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By

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF LECTINS
FROM MAMMALIAN FIBROBLASTS

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Three distinct carbohydrate-binding proteins (CBPs) were purified by affinity chromatography on asialofetuin-Sepharose from 3T3 fibroblasts: CBP35 (M_r = 35,000); CBP16 (M_r = 16,000); and CBP13.5 (M_r = 13,500). These CBPs were similar in several key properties: (a) they showed agglutination activity when assayed with rabbit erythrocytes; (b) they all appear to specifically recognize galactose-containing glycoconjugates; (c) they have low isoelectric points, pIs 4.5-4.7; (d) their binding activities are rapidly lost in the absence of β-mercaptoethanol; (e) the CBPs do not interact with each other and the fractionated proteins can bind to asialofetuin independent of associated polypeptides; and (f) none of the proteins tend to self-associate to form oligomers of identical subunits. CBP16 and CBP13.5 may be the murine counterparts of lactose-specific lectins previously identified in electric eel and in several bovine and avian tissues. In contrast, it appears that CBP35 represents a newly identified protein capable of binding to galactose-containing carbohydrates.

Chemically defined carbohydrates were covalently coupled to polyacrylamide beads. These saccharide-containing beads were used to

demonstrate the carbohydrate-binding capacity of CBP35, CBP16, and CBP13.5, isolated from cultured 3T3 mouse fibroblasts on the basis of their binding to asialofetuin-Sepharose. All three proteins bound to polyacrylamide beads containing the disaccharide DGalβ(1⟶4)βDGlcNAc but not to beads containing the monosaccharide βDGal. We have also purified, in a single step, a carbohydrate-binding protein from extracts of human foreskin fibroblasts using an affinity column of polyacrylamide beads derivatized with DGalβ(1⟶4)βDGlcNAc.

Immunologically cross-reactive proteins of the same molecular weight (M_r = 35,000) were found in lung, thymus, spleen and arteries. Fractionation of extracts of mouse lung on affinty columns of asialofetuin-Sepharose yielded CBP35 (lung). The binding of ^{125}I-labeled CBP35 (lung) to rabbit erythrocytes was quantitated in the presence of various carbohydrates. It was found that only saccharides containing galactose were inhibitors of the binding of CBP35 (lung) to erythrocytes; the disaccharide lactose was 100-fold more potent as an inhibitor than the monosaccharide galactose.

TO MY WIFE AND CHILDREN WHO HAVE MADE MANY SACRIFICES

AND HAVE GIVEN THEIR SUPPORT THROUGHOUT THESE YEARS

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ABBREVIATIONS

CBP	carbohydrate binding protein
CHO	carbohydrate
LE	lysosomal enzyme
M6P	mannose 6-phosphate
CLL I	chicken lactose lectin I
CLL II	chicken lactose lectin II
DMEM	Dulbecco-Modified Eagle's Medium
ASF	asialofetuin
PMSF	phenyl methyl sulfonylfluoride
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
HA	hexanolamine
PA	polyacrylamide
PBS	phosphate buffered saline
Hepes	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
EDTA	(ethylenedinitrilo)-tetraacetic acid
Tris	Tris (hydroxymethyl)aminoethane

INTRODUCTION

This thesis describes the purification and characterization of three carbohydrate binding proteins (CBPs) from Swiss 3T3 fibroblasts. Since relatively little is known about these lectins, I have chosen to review the literature on CBPs, isolated from other sources, which have been studied in more detail. As will become apparent, certain of these CBPs may be related to those isolated from 3T3 cells and, therefore, may serve as paradigm systems for the initial studies concerning the cellular functions of the fibroblast CBPs. However, it will also become apparent that in very few instances are the functions of the CBPs well understood.

We have chosen to search for CBPs in Swiss 3T3 fibroblasts for numerous reasons. The foremost is that this cell line has been extensively characterized in terms of its genetic stability (1), growth control (2), nutrient requirements (3), differentiation (4) and transformation (5) as well as having had many molecular components identified. This allows for the study of the functions of the CBPs in a relatively defined system.

Although a variety of fibroblast cellular events are affected by reagents which alter the cellular state of glycosylation, very little is known about the nature of the molecules which are affected. One class of examples of the effects of reagent that affect glycosylation include the regulation of cell proliferation by uridine

diphospho-2-N-acetyl-glucosamine (83) and by tunicamycin (84) in the 3T3 system. One possible explanation for these effects is that interactions between glycoconjugates and CBPs are prevented. Therefore we have proceeded to purify and characterize CBPs from 3T3 fibroblasts. This will allow us to investigate the importance of glycoconjugate-CBP interactions in fibroblasts.

Chapter I

LITERATURE REVIEW

(I) Carbohydrates as Recognition Markers.

Until recently the function of the CHO moieties of glycoconjugates has not been well understood. Eylar (6) and Melchers (7) proposed that glycosylation is a means by which a protein is targeted for export from the cell. Inconsistent with this idea, however, are the findings that many glycoproteins are found within the cell. Moreover, Blobel (8) has shown that the instructional information for export resides in the polypeptide and is independent of glycosylation.

Winterburn and Phelps (9) proposed that the CHO determines the fate of the protein after it is secreted. Envisaged in their scheme is a direct effect of CHOs on the interaction of the glycoprotein with receptors on the plasmalemma. Indeed, the data supporting this notion are accumulating. Not only do the CHO moieties affect interactions of glycoproteins with receptors on the plasmalemma but they also affect interactions of this class of proteins with receptors residing on intracellular membranes. Many mechanisms by which the CHO can affect these interactions with receptors are possible. However, only those interactions which result directly from the binding of the CHO moiety will be discussed in this thesis.

The heterogeneity of sugar sequence, linkage and conformation makes possible for a large repetoire of encoded "signals". However there appear to be limitations on the structures which are actually found in

nature. This is most probably due to the mechanisms by which the oligosaccharides are constructed (e.g. formation of high mannose oligosaccharides on dolichol phosphate). This limitation may also result directly from the relative abundance of the various oligosaccharides (i.e. there may be many structures which are minor components). It should be noted, however, that the variety of known CHO structures is rapidly increasing and that the potential for a tremendous amount of "signal" information in the CHO structure exists. Thus alteration of some structural feature of the oligosaccharide such as addition or removal of a monosaccharide (sialic acid), change in linkage (1⟶4 vs. 1⟶3), anomeric change (α to β) or chemical modification (phosphorylation) can lead to dramatic effects on the interaction of a glycoprotein with its receptor.

(II) Carbohydrate-Binding Proteins

If the CHO moiety serves as a recognition marker, then the cell must possess a complementary set of molecules which have the inherent ability to specifically recognize and bind distinct structural features of the oligosaccharide chains. These complementary molecules are referred to as carbohydrate binding proteins (CBPs). If a major function of the CHOs on glycoproteins is to serve as "signals" it would be expected that there be many CBPs, each of which recognizes some special feature of the oligosaccharide. Actually very few CBPs relative to the variety of CHO structures have been isolated. Undoubtedly there exists as yet unidentified CBPs. It is also possible that it is not necessary for a cell to possess a large variety of CBPs. Instead the CBP-glycoprotein interactions can be regulated by developmental controls (either

by time dependent expression of the CBP and/or complementary glycoprotein ligand), compartmentalization as well as by many other mechanisms.

Many functions have been attributed to CBPs. Here they are arbitrarily divided into three classes: (a) transport glycoproteins, (b) proteins that organize cellular domains, and (c) proteins that mediate cell recognition and aggregation. In discussing each class of functionally defined CBPs the difficulty of determining the function of a CBP should be considered. This is due, in part, to the difficulty of identifying the in vivo ligand. It is also evident that the function ascribed to a certain CBP is depenent on the assay employed. In the following discussion these pitfalls will be addressed when they are relevant.

(III) Protein Transport: Endocytosis - Asialoglycoprotein Receptor

Pinocytosis of desialylated glycoproteins is mediated by CBPs. The first major breakthrough in this area was the finding that removal of terminal sialic acids on glycoproteins results in their rapid clearance from the circulatory system (10). The liver is the major organ responsible for this clearance (11). Both perfused liver and cultured hepatocytes show this ability to endocytose asialoglycoproteins.

In mammals, a CBP has been shown to bind and to internalize galactose bearing compounds. Direct evidence for the involvement of these CBPs in this clearance are: (i) antibodies directed at the CBP inhibit this clearance (12), (ii) insertion of the CBP into fibroblasts which do not contain it, endows them the ability to endocytose galactose bearing ligands at relatively high rates (13), and (iii) when these

galactose terminating ligands are bound to hepatocytes and crosslinked with a bifunctional reagent it is the CBP to which they are crosslinked (14). These as well as other less direct evidence have firmly established the roles of CBPs in the removal of glycoproteins from the circulatory system.

