paper with anti-CBP35 (3T3) and ^{125} I-goat anti-rabbit IgG. Lane a is from a 9-cm-long slab gel, and lane b is from an 11-cm-long slab gel. The arrow marks the position of migration of CBP35 from 3T3 cells. Molecular weight markers (▲) are as described for Fig. 1.

TABLE 1. Amino acid compositions of lactose-inhibitable lectins

Amino acid	Amino acid composition of ^a :						
	CBP35 (lung) (35,000)	CBP16 (liver) (16,000)	Rat lung lectin (14,500)	Human lung lectin (14,500)	Calf spleen lectin (12,000)	Chicken heart lectin (13,000)	Electrolectin (16,500)
Aspartic acid	3.4	4.6	9.5	7.5	15.2	11.1	11.8
Threonine	19.4	10.9	5.2	5.0	2.0	5.2	3.5
Serine	9.4	13.8	14.7	15.8	4.9	6.7	6.8
Glutamic acid	7.4	8.0	17.2	15.0	8.7	10.8	14.0
Proline	5.4	4.3	4.3	4.2	4.4	5.6	3.5
Glycine	8.7	11.9	12.9	15.8	8.2	9.1	11.0
Alanine	7.8	13.4	7.8	8.3	9.4	3.9	4.8
Cysteine	ND	ND	0.9	0.8	4.5	2.3	0.0
Valine	5.7	4.6	4.3	5.0	6.6	7.8	5.9
Methionine	ND	ND	0.9	0.8	0.0	1.4	2.1
Isoleucine	5.4	9.6	2.6	3.3	3.4	3.8	7.0
Leucine	5.1	2.6	5.2	5.0	10.2	7.7	3.4
Tyrosine	3.5	0.6	2.6	1.7	1.5	0.8	2.1
Phenylalanine	2.9	1.7	1.7	1.7	7.2	7.5	8.3
Histidine	3.3	2.8	3.5	3.3	1.5	3.4	4.2
Lysine	2.8	6.6	3.5	3.3	5.8	6.6	7.2
Arginine	5.4	4.2	2.6	2.5	3.6	3.2	4.2
Tryptophan	ND	ND	0.9	0.8	2.9	3.1	0.7

^a Expressed as number of residues of amino acid per 100 residues of protein. ND, Not determined. The reference or source for the lectins are: CBP35 (lung), this work; CBP16 (liver), this work; rat and human lung lectins, reference 17; calf spleen and chicken heart lectins, reference 1; electrolectin, reference 14. The values for M_r are within parentheses.

Because these lectins have different molecular weights, ranging from 12,000 to 35,000, the data are expressed in terms of number of residues of a given amino acid per 100 residues of protein. Such a comparison indicates that CBP35 (lung) is quite distinct from the other lectins.

Isolation of CBP16 from the mouse liver. We have also carried out the isolation of lectins from the mouse liver by using identical procedures applied to homogenates of the mouse lung. After two cycles of affinity chromatography on asialofetuin-Sepharose, the material eluted by lactose (Fig. 6, component B) yielded a single Coomassie brilliant blue-stained band on polyacrylamide gels (Fig. 6, inset). The position of migration of this polypeptide corresponded to an M_r of 16,000; this protein is designated CBP16 (liver). The amino acid composition of CBP16 (liver) is listed in Table 1.

Although the procedures used on the liver extracts were identical to those carried out in the purification of CBP35 (lung), no polypeptide corresponding to CBP35 was observed. These results corroborate the immunoblotting data (Fig. 2A), which failed to detect any CBP35 in the adult mouse liver.