The CBP responsible for glycoprotein clearing has been purified from a number of mammalian livers. The intact proteins are constructed from polypeptides with apparent molecular weights of 48,000 and 40,000 for rabbit (15), 52,000 for rat (Table I) (16) and 41,000 for human (17). Although isolated from different sources the CBPs share many common characteristics. They are all galactose binding proteins. In aqueous solution they form large aggregates which, in the case of the rabbit CBP, can be dispersed into 260,000 dalton species upon the addition of the detergent, Triton X-100 (18). It is presumed that this 260,000 dalton species is comprised of two large and four small subunits. Based on target analysis from radiation inactivation of isolated rat liver plasma membranes, a molecular weight of 105,000 dalton (two 52,000 dalton subunits) has been assigned to the rat CBP (19). Thus it appears that the active CBPs are oligomers. All these CBPs are glycoproteins and have an absolute requirement for divalent cations for expression of binding activity (20). Avian species possess a similar system with one major exception, the CHO specificity differs (21).

Following binding and endocytosis the asialoglycoprotein is degraded in the lysosomes (22). Initially the ligands are bound to CBPs which appear to be diffusely distributed on the plasmalemma. The ligand-CBP complexes then translocate laterally through the membrane and aggregate in coated pits (23). The coated pits subsequently bud

	HEPATIC	M6P	CLL I	CLL II	LIGATIN	DISCOIDIN I	BINDIN
SOURCE	RAT	HUMAN BOVINE	CHICKEN	CHICKEN	CHICKEN RAT	SLIME MOLD	SEA URCHIN
TISSUE DISTRIBUTION	LIVER	UBIQUITOUS	E-MUSCLE E-NERVOUS A-LIVER	E-LIVER E-KIDNEY A-INTESTINE	RETINA ILEUM	—	SPERM
SPECIFICITY	B-D-GAL	MAN6P	D-GAL	D-GAL	GLCIP	D-GAL	?
SUBUNIT M_r	52K	215K	16K	14K	10K	25K	30.5K
OLIGOMER	DIMER	MONOMER	DIMER	MONOMER	POLYMER	TETRAMER	?
SUBCELLULAR DISTRIBUTION	PM, G, ER	PM, G, ER, LY	VARIABLE	VARIABLE	PM	VARIABLE	ACROSOME
FUNCTION	ENDO-CYTOSIS	PROTEIN TRANSPORT	ORGANIZE GPs	ORGANIZE GPs	ORGANIZE SURFACE	AGGRE-GATION	SPERM-EGG BINDING

Table I: Properties of Lectins Purified from Various Sources.

Abbreviations used: CLL, chicken lactose lectin; E, embryonic; A, adult; PM, plasma membrane; G, golgi; ER, endoplasmic reticulum; LY, lysosome; GP, glycoprotein.

into the cytoplasm and are directed towards the lysosomes. Once internalized the ligand-CBP complexes become associated with smooth membranes termed endocytotic vesicles. The ligands are ultimately delivered to the lysosome where they are degraded to amino acids and monosaccharides. During this delivery the CBP becomes separated from the ligand (24) and is spared from degradation.

A number of elegant experiments have shown that the CBPs originally found at the cell surface can participate in many cycles of endocytosis (25). In the absence of protein synthesis hepatocytes can endocytose a quantity of ligand in great excess of their number of CBPs (26). Furthermore they continue to endocytose these ligands at or near a rate equal to the initial rate even while degradation of the ligand is occuring (27). These results have been interpreted to mean that receptors recycle. However, the CBPs may be segregated into two or more functionally distinct pools, one pool which recycles and others which do not. It has been proposed that the majority of CBPs found on intracelluar membranes do not cycle nor do they participate in endocytosis (28). The mechanism(s) by which these CBPs are segregated and by which they recycle remain to be determined.

Subcellular distribution and binding studies further support the idea that these CBPs are recycled. Of the total mammalian liver, galactose-specific CBP only 5% is found on the plasma membrane. The remaining binding activity resides on internal membranes (43% on microsomes, 32% on golgi and 20% on lysosomes) (29). The CBP is oriented towards the lumen in all the intracellular organelles except the lysosome where it has its binding site oriented outward. This exterior orientation on the lysosome, which would require trans-bilayer

displacement, may explain in part, the mechanism by which the CBP is spared degradation. More recently it has been demonstrated that the CBP and the ligand are separated into different membranous compartments prior to ligand delivery to the lysosome (30). Thus the ligand proceeds to the lysosome, whereas the CBP is free to recycle.

Although the studies demonstrating the involvement of CBPs in the clearance of asialoglycoproteins from the circulatory system have been extensive, there remains the possibility that these CBPs are also involved in the intracellular transport of endogenous glycoproteins. Of major concern in this regard is the assay which is the mainstay of this particular field, i.e. the use of an exogenous ligand. It appears that many plasmalemma receptors deliver their ligands to the lysosome. This list includes those receptors specific for polypeptide hormones, low density lipoprotein, mannose 6-phosphate bearing proteins and α_2-macroglobulin (31). It is possible that those receptors found on the cell surface have one major "artery" into the cell and this "artery" goes through/to the lysosome. Therefore ligands which cannot survive the lysosomal milieu are degraded. An endogenous ligand such as a newly synthesized glycoprotein may never see the lysosome. Instead it could be delivered to an organelle prior to seeing the lysosome or may have an intracellular pathway which excludes the lysosome.

If the CBP is involved in transport/sorting mechanisms it would be expected to be ubiquitous. Reticuloendothelial cells possess a CBP which mediates the endocytosis of glycoconjugates bearing terminal mannose or N-acetylglucosamine residues (32). A man/glcNAc specific CBP has been isolated from rabbit liver (33), although no evidence identifying this CBP as the one involved in endocytosis has been presented.

Recently a CBP which, based on subunit molecular weight and immunological crossreactivity, appears to be identical to that isolated from liver, has been isolated from hepatocytes (34). Whereas reticuloendothelial cells can endocytose man/glcNAc terminating glycoconjugates, hepatocytes can not. Furthermore, the hepatocyte CBP appears to be an intracellular protein and is not an integral membrane protein. If the CBP isolated from whole liver turns out to be responsible for endocytosis then cell specific roles must be postulated. These would have to include the possibility that a given CBP can play different roles, such as involvement in endocytosis when it is expressed on the cell surface and some other function when it is located intracellularly.

(IV) Protein Transport: Intracellular Sorting - Mannose 6-Phosphate Receptor

Glycoprotein synthesis occurs on the rough endoplasmic reticulum where the polypeptides are deposited in the lumen (35). Subsequently the proteins must be segregated and delivered to their respective subcelluar sites. In the case of fibroblast lysosomal enzymes (LEs) it is a CBP which is responsible for this segregation and delivery.

In the classical studies of Neufeld and coworkers (36), to show that a CBP was involved in delivery of LEs, advantage was taken of the wide range of human mutant fibroblasts which are available. These mutants have single mutations which results in the absence of a particular lysosomal enzymatic activity. Early studies demonstrated the ability of these mutant cells to acquire corrective factors (i.e. enzyme replacement) which were supplied to the media of cultured

fibroblasts. This uptake of the lysosomal enzymes was demonstrated to be a receptor mediated transport process (37).

The CHO moieties of the LEs were shown to be essential components for this endocytosis (38). Specifically, mannose 6-phosphate (M6P) residues are bound by a CBP. Their uptake is inhibitable by yeast phosphomannans (39), mannose 6-phosphate and fructose 1-phosphate (37). Furthermore analysis of the LE oligosaccharides revealed high mannose types which are phosphorylated on certain 6-hydroxyls. There appears to be at least five different sites of phosphorylation and the degree of phosphorylation of each oligosaccharide chain is variable (40). The acid hydrolases are phosphorylated by the transfer of N-acetylglucosamine 1-phosphate from UDP-N-acetylglucosamine to the 6-hydroxyls on the high mannose oligosaccharide. The blocking α-N-acetylglucosamine is subsequently removed exposing the mannose 6-phosphate residues (Fig. 1). Both of these enzymatic activities are localized in the cis-golgi and endoplasmic reticulum (41). This is the expected location of these enzymatic activities if the M6P moieties are involved in targeting these enzymes.

The most convincing evidence which suggests that the M6P binding protein serves to sort newly synthesized endogenous LEs comes from studies of fibroblasts from patients with mucolipidosis II disease. These cells do not possess the UDP-N-acetylglucosamine: glycoprotein N-acetylglucosamine 1-phosphotransferase activity (42). This results in LEs which lack phosphates on their oligosaccharides. The consequence of this is that the enzymes are secreted and the cells exhibit a deficiency of LEs in their lysosomes. Thus, at least in fibroblasts, the failure to phosphorylate the oligosaccharides results in the

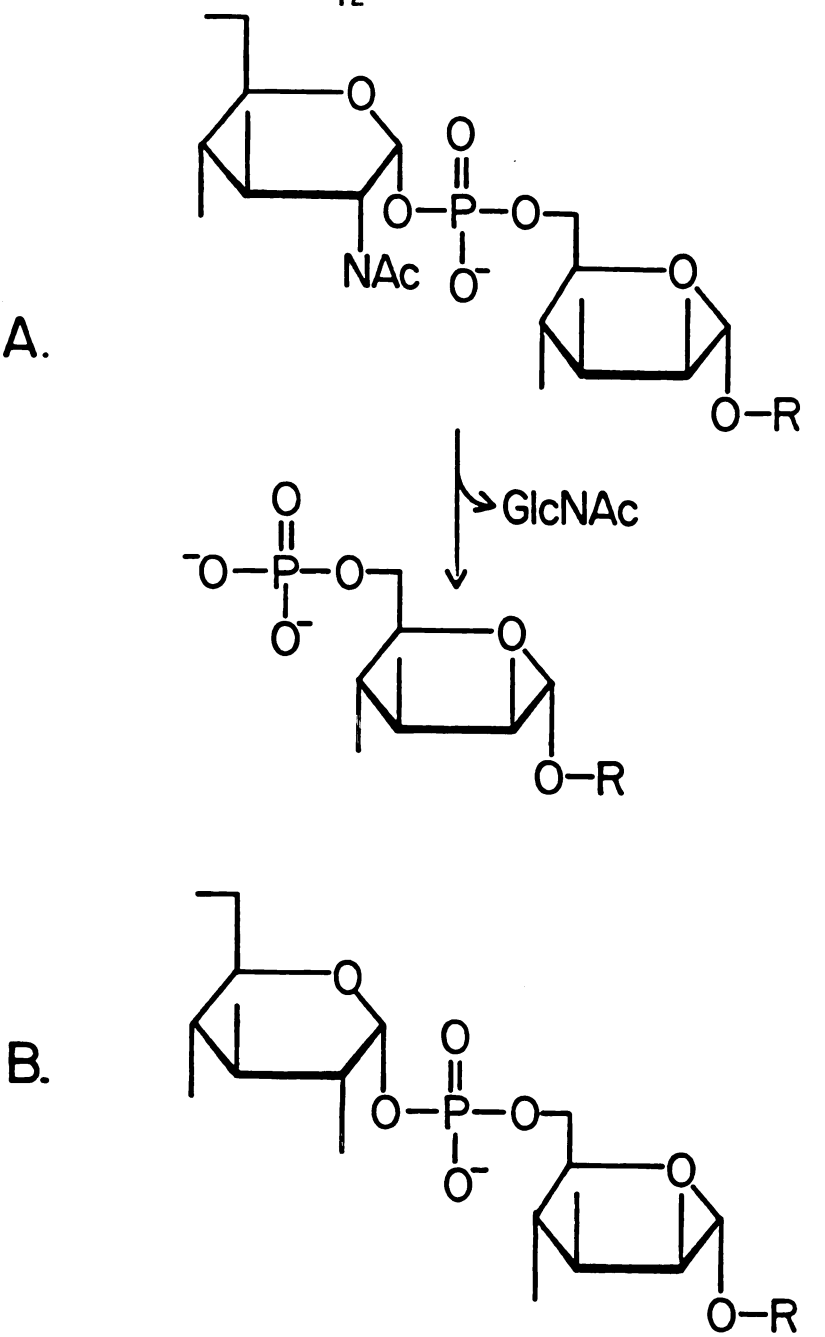

Figure 1: Schematic representation of: (A) the conversion of GlcNAc-covered M6P to uncovered M6P; and (B) the structure of Glc-covered M6P.

diversion of newly synthesied LEs from their normal intracellular pathway.

The M6P receptor has been purified from bovine liver (43), human fibroblasts and Swarm rat chondrosarcoma cells (44) (Table I). The receptor is a glycoprotein with subunit molecular weight of 215,000. The receptor binding does not require divalent cations for CHO binding and this binding is reversed below pH 6. It is an integral membrane protein since it requires detergent for solubilization and can be adsorbed into liposomes.

The distribution as well as the degree of occupancy by LEs of the receptor in rat liver has been determined. Of the total phosphomannosyl enzyme receptors, 90% were found in endoplasmic reticulum, Golgi apparatus, and lysosomes (78%, 7%, 5% respectively) and 10% was found in the plasma membranes (45). Other organelles had negligable binding activity. The receptors appear to be on the luminal surfaces of all the organelles except the plasma membrane where they are oriented outward from the cell. Furthermore, of the total receptors in the endoplasmic reticulum and Golgi more than 80% and 50%, respectively, of the binding sites are occupied. Only 10% of the binding sites in the lysosomes and plasma membranes were so occupied. Thus the receptors are distributed and occupied in a gradient which would suggest their involvement in the delivery of newly synthesized enzymes from the endoplasmic reticulum to the lysosome.

An affinity column covalently derivatized with the M6P receptor has been used to determine the structural requirements for binding to the CHOs (46). Oligosaccharide bearing covered M6P residues (the N-acetylglucosamine has not been removed, Fig. 1), do not bind, those

with one exposed M6P are slightly retarded and oligosaccharides containing two or more exposed M6P residues require the addition of M6P to the buffer to elute them from the column. Thus the receptor binds to exposed M6P moieties and increasing the number of M6P residues results in tighter binding. These results were found to correlate with the ability of human fibroblasts to endocytose these oligosaccharides (47).

Although these studies clearly indicate that oligosaccharides with two or more exposed M6P moieties is optimal for binding and endocytosis, the situation is more complex when considering the binding and uptake of an intact enzyme. Most acid hydrolases are multimeric which results in multiple oligosaccharide moieties. In addition certain of the polypeptides have multiple sites of glycosylation. The effect on uptake of multiple recognition sites on an intact protein can clearly cause synergistic effects such that what may be weak interactions between the receptor and oligosaccharides can be strong interactions between the acid hydrolase and components of the endocytotic mechanism.

The involvement of the M6P receptor in the intracellular transport of LEs may be a specialized function in fibroblasts and other cell types. As noted above patients afflicted with mucolipidosis II disease fail to phosphorylate their LEs and thus cultured fibroblasts from these patients have very deficient levels of intracellular LEs. However it has been found that certain tissues (liver, kidney, brain and spleen) from these patients acquire near normal amounts of lysosomal activities except for β-galactosidase (48). It appears that these tissues contain an alternate mechanism for the targeting of LEs to the lysosome. It is possible that other CHO receptors may be responsible

for their uptake from the circulatory system or that an as yet unknown mechanism is responsible for these tissues ability to target the enzymes to the lysosomes. It may also be possible that the targeting of these enzymes via the M6P receptor to the lysosome in fibroblasts is a consequence of culture conditions. This is unlikely when the clinical aspects of mucolipidosis II disease are considered. In these patients the connective tissues are most severly affected (49). This correlates well with the high content of fibroblasts in these tissues.

The role the fibroblast M6P receptor plays in the delivery of LEs is firmly established. However there is evidence that certain of the ligands are unique, thus providing a very specialized function of the M6P receptor-LE system. Uteroferrin is a protein which is: (i) iron containing, (ii) secreted in large amounts by the porcine uterus during pregnancy, (iii) is absorbed by the developing fetus, (iv) is endocytosed by a mannose receptor mediated event in reticuloendothelial cells, (v) has acid phosphatase activity, (vi) bears glcNAc covered M6P moeities and (vii) is located in intracellular vacuoles resembling lysosomes (50). Therefore uteroferrin appears to be an overproduced LE which has both acid phosphatase and iron transport activity, resulting in a LE with specialized functions.

It has also been shown that M6P, fructose 1-phosphate and fructose 6-phosphate inhibit <u>in vitro</u> human natural cell-mediated cytoxicity (51). Although no molecular evidence has been presented it is suggested that this effect may be due to either the inhibition of binding of the effector cell to the target cell or inhibition of binding of a cytolytic effector molecule to the target cell. If it is a cytolytic factor there must be specific mechanisms such that the natural killer

cells are not exposed to the factors activity which would result in suicide.

When mouse fibroblasts are either virally transformed or treated with growth factors the major excreted protein (MEP) is a 35,000 dalton glycoprotein (52). MEP contains M6P moeities and binds to immobilized M6P receptor in an M6P inhibitable manner (53). However the phosphoglycoprotein is unique in that it is not endocytosed by cells. The paradox that it binds to the receptor but is not internalized remains to be clarified. The possibility that MEP is a hydrolase, localized to the plasma membrane by the M6P receptor, and is involved in the maintenance of the transformed phenotype is an intriguing one.

There is recent evidence which supports the general idea that the M6P receptor may serve to localize certain hydrolases at the cell surface (54). When human fibroblasts are cultured in the presence of M6P, there is a decrease in the amount of glycosaminoglycans released into the media as compared to fibroblasts grown in its absence. This is accompanied by an increase in cell surface glycosaminoglycans in cultures grown in the presence of M6P (55). Furthermore there are distinct molecular differences between the glycosaminoglycans isolated from M6P treated cultures and control cultures. Based on specificity, dose response and cell specificity it was concluded that this effect was due to the dissociation of LEs from cell surface M6P receptors. It was proposed that M6P receptors serve to anchor LEs proximate to specific substrates, such as glycosaminoglycans, so that they can cleave them.

(V) Organization of Domains-Ligatin

Just as a cell is divided into regions of metabolic activity so are these organelles further subdivided into specific domains. These domains consist of the segregation of macromolecules to a specific region of a membrane. An involvement of CBPs in the formation of these domains has been suggested by a number of laboratories.