In the isolation of CBP16 (liver), we failed to recover the galactose-specific asialoglycoprotein receptor, a well-characterized protein found on the plasma membrane as well as on intracellular membranes of adult mammalian livers (25). Although the reason for this remains to be determined, three differences between the isolation protocols for the asialoglycoprotein receptor and for CBPs should be noted. First, the isolation of CBPs is carried out in the presence of β -mercaptoethanol, which may alter the properties of the asialoglycoprotein receptor. Second, some of the conditions for affinity chromatography in the isolation of the asialoglycoprotein receptor (pH 7.8, 500 mM KCl) and of CBPs (pH 7.1, no KCl) are different. Finally, the detergent Triton X-100 is removed early during the isolation of CBPs, possibly contributing to the precipitation or aggregation of the asialoglycoprotein receptor on the first affinity column.

Iodination of CBPs with retention of carbohydrate-binding activity. We have previously noted that all three fibroblast CBPs are sensitive to oxidation (20). This oxidation is most

probably the cause of loss of binding activities of CBPs upon iodination by several different methods (9, 10). We have found that one method, in which glycoluril is used, does not destroy their activity. Mouse lung CBPs purified by two cycles of affinity chromatography were iodinated by the method of Fraker and Speck (8). The majority of unincorporated ^{125} I was removed by chromatography over Sephadex G-15. CBP35 (lung) was then fractionated from CBP16 (lung), CBP13.5 (lung), and residual ^{125} I by chromatography

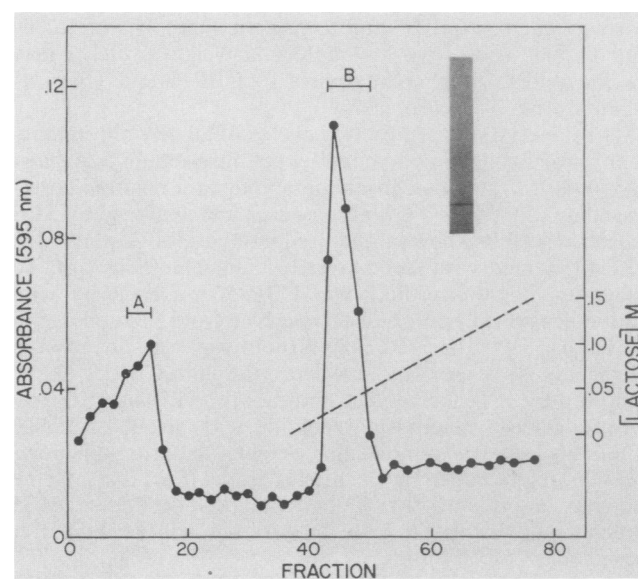


FIG. 6. Representative column profile showing the affinity chromatography of mouse liver extracts on an asialofetuin-Sepharose column (1.2 by 15 cm). Liver extracts from 100 mice were subjected to the isolation protocol described in the text. The conditions for rechromatography were the same as those described in the legend to Fig. 4. The horizontal bars, designated components A and B, indicate fractions which were pooled for gel electrophoretic analysis. The inset shows the Coomassie brilliant blue-stained gel of component B.

over Sephadex G-150 (20). The material which migrated to a position corresponding to a molecular weight of 35,000 was rechromatographed on an asialofetuin-Sepharose column (Fig. 7). More than 90% of the material loaded was bound; this bound material could be eluted with lactose. Polyacrylamide electrophoresis of the material loaded onto this column revealed CBP35 (lung) (Fig. 7, inset).

Binding of CBP35 (lung) to erythrocytes and inhibition by specific saccharides. CBP35 (lung) labeled with ^{125}I bound to trypsinized, glutaraldehyde-fixed rabbit erythrocytes. This binding was inhibited by unlabeled CBP35 (lung); the addition of an equal amount of the unlabeled protein (~ 150 ng) reduced the binding by ca. 45%, which is consistent with the result expected due to the dilution of specific radioactivity. The extent of binding depended on the amount of lectin added, the concentration of erythrocytes, and the presence or absence of the inhibitor, lactose. Therefore, this binding provided an opportunity to explore the specificity of the saccharides in terms of their capacity to inhibit the lectin-erythrocyte interaction. For these studies, the amount of ^{125}I -labeled CBP35 (lung) bound to the erythrocytes saturated at a final erythrocyte concentration of 5% (vol/vol). Further increases in the number of erythrocytes did not increase binding, suggesting that there was an excess of binding sites under these assay conditions. Approximately 48% of the total radioactivity added to the assay was bound by the cells in the absence of any hapten inhibitor.