Ligatin (Table I) is a protein which forms regular arrays of 4.5 nm filaments on suckling rat intestinal mucosa (56) and on the cell surface of chick neural retina (57), mouse macrophages, sea urchin sperm and human fibroblasts (58). The protein, with associated lipids, is released from the cell surface by treatment with 30 mM calcium or by alkaline pH. When the calcium is removed these filaments dissociate into monomers of M_r = 10,000. Readdition of calcium causes the monomers to polymerize back to filaments of 3 nm diameter. This difference in diameter is thought to result from the absence of associated polypeptides in the reconstituted filaments. This suggests that ligatin serves as a baseplate at the cell surface for the attachment of other proteins.

One protein which is associated with neonatal rat ileum ligatin is N-acetyl-β-D-glucosaminidase. This protein is bound to ligatin via glucose 1-phosphate residues. Whereas the M6P receptor does not bind to covered M6P residues, ligatin preferentially binds to M6P residues covered by glucose in a phosphodiester linkage (Fig. 1). Recently an enzymatic activity which transfers glucose 1-phosphate to high mannose oligosaccharides has been identified in embryonic chick neural retina (59).

Additional evidence is accumulating which supports the idea that ligatin serves to localize certain glycoproteins to the plasmalemma. When ligatin is extracted from cerebrum membranes, acetylcholinesterase is cosolubilized. This acetylcholinesterase binds to ligatin affinity columns and can be eluted with either M6P or glucose 1-phosphate (60). Marchase's laboratory has found that washing neural retina cells with glucose 1-phosphate releases a glycoprotein of 48,000 daltons (Marchase, personal communication). Although this glycoprotein has not been identified it may be the protein cognin (50,000 daltons) which has been shown to promote the aggregation of neural retina cells (61). Consistent with this possible localization of cognin by ligatin is the finding that addition of ligatin to dispersed neural retina cells inhibits their aggregation (62). This may possibly be due to the binding of cognin molecules, thus removing them from the surfaces of cells. It has also been reported that neural retina cells release glycoprotein complexes, termed adherons, into the culture medium (63). Adherons have been shown to increase cell-cell aggregation and cell-substrate adhesion. Recently Marchase's group has found that these adherons contain glucose 1-phosphate bearing components (Marchase, personal communication). When all the preliminary data are combined, a role for ligatin in the localization of cell-cell adhesion factors in neural retina cells is suggested.

(VI) Organization of domains - Low Molecular Weight Galactose Specific Lectins.

There is a group of lectins whose functions have been very elusive. Teichberg et al. first identified galactose specific agglutination

activity in a number of tissues from rat, chicken, eel and mouse (64). They subsequently purified a galactose specific lectin from eel and others have isolated similar lectins from bovine (65) and chicken (66) tissues. These lectins have many common properties (Table 1): (I) have subunit molecular weights of 10,000- 16,000 daltons, (ii) are sensitive to air oxidation, (iii) do not require divalent cations for binding, (iv) are extractable from disrupted cells in the absence of detergent, (v) have low isoelectric points of 3-5, ard (vi) most appear to form dimers in aqueous solution. Although these lectins have been studied in a number of systems their functions are not known. Since the most recent studies implicate their involvement in the organization of domains they have been included in this section. However a number of other functions have been proposed.

Chicken-lactose-lectin I (CLL I) is a 32,000 dalton protein comprised of two identical 16,000 dalton monomers (Table I). It is found in embryonic muscle and is developmentally regulated. The activity increases in early developmental stages and has maximal activity at a time which coincides with the fusion of myoblasts to form myotubes. The activity subsides at later times and in adult muscle is quite low (67). This temporal correlation of activity with morphological events spurred investigations into the involvement of this lectin in the fusion process. Inclusion of either thiodigalactose (68) or CLL I (69) in cultures of myoblasts has been reported to inhibit myoblast fusion. These results suggest that CLL I is involved in the fusion process. However these results are subject to debate since a series of reports (70) which claim that neither thiodigalactose nor CLL I has an effect on fusion, have appeared.

Recent reports have suggested a role for CLL I in the organization of glycoconjugates during the formation of T-tubules (71). In myoblasts the lectin is predominatly intracellular. The lectin is concentrated in longitudinal lines and perpendicular spokes radiating from these lines as myoblasts fuse and begin synthesis of contractile proteins. This pattern is similar to that observed with T-tubules. Later in development as the sarcoplasmic reticulum fuse with the plasma membrane, the lectin becomes localized extracellularly. Thus, it appears that CLL I may serve to organize glycoconjugates which are involved in the formation of T-tubules during differentiation.

A similar involvement of lectins in the organization of glycoconjugates has been proposed for chicken-lactose-lectin II (CLL II), a monomeric protein of M_r = 14,000 (66). CLL II (Table I) is very abundant in intestine and is located in secretory vesicles of mucin-secreting goblet cells (72). It is also located on the lumenal surfaces of the epithelial cells which line the intestine. It is presumed that the lectin found on the mucosal surface is bound to either multiple mucins or crosslinks mucin to glycoproteins on the intestinal epithelial surface. Furthermore, it was demonstrated that CLL II is secreted in conjunction with mucin. The findings are similar to those concerning CLL I and suggest that lectins in animal tissue may play an important role in the organization of glycoconjugates.

The identification of the functions of CLL I and CLL II are complicated by their tissue distribution. Extracts of many embryonic and adult chicken tissues contain substantial amounts of both CLL I and CLL II (73). Both lectins show striking changes in concentration at different stages of development. Whereas CLL I concentration is greatly

decreased in adult muscle as compared to embryonic muscle it is greatly increased in adult liver when compared to the embryonic liver. CLL II is very abundant in embryonic kidney but is present at less than one tenth that level in the adult organ. Conversely CLL II increases 30 fold in the intestine in the adult as compared to the 15 day embryo. These results suggest multiple functions for these CBPs. Furthermore the functions may be specific to the cell types in which they are found.

The involvement of a galactose specific lectin (M_r = 13,000) has been implicated in erythroid development (74). In adult mammalian bone marrow developing erythroblasts are clustered closely together in "erythroblastic islands" around a central macrophage nurse cell. It is proposed that the lectin is involved in the bridging of these erythroblasts to form these clusters. Consistent with this notion is the finding that the lectin is found on the cell surface of the erythroblasts but not on cells which have undergone further differentiation.

It has been found that as these cells mature their susceptability to agglutination by this lectin decreases, indicating a decrease in the amount of cell surface galactose terminating glycoconjugates. Indeed the levels of galactose terminating glycoconjugates has been shown to change as a function of development (75). Not only is there a quantitative change but there also appears to be an organizational change. That is, there are patches or domains of these galactose bearing compounds on the cell surface. This raises the possibility that the lectin may be involved in the formation of these microdomains rather than bridging molecules between two adjacent cells.

The major drawback in these studies is the lack of information concerning their natural ligand(s). As previously noted, this is a difficulty not easily overcome. Since many glycoconjugates contain galactose moeities, it is impossible to identify the in situ ligand solely on the basis of their binding to the lectins. Thus an important breakthrough in this field will be the establishment of procedures to identify the in situ ligand.

(VII) Cell Aggregation: Slime Molds -Discoidin

Certain homotypic and heterotypic cell aggregation processes have been implied to be mediated by lectins. This type of activity was originally identified in the symbiotic interactions of nitrogen fixing bacteria with legumes (for review see Ref. 76). It is generally accepted that these species specific lectins are responsible for the initial recognition and binding events between the bacterium and the root hairs. Thus the lectins confer the specificity seen in these processes. These studies have prompted investigations into the possibility that lectins are involved in the cell-cell aggregation/ recognition processes observed in other organisms.

The most convincing results concern the aggregation of the slime molds. When supplied with adequate nutrients the slime molds exist as independent amoebae. Upon starvation the cells display a mutual cohesiveness and aggregate into multicelluar pseudoplasmodia (slugs). Further morphogenetic events result in the formation of mature fruiting bodies. Lectins have been shown to mediate the initial aggregation process.

This event is species specific in that if two or more species of slime molds in the amoeboid stage of their life cycle, are mixed prior to starvation, they seek out members of their own species to form slugs of a homogenous cell type upon removal of nutrients. Therefore if lectins are the molecules responsible for this species specificity the different slime molds should have distinct lectins. To date identical lectins have not been identified to be present in more than one species of slime mold. Moreover different species appear to possess unique lectins.

It has been shown that in Dictyostelium discoideum an endogenous lectin, discoidin I (Table I), is absolutely required for these cells to form aggregates. The lectin is a tetrameric protein consisting of subunits of M_r = 25,000 (76). The lectin binds to terminal and possibly internal galactose residues. When the slime mold is in the vegatative state (single cell) the lectin is virtually absent. In the aggregating amoebae it may comprise more than 1% of the total cellular protein (78) and this increase is under transcriptional control. The initial increase in lectin activity precedes cell cohesiveness by approximately two hours and has its most rapid increase at a time coincidental with the rapid increase in the aggregation of the amoebae (79).