At a concentration of 0.1 M, all haptens which contained galactose or derivatives of galactose exhibited between 63 and 79% inhibition as compared with controls (Table 2). At this concentration, both α and β anomers as well as a variety of glycosidic linkages of galactose were able to compete for the binding site on CBP35. All other mono- and disaccharides which did not contain galactose showed little or no inhibition.

The effects of galactose and lactose on the binding of CBP35 were analyzed in greater detail. Galactose inhibited the binding at a concentration of 10 mM and above. Half maximal inhibition (50%) was achieved at 32 mM. Lactose was a more potent inhibitor; it showed inhibition at 10 μM and above, with 50% maximal inhibition at 250 μM . We also found that asialofetuin (M_r , 50,000) and asialo α_1 -acid glycoprotein (M_r , 40,000) were even more potent inhibitors; 45 and 42% inhibition were obtained with 10 μM of each protein, respectively. These results suggest that the carbohydrate-binding site on CBP35 (lung) recognizes structural features more complex than the monosaccharide moiety, galactose.

DISCUSSION

We have previously reported the purification, from 3T3 fibroblasts, of three distinct carbohydrate binding proteins: CBP35, CBP16, and CBP13.5. Based on their ability to agglutinate rabbit erythrocytes, to bind asialofetuin (20), and to bind to polyacrylamide beads derivatized with β -galactose(1 $\rightarrow$ 4)-*N*-acetyl- β -glucosamine (19), all in a lactose-inhibitable manner, these proteins appear to be fibroblast lectins.

When extracts of 3T3 cells (100 μg of total protein) were electrophoresed and immunoblotted with a rabbit antiserum directed against CBP35 (3T3), a single polypeptide (M_r , 35,000) was observed. The same single band was obtained when the amount of 3T3 cell protein was subjected to electrophoresis and immunoblotting was varied from 100 to 500 μg . These results, carried out on SDS-denatured proteins, corroborate the results of immunoprecipitation experi-

ments on native proteins (20), which indicated that anti-CBP35 (3T3) recognizes specifically CBP35 but not CBP16 and CBP13.5. We had previously estimated that CBP35, CBP16, and CBP13.5 accounted for $<0.01\%$ of the total protein of 3T3 cells and that these three lectins were isolated in a ratio of 3:1:1 (20). Using these values and the fact that we could easily detect CBP35 in immunoblots containing 100 μg of 3T3 cell extract (e.g., Fig. 1), we estimate the lower limit of detection of CBP35 in the present immunoblotting protocol to be in the range of 5 ng of the polypeptide.

We have also found proteins (M_r , 35,000) cross-reactive with rabbit antibodies directed against CBP35 (3T3) in fibroblasts of human and avian origin. The result obtained with human fibroblasts (SL66) is consistent with our previous isolation of CBP35 from the same cell line (19). Moreover, the cross-reactivities of the antisera with proteins derived from different species suggest that the failure to detect CBP35-like proteins in cultured liver and intestine cells of human origin and in chicken myoblasts and myotubes cannot be due to species-specific reactivity of the antibody raised against CBP35 (3T3).