The lectin has been demonstrated to reside at the cell surface by a number of procedures: (i) it can be labeled with ^{125}I by cell surface labeling procedures, (ii) is immunoreactive with fluorescent antibodies when intact cells are used, and (iii) aggregating cells agglutinate erythrocytes in a galactose inhibitable fashion; however nonaggregating cells do not agglutinate erythrocytes. Although the

lectin is a cell surface protein it is not an integral protein since it can be extracted from disrupted cells in the absence of detergents.

Of the total cellular lectin in aggregating cells only about 2% (i.e. 1×10^5 molecules per cell) is detectable on the cell surface; the remainder is intracellular. It has been shown that the intracellular lectin can be elicited to the cell surface. When monovalent Fab fragments against the lectin was used to quantitate surface lectin less than 1×10^5 molecules per cell were detected. However if divalent antibodies were used then at least 1×10^6 molecules per cell were seen. Polyvalent glycoproteins and conconavalin A can also elicit the appearance of additional cell surface lectin. Although the relevance of this elicitation to the in vivo situation is still somewhat obscure, there does appear to be a possible correlation between the induced movement of the lectin to the cell surface and the progressive externalization of the lectin during differentiation. Towards later stages of aggregation, the lectin is no longer detectable within the cells, but is confined to the aggregate's periphery. It finally becomes associated with the amorphous material associated with the surface of the slug as well as with material which is shed (80).

The most convincing evidence suggesting an involvement of lectins in this aggregation process comes from work with mutants. Mutants which have discoidins containing defective binding sites fail to aggregate and differentiate past this step (81). The mutant lectin is synthesized upon starvation and is localized to the correct subcellular site. However it fails to agglutinate erythrocytes. Revertants of this mutant acquire aggregation competence. It is concluded from these

studies that the lectin plays an essential part in this differentiation process.

Whether the lectin serves to bridge molecules between adjacent cells or aggregates glycoconjugates into a domain on a single cell is unknown. It is most likely that there is a sequence of events which lead to interactions which provide for the avidity required to maintain these tight cell-cell contacts. A number of glycoproteins have been suggested to be involved in this formation of cell-cell contacts, but the ligand-receptor relationship of these glycoproteins and lectin remain to be determined.

(VIII) Cell Aggregation: Sperm Egg Interaction-Bindin

The process of fertilization requires that sperm bind to the egg's surface. It has been proposed that a lectin mediates this initial binding event. A lectin, bindin, (M_r = 30,500) has been isolated from the acrosomal processes of sperm from the sea urchin, Strongylocentrotus purpuratus (82). Bindin agglutinates eggs in a species specific manner and this agglutination is inhibitable by mild periodate oxidation of the egg as well as by glycopeptides derived from proteolyzed vitelline layers. As the sperm binds to the egg the lectin is localized to the point of attachment on both the acrosomal process and the vitelline layer adjacent to this site of attachment. Although there is no direct evidence as to the function of this lectin it appears that it is involved in the fertilization process.

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Chapter II

ENDOGENOUS LECTINS FROM CULTURED CELLS

I. Isolation and characterization of carbohydrate - binding proteins from 3T3 fibroblasts*

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Running Title: Carbohydrate-Binding Proteins from 3T3 Cells

SUMMARY

Extracts of cultured 3T3 fibroblasts, obtained by homogenization and Triton X-100 solubilization, were fractionated on Sepharose columns covalently derivatized with asialofetuin. Three distinct carboyhdrate-binding proteins (CBPs) were purified from the material bound to the affinity column: CBP35 (M_r=35,000), CBP16 (M_r=16,000); and CBP13.5 (M_r=13,500). These CBPs were similar in several key properties: (a) they showed agglutination activity when assayed with rabbit erythrocytes; (b) they all appear to specifically recognize galactose-containing glycoconjugates; (c) they have low isoelectric points, pIs 4.5-4.7; (d) their binding activities are rapidly lost in the absence of β-mercaptoethanol; (e) the CBPs do not interact with each other and the fractionated proteins can bind to asialofetuin independent of associated polypeptides; and (f) none of the proteins tend to self-associate to form oligomers of identical subunits. Comparisons of these and other properties of the CBPs suggest that CBP16 and CBP13.5 may be the murine counterparts of lactose-specific lectins previously identified in electric eel and in several bovine and avian tissues. In contrast, it appears that CBP35 represents a newly identified protein capable of binding to galactose-containing carbohydrates.

The purification of carbohydrate-binding proteins (CBPs) , including lectins and enzymes such as glycosyl transferases and glycosidases, has made much use of the powerful technique of affinity chromatography. Because of the similarity of saccharide structures found on various serum glycoproteins such as fetuin and those found on the cell surface (1-3), CBPs can potentially recognize similar monosaccharide units, oligosaccharide structures, or the entire carbohydrate complex on glycoproteins and on cell surface heterosaccharides. This suggests that affinity columns containing Sepharose covalently coupled to a glycoprotein such as fetuin might be used for the isolation of CBPs. Indeed, this approach has been successfully applied in the purification of the hemagglutinin receptor of influenza virus (4), carbohydrate-specific antibodies (5), as well as lectins from both plant (6) and animal (7,8) sources.

We have undertaken a study of CBPs from an established tissue culture cell line, Swiss 3T3 fibroblasts. This cell line has well-defined growth and morphological characteristics (9, 10). Because it is thought to be derived originally from mouse embryo fibroblasts, 3T3 cells presumably represent cells of a rather ubiquitous distribution. In the present communication, we report the purification and characterization of three distinct CBPs, all of which were isolated on the basis of their binding to asialofetuin (ASF) - Sepharose and subsequent elution with the disaccharide, lactose. The CBPs exhibited agglutination activity for rabbit erythrocytes and therefore, are probably fibroblast lectins.

EXPERIMENTAL PROCEDURES

Materials - Swiss 3T3 cells were obtained from American Type Culture Collection (CCL92). Dulbecco modified Eagle's medium (DMEM) was from K.C. Biologicals, calf serum from M.A. Bioproducts and fetuin from Gibco. All carbohydrates, cyanogen bromide and phenyl methyl sulfonylfluoride (PMSF) were products of Sigma, Aquacide III of Calbiochem, and Sepharose 4B and Sephadex G-150 of Pharmacia. [^{35}S]Methionine (1012 C_i/mmol) was bought from New England Nuclear. Ampholines were purchased from LKB.

Culture and Radiolabeling of 3T3 Cells - Maintenance of Swiss 3T3 cells has been described elsewhere (11). Swiss 3T3 cells were grown to confluent monolayers (4-5 x 10^4 cells/cm^2). The medium was replaced with fresh growth medium for 24h. This medium was removed and the cells were cultured in serum-free DMEM (10 ml/150 cm^2 growth area) containing 3 µg/ml unlabeled methionine (one-tenth of the concentration normally found in DMEM) and 20 uC_i/ml [^{35}S]methionine (12). After 24 h, the medium was removed and the cells were washed prior to the extraction and isolation of the proteins.

Preparation of Asialofetuin - Sepharose - Fetuin was desialylated as described by De Waard et al. (13) and coupled to Sepharose 4B by the method of Cuatrecasas (14). Fetuin (500 mg) dissolved in 25 ml H_2O (pH 2.0) was heated at 80° for 1h. The solution was then cooled to

25°, neutralized with NaOH, and dialyzed against 0.2 M $NaHCO_3$, pH 7.9. The ASF was coupled to 150 ml of CNBr (20 g) activated Sepharose 4B in a combined volume of 300 ml of 0.2 M $NaHCO_3$, pH 7.9. After 24 h at 4°, 150 ml of 2 M ethanolamine, pH 8.0, was added for an additional 24 h. The resin was washed extensively with 1 M NaCl and then washed with Buffer A (see below). Routinely, greater than 80% of the ASF was coupled as determined by the difference in absorbance at 280 nm of the ASF solution before and after the coupling reaction.

Isolation of Asialofetuin Binding Proteins - The purification of ASF-binding proteins used the following buffers: Buffer A - 50 mM $CaCl_2$, 1 mM NaN_3, 75 mM Tris(hydroxymethyl)aminomethane, pH 7.2; Buffer B - Buffer A supplemented with 1% Triton X-100 and 1 mM PMSF; and Buffer C - Buffer A containing 2 mM β-mercaptoethanol.

Extracts of 3T3 cells were prepared from [^{35}S]methionine-labeled monolayers (in 150 cm^2 flasks) by decanting the labeling medium and washing with 15 ml of Buffer A containing 1 mM PMSF. The cells in each flask were scraped with a rubber policeman into 2 ml Buffer B. The pooled cellular material was homogenized in a 2 ml Potter homogenizer (102-152 um clearance) at five strokes/2 ml. Insoluble material was pelleted by centrifugation at 3,000 x g for 15 min. and then the supernatant was cooled to 4°. All previous operations were performed at 25° and subsequent steps at 4°.