Of particular interest is the finding that antibodies directed against CBP35 (3T3) revealed an analogous protein in P388D1, a murine lymphoblastoid derivative with macrophage-like properties (11). Therefore, CBP35 can be found in at least two cell types: fibroblasts and lymphoblastoid cells. This is consistent with the tissue distribution of CBP35. The protein is abundant in the lung, artery, thymus and spleen, tissues which are rich in both cell types. It was also present to a lesser extent in other tissues: bone, skin, eye, and cartilage. However, due to the nature of these tissues,

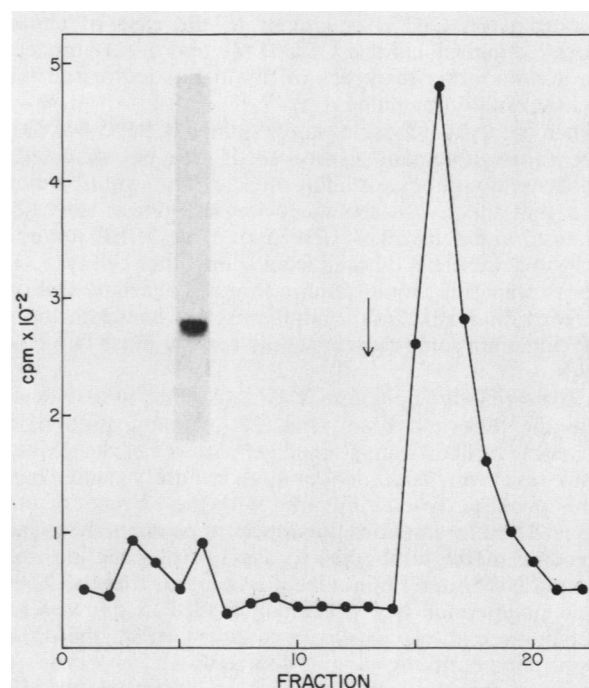


FIG. 7. Chromatography of ^{125}I -labeled CBP35 on asialofetuin-Sepharose (0.75 ml). ^{125}I -labeled CBP35 (0.1 ml, 1,000 cpm) in buffer A was loaded onto an asialofetuin column which was preequilibrated in the same buffer. Fractions (0.5 ml) were collected, and radioactivity was determined by gamma counting. The arrow marks the start of the elution with buffer A containing 0.15 M lactose. The inset shows polyacrylamide gel analysis of CBP35 loaded onto the column.

TABLE 2. Effect of saccharides on the binding of CBP35 (lung) to erythrocytes

Hapten ^a	cpm bound ^b	% Inhibition
None	754	0
Lactose	161	79
N-Acetylglucosamine	164	78
Stachyose	182	76
Methyl- α -D-galactoside	230	70
Methyl- β -D-galactoside	236	69
Galactose	263	65
Melibiose	263	65
N-Acetylgalactosamine	260	65
Galactosamine	277	63
Fucose	597	21
Sucrose	603	20
Mannose	623	17
Cellobiose	632	16
Methyl- α -D-mannoside	633	16
Methyl- α -D-glucoside	658	13
N-Acetylglucosamine	692	8

^a All monosaccharides were of the D configuration except for L-fucose. The final concentrations of haptens in the binding assay were 0.1 M except N-acetylglucosamine which was 6 mM.

^b Each assay received 100 μ l of the radioactive lectin (1.4×10^4 cpm/ μ g; 1.6×10^3 cpm per assay). Values reported represent averages of duplicate determinations.

extracts were difficult to prepare, and therefore these tissues may contain more CBP35 which was not extracted.

There were drastic differences between embryonic and adult tissues when the presence of CBP35 was compared in the liver, muscle, and skin. Whereas a cross-reactive protein was abundant in the embryonic tissues, a similar protein was not found or was hardly detectable in the adult. This observation on CBP35 is similar to the case of chicken lactose lectins, CLL I and CLL II (2), and of rat lung lectin (18), in which the quantities of the lectins expressed were developmentally regulated.

When embryonic muscle was examined, CBP35 was found to be rather abundant. However, it was not detected in myoblasts, myotubes, or adult muscle. This would indicate either that there is a specific event at or near day 17 of gestation which involves CBP35 or that CBP35 found in embryonic muscle is derived from some other cell type. The latter explanation would require that the alternate cell type be greatly diminished in the adult muscle. The localization of CBP35 in embryonic muscle should resolve these two possibilities.