The supernatant was applied to an ASF-Sepharose column (1.4 x 15 cm) and the column was washed extensively with Buffer B containing 2 mM β - mercaptoethanol. To remove detergent, the column was washed with 2-4 column volumes of Buffer C. Protein bound on the ASF-Sepharose column was eluted with a 0-0.15 M lactose gradient (100 ml total

volume). Aliquots from the column effluent were assayed for radioactivity due to [^{35}S]methionine-labeled proteins using scintillation counting (12).

The material eluted by lactose was pooled and dialyzed against Buffer C in tubing impermeable to molecules of molecular weight greater than 3,500. The dialysis tubing also contained ASF-Sepharose. The dialyzed contents were poured into a column and the resin was allowed to settle before washing with Buffer C. The bound proteins were eluted with a lactose gradient as described above.

The material eluted with lactose from the second affinity column was concentrated by reverse dialysis against Aquacide III to a final volume of 1-2 ml. This concentrate was chromatographed on a column (1 x 150 cm) of Sephadex G-150. Fractions from the Sephadex G-150 column were pooled and tested for their ability to rebind to another ASF-Sepharose column.

Gel Electrophoretic Characterization of CBPs - Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) was performed according to the procedure of Laemmli (15) on a 1 mm thick, 9 cm long, 5-16% gradient slab gel (.21-.67% bisacrylamide) with a 1 cm long 4% stacking gel. Samples were prepared by dialysis against water followed by lyophilization. They were dissolved in 1% SDS, 4% β-mercaptoethanol and boiled for 1 minute. 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 as described by Bonner and Laskey (16), using Kodak X-Omat AR (XAR-5) film.

Two-dimensional gel electrophoretic analysis was performed according to the method of O'Farrell (17). Samples were first subjected to isoelectric focusing in 1 mm X 10 cm tube gels containing pH 3-10 ampholines. The second dimension was electrophoresed on 5-16% polyacrylamide slabs as described above.

Assays of Agglutination and Enzymatic Activities

Fresh rabbit erythrocytes were isolated following the method of Lis and Sharon (18) and trypsin-treated, glutaraldehyde-fixed rabbit erythrocytes were prepared by the method of Nowak et al. (19). The cells were used as a 4% stock suspension in 0.9% NaCl containing 0.3% bovine serum albumin (pH 7.4). Hemagglutination assays were carried out in microtiter V-plates; each well contained 25 µl of erythrocyte suspension and 25 µl of the test sample. To study the effects of saccharides on hemagglutination, 10 µl of a stock solution of saccharide in 0.9% NaCl was added; control wells received 10 µl of 0.9% NaCl. In addition, the effect of the various saccharides on the erythrocytes were tested in the absence of any agglutinin sample. All agglutination assays were scored after 1 h at room temperature.

β-Galactosidase activity was determined by the method of Bishop and Desnick (20). To 150 µl of 1.5 mM 4-methylumbelliferyl-β-D-galacto-pyranoside (Pierce Chemical) in 0.03 M citrate, 0.05 M phosphate, pH 4.6, was added 50 µl of the test sample. After incubation for 1 h at 37°, the reaction was terminated by the addition of 2.4 ml of 0.1 M ethylenediamine. Fluorescence was monitored on a Perkin-Elmer 650-40 fluorimeter using excitation and emission wavelenghts of 360 nm and 440 nm, respectively. β-Galactosidase activity was also determined at

neutral pH using 1.5 mM 4-methylumbelliferyl-β-D-galactopyanoside in Buffer B.

Sialyl transferase activity was determined by the method described by Bosmann (21) using ASF as a potential acceptor. To a solution of 10 mM $MgCl_2$, 10 mM $MnCl_2$, 400 µg ASF and 8.6×10^4 dpm of cytidine 5'-monophospho-N-acetyl-[4,5,6,7,8,9-^{14}C]-neuraminic acid (Amersham, 247 mCi/mmol) in 100 µl of Buffer B was added 50 µl of test sample. After 4 h at 37°C, an aliquot was removed and 5 volumes of cold 1% phosphotungstic acid in 0.5 N HCl was added. The mixture was centrifuged and the precipitate was washed twice with 1% phosphotungstic acid in 0.5 N HCl, resuspended in 0.5 ml H_2O and neutralized with 1 N NaOH. The radioactivity was then determined by scintillation counting. Alternatively, the reaction mixture was chromatographed on columns (100 x 1.5 cm) of Sephadex G-25 and the effluent fractions were monitored for radioactivity. The test sample has also been assayed for transferase activity in the presence of 2 mM unlabeled CMP-sialic acid.

Preparation of antisera and Immunoprecipitation

Antisera directed against CBP 35 were raised in New Zealand White female rabbits. CBP35 isolated from 17 flasks (150 cm of confluent 3T3 fibroblasts) was mixed in 200 µl complete Freunds adjuvant and injected near the popliteal lymph node (~100 µl/node) of an etherized rabbit. After 10 days, the rabbit was injected at the same site with CBP35 (isolated from 10 flasks of confluent 3T3s) which was suspended in incomplete Freunds adjuvant. The rabbit was bled 10 days after the second immunization. Subsequent bleedings were also made 10 days after boosting the rabbit. Antiserum against chicken lactose lectin I (CLL

I) was prepared by injecting CLL I (M_r = 16,000) isolated from female adult chicken liver into a rabbit (22). It was a gift of Dr. Steven Ullrich (Michigan Molecular Institute, Midland, MI).

Material used for the immunoprecipitation was ^{35}S-methionine labeled 3T3 cell extract partially purified by affinity chromatography over one ASFSepharose column. The material was concentrated by reverse dialysis against Aquacide III to a volume of approximately 2 ml. The concentrated material was split into 4 aliquots, 450 µl per tube, placed on ice for 1 hour, after which it was centrifuged for 15 minutes at 12,000 x g. The supernatants were transferred to new tubes and 5 µl of antisera was added. After incubation at 37°C for 1 hour, they were incubated at 4°C for 8-12 hours. Then, 150 µl of goat anti-rabbit IgG serum (Gibco) was added to each sample and they were placed at 4° for an additional 14 h.

The precipitates were pelleted by centrifugation at 10,000 x g for 15 min. The supernatant was discarded and the pellet was washed twice in .05 M TrisHCl, 1.2 M KCl, 1% (v/v) Triton X-100, pH 7.4, followed by two washings in .05 M Tris-HCl, .1 M NaCl, pH 7.4, and finally with water. The pellet was dissolved in 100 µl of buffer for polyacrylamide gel electrophoresis, and boiled for 2 minutes prior to electrophoresis on 10% polyacrylamide gels.

RESULTS

Asialofetuin-Binding Proteins from 3T3 Cells - 3T3 fibroblasts were cultured in the presence of [^{35}S]methionine to label the cellular proteins. After washing, confluent monolayers of these labeled cells were extracted with Triton X-100 and fractionated by affinity chromatography on a column of ASF-Sepharose (Fig. 1). The majority of the radioactive material was not bound by the column (Component A, Fig. 1). After extensive washing in buffer containing Triton X-100, the column was further developed with detergent free buffer (position of arrow 1, Fig. 1). Finally, the column was eluted with a linear gradient of lactose (position of arrow 2, Fig. 1), which resulted in the appearance of a peak of radioactivity (Component C, Fig. 1). The radioactivity in Component C (Fig. 1) accounted for < .01% of the total radioactivity applied to the affinity column.

Polyacrylamide gel electrophoretic analysis in SDS was carried out on Component C (Fig. 1), as well as on ^{35}S-labeled material from pooled fractions immediately before (Component B, Fig. 1) and immediately after (Component D, Fig. 1) the radioactive peak. Component B (Fig. 1) yielded a heterogeneous mixture of polypeptides on SDS gel analysis (lane b, Fig. 2). In contrast, Component C (Fig. 1) yielded three predominant bands, corresponding to molecular weights of 35,000, 16,000, and 13,500 (lane c, Fig. 2). Several other bands were noticeable; they corresponded to molecular weights of 30,000, 20,000, 11,000

Figure 1. Affinity chromatography of [^{35}S]methionine-labeled, Triton X-100 solubilized, extracts of 3T3 fibroblasts on a column (1.4 x 15 cm) of asialofetuin-Sepharose. The column was equilibrated with Buffer B and was eluted as described in Materials and Methods. The arrows mark the fractions at which the buffers were changed: 1, buffer C; and 2, 0-0.15 M lactose gradient (100 ml total volume). The gradient is marked (---) so as to indicate the concentration of lactose at the top of the resin bed. Fractions (2.5 ml) were collected and aliquots (0.2 ml) were assayed for radioactivity.

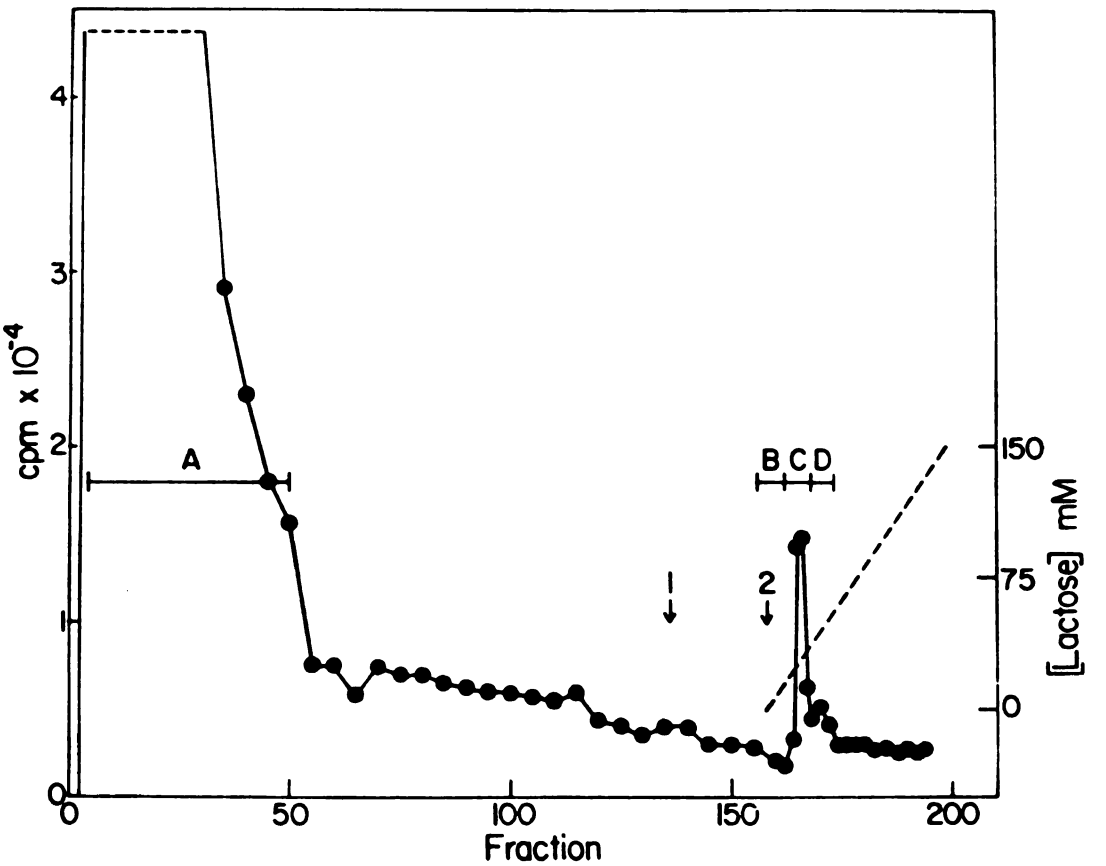

cpm x 10^{-4}
4
3
2
1
0
A
B C D
2
150
75
0
[Lactose] mM
0
50
100
150
200
Fraction

Figure 1

Figure 2. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of fractions derived from affinity chromatography of [^{35}S]-methionine-labeled extracts of 3T3 cells on asialofetuin (ASF)-Sepharose columns. Effluent fractions from the first ASF-Sepharose column: lane a, component A of Fig. 1; lane b, component B of Fig. 1; lane c, component C of Fig. 1; and lane d, component D of Fig. 1. Effluents from rechromatography of component C (Fig. 1) on a second ASF-Sepharose column: lane e, component A of Fig. 3; and lane f, component B of Fig. 3. Lane g consists of material corresponding to Component B of Fig. 3 and shows the M_r 34,000 polypeptide which occurs in some preparations. Radioactivity in the samples: lane a, 40,000 cpm; lanes b-f, 10,000 cpm; and lane g, 25,000 cpm. The fluorogram was exposed at -70° for 72 hours.


220
68
40
12,5
a
b
c
d
e
f
g

and <10,000. Finally, Component D (Fig. 1), which consisted of material eluted as a shoulder of the main radioactive peak, yielded a gel pattern that contained at least four prominent bands (M_rs of 100,000, 36,000, 16,000 and 11,000), as well as many minor contaminants.

Component C (Fig. 1) can be further purified by another cycle of affinity chromatography. The pooled material was dialyzed to remove the lactose. Two important requirements were noted concerning the dialysis: (a) the presence of β-mercaptoethanol (2 mM) preserved the ASF-binding capacity of the proteins; in its absence, the binding activity of the preparation was completely lost; (b) the inclusion of the affinity matrix (ASF-Sepharose) in the dialysis bag prevented the nonspecific absorption of ^{35}S-labeled proteins to the tubing and therefore, increased the recovery of material after dialysis. After dialysis, the contents of the bag was packed directly into a column and washed (Fig. 3). Approximately 20% of the dialyzed material did not bind to ASF-Sepharose (Component A, Fig. 3). The bound material (Component B, Fig. 3) was eluted with lactose at a position similar to that found in the first affinity column (20 mM lactose).

Molecular Weights and Isoelectric Points of Asialofetuin-Binding Proteins- Polyacrylamide gel electrophoretic analysis in SDS of Component B (Fig. 3) yielded three polypeptide bands, with molecular weights of 35,000, 16,000, and 13,500 (lane f, Fig. 2). The material corresponding to these three bands will be referred to hereafter as CBP35, CBP16, and CBP13.5. Densitometric tracing of the fluorogram (lane f, Fig. 2) showed that these three bands accounted for > 99.9% of the total radioactivity and that the relative proportions of the

Figure 3. Rechromatography of asialofetuin-binding proteins on a second ASF-Sepharose column. Material eluted with lactose from an ASF-Sepharose column (component C, Fig. 1) was applied to a second ASF-Sepharose column (1.8 x 5 cm), washed with Buffer C, and eluted with a lactose gradient (0-0.15 M, ---). Fractions (2.1 ml) were collected and aliquots (0.2 ml) were assayed for radioactivity.

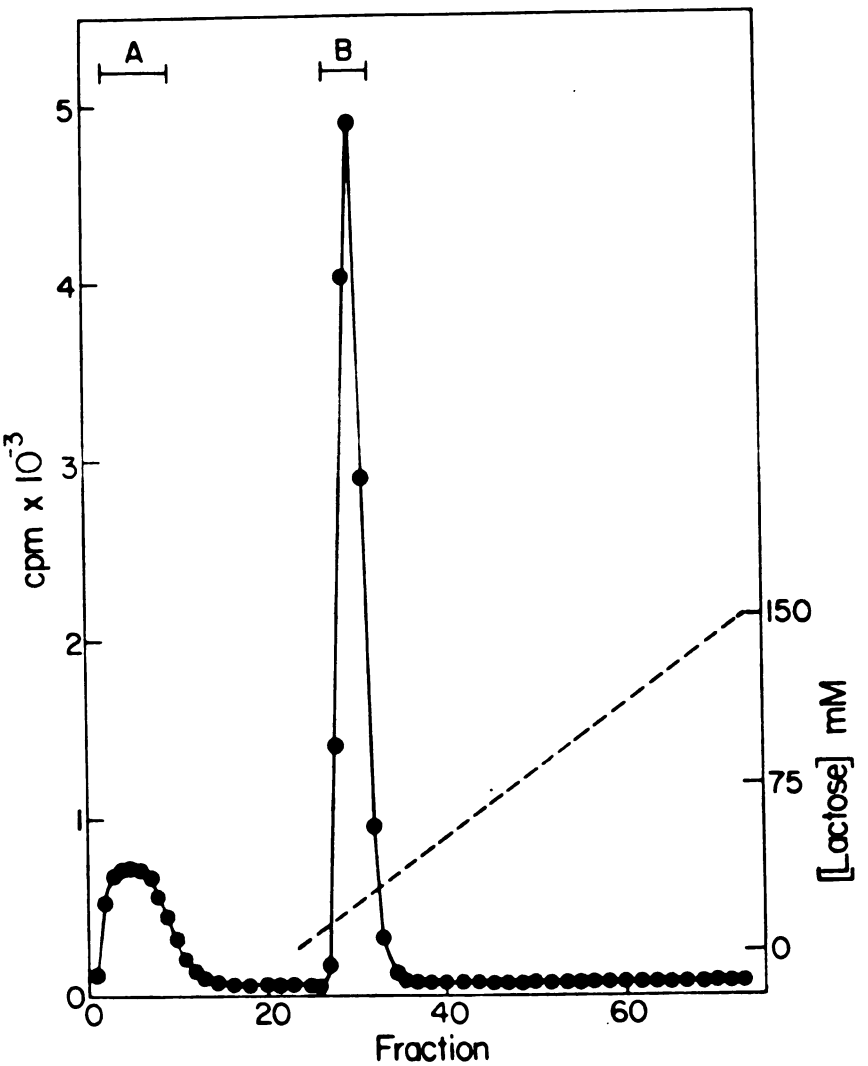


Figure 3

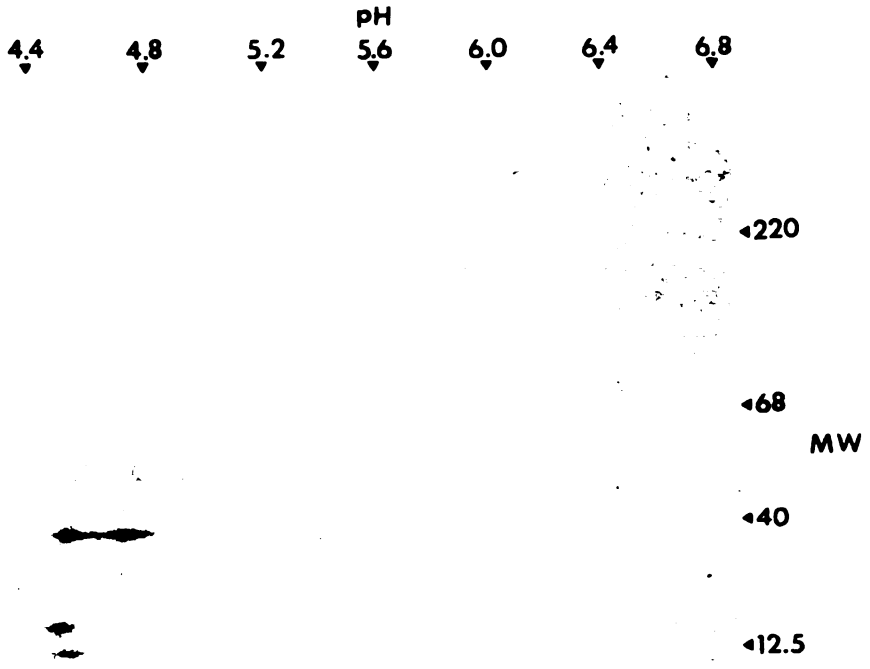
individual bands were: 2.9 (CBP35): 1.1 (CBP16): 1(CBP13.5). Similar results were obtained both in the presence and absence of β-mercaptoethanol during the electrophoresis.

In some preparations, the material corresponding to Component B (Fig. 3) yielded a fourth band on polyacrylamide gels (lane g, Fig. 2). It accounted for no more than 2-3% of the total radioactivity on the gel. The molecular weight of this fourth band was estimated to be 34,000. Although the origin of this band is not known at present, this polypeptide also has the capacity to bind to ASF.

In order to determine the isoelectric properties of the polypeptides corresponding to Component B (Fig. 3), a sample containing only CBP35, CBP16, and CBP13.5 was subjected to two dimensional gel electrophoretic analysis (Fig. 4). The results showed that CBP16 and CBP13.5 had similar pI's of approximately 4.5. CBP35, which corresponded to a single band on one dimensional electrophoresis, yielded two spots that had the same molecular weight (M_r = 35,000) but different isoelectric points (pI's 4.5 and 4.7) (Fig. 4). These results indicate that the ASF-Sepharose column could be used to isolate a minimum of three, and possibly four, polypeptides from 3T3 cells.

Effect of Saccharide Ligands and EDTA on Asialofetuin-Binding Proteins - In order to probe the sugar-binding specificity of the ASF-binding proteins, Triton X-100 extracts of 3T3 cells were chromatographed on columns of ASF-Sepharose. Various saccharides were tested for their capacity to elute the bound radioactive polypeptides. When the column was developed sequentially with mannose (position of arrow 2) sucrose (position of arrow 3) and lactose (position of arrow 4), a prominent radioactive peak was observed only upon the addition of

Figure 4. Two dimensional gel electrophoretic analysis of CBP35, CBP16, and CBP13.5. Material eluted with lactose (component B, Fig. 3; lane f, Fig. 2) was subjected to isoelectric focusing in the first dimension and sodium dodecyl sulfate polyacrylamide electrophoresis in the second dimension. Approximately 8,000 cpm were electrophoresed and the fluorogram was exposed for 16 days.


pH
4.4
4.8
5.2
5.6
6.0
6.4
6.8
220
68
MW
40
12.5

lactose (Fig. 5a). Polacrylamide gel analysis in SDS of Components A, B, and C (Fig. 5a) showed that CBP35, CBP16 and CBP13.5 all were eluted with lactose (lanes a-c, Fig. 6).

When the ASF-Sepharose column was developed sequentially with N-acetyl-Dglucosamine (position of arrow 2), galactose (position of arrow 3), and lactose (position of arrow 4), no radioactive material was eluted with the first monosaccharide (Fig. 5b). A peak of radioactivity was eluted, however, upon the addition of galactose (Component B, Fig. 5b). Polyacrylamide gel analysis in SDS of this material yielded two predominant polypeptides, corresponding to CBP35 and CBP13.5 (lane e, Fig. 6). When lactose was used to develope the column after galactose, some radioactivity was eluted in a rather ill-defined peak (Component C, Fig. 5b). This material yielded CBP16 upon SDS gel electrophoresis (lane f, Fig. 6). These results indicate that the ASF-binding polypeptides are carbohydrate-binding proteins (CBPs).

The question arose whether any of the CBPs had an intrinsic requirement for Ca^{2+} ion in order to bind the saccharide. To test this, the CBPs bound on ASF-Sepharose were eluted with Ca^{2+} free buffer containing 10 mM EDTA (position of arrow 1, Fig. 7). No distinct peak of radioactivity was observed (component B, Fig. 7). Moreover, SDS gel analysis of Component B (Fig. 7) revealed trace amounts of CBP35, CBP16, and CBP13.5. There was no apparent enrichment of any one of the polypeptides relative to the other two.

In contrast, the addition of lactose to the ASF-Sepharose column after EDTA (position of arrow 2, Fig. 7) yielded a substantial peak of radioactivity (Component C, Fig. 7). All three of the ASF-binding

Figure 5. Effect of various saccharides on the elution of CBP35, CBP16, and CBP13.5 from ASF-Sepharose. An extract of [^{35}S]methionine labeled cells was divided into two aliquots, each of which was applied to an ASF-Sepharose column (1.2 x 7 cm). After washing, the column was eluted sequentially with mono- and disaccharides of the D-configuration. Fractions (2 ml) were collected and aliquots (0.5 ml) were assayed for ^{35}S-radioactivity. The arrows indicate changes in carbohydrate (0.15 M) included in the developing buffer. Panel a: 1, Buffer C; 2, mannose; 3, sucrose; and 4, lactose. Panel b: 1, Buffer C; 2, N-acetylglucosamine; 3, galactose; and 4, lactose.

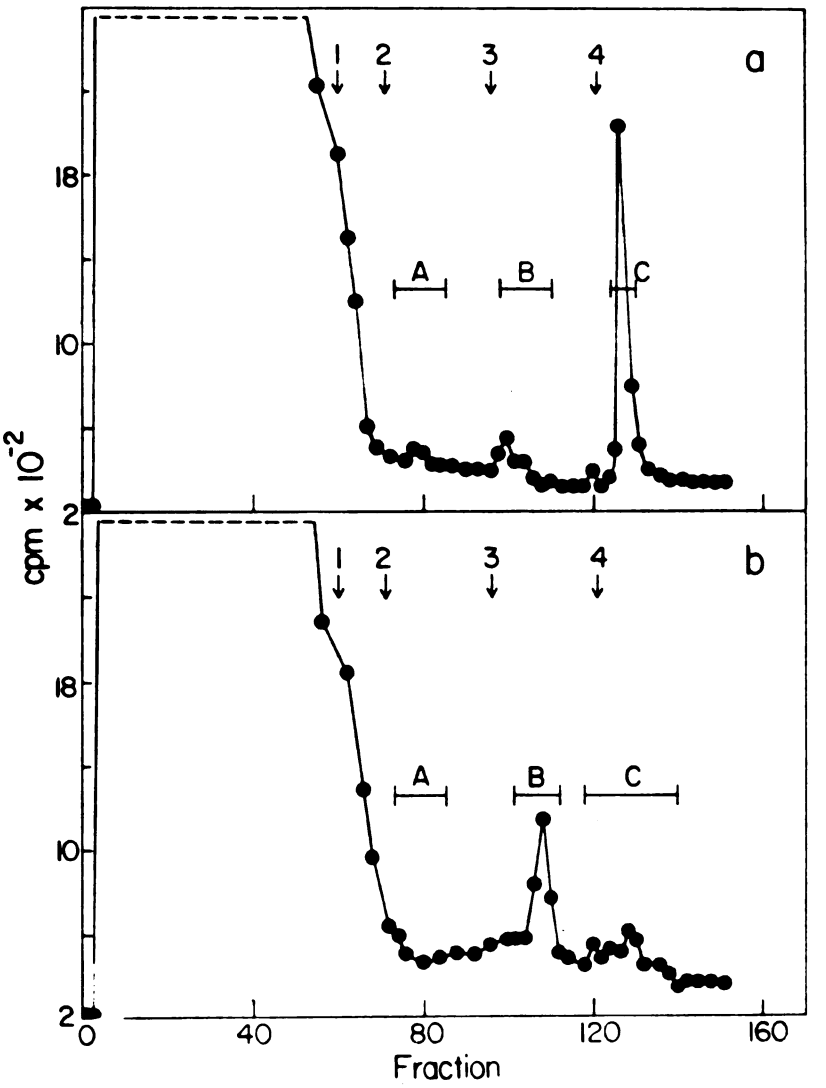