A cross-reactive polypeptide (M_r , 42,000) was also detected in the lung and brain (Fig. 2A). Examination of the Coomassie brilliant blue-stained gel patterns of these tissues did not reveal any additional or more intensely stained bands at this position, when compared with the extracts of other tissues. Therefore, it does not appear to be due to nonspecific binding of the antibodies to a major polypeptide found only in the lung and brain. One possibility is that the 42,000-dalton polypeptide is a precursor of CBP35. However, in other tissue extracts which do contain CBP35, the 42,000-dalton polypeptide was not detected. In any case, the identity of this polypeptide and its relationship to CBP35 remain to be determined.

Since the immunoblotting results demonstrated that CBP35 was most abundant in lung tissue, we have used it as a source for large-scale preparation of the lectin. The purification scheme worked out for the isolation of CBPs from cultured cells (20) was found to be applicable to the isolation of the corresponding proteins from the lung. It is important

to note that all three CBPs (CBP35, CBP16, and CBP13.5) were isolated from the mouse lung by using asialofetuin-Sepharose affinity chromatography. In contrast, when extracts of lungs from calf (7) and rat and human (17) tissues were fractionated by essentially the same method, only lectins with molecular weights of 12,000 to 14,000 were observed.

Several lines of evidence have now been accumulated to indicate that CBP35 is a unique lectin, distinct from CBP16 and the chicken lactose lectins, CLL I and CLL II. First, rabbit anti-CBP35 (3T3) reacts with CBP35 but not with CBP16 and CBP13.5 in precipitation experiments with native proteins and in immunoblotting experiments with SDS-denatured proteins. Conversely, an antiserum directed against CLL I precipitated only CBP16 (20). Second, the distribution of CBP35 in adult mouse tissues was different from the distributions of CBP16 and of CLL I. Whereas CLL I was found in the liver and kidney of adult chickens (2), CBP35 was not detectable in either tissue of the adult mouse. When extracts of adult mouse liver were fractionated by asialofetuin-Sepharose chromatography, only a protein corresponding to CBP16 was isolated. The failure to find CBP35 by affinity chromatography of liver extracts corroborates the immunoblotting results which indicated that no protein cross-reactive with anti-CBP35 (3T3) was observable in the liver extracts. Finally, the amino acid compositions of CBP35 (lung) and CBP16 (liver) were distinct from each other.

The availability of ^{125} I-labeled CBP35 permitted the determination of the effect of sugar haptens on the binding of the lectin to erythrocytes. All terminal galactose-containing haptens inhibited the binding. At the concentration used (0.1 M), the anomeric or glycosidic linkage did not alter their inhibitory activity. All other mono- and disaccharides were noninhibitory. This is in good agreement with our previous reports of the inability of sucrose, mannose, and N-acetylglucosamine to elute the CBPs from the affinity columns (20). Therefore, CBP35 binds specifically to galactose-containing glycoconjugates. The ability of galactose to inhibit the binding of CBP35 was 100-fold less potent than that of lactose in this assay. These results are very similar to the differences seen in the ability of the same sugars to inhibit the binding of the lectins from chicken (3, 16) and bovine (1, 7) tissues to erythrocytes.

The quantity of CBP35 per mouse lung has been estimated in the following manner. The peak fractions from the second asialofetuin column (Fig. 4) contain ~ 140 μ g of protein as determined by the method of Bradford (5), using bovine serum albumin as a standard. By using a ratio of 3:1:1 for CBP35:CBP16:CBP13.5 (as determined for fibroblasts [20]), ca. 84 μ g of CBP35 was isolated from the lungs of 100 mice. Therefore, the lungs of each adult mouse contain ca. 0.84 μ g of CBP35. Such amounts should allow us to carry out structural studies on the polypeptide, to search for its endogenous ligand, and to probe its possible function in the physiology of the cell.

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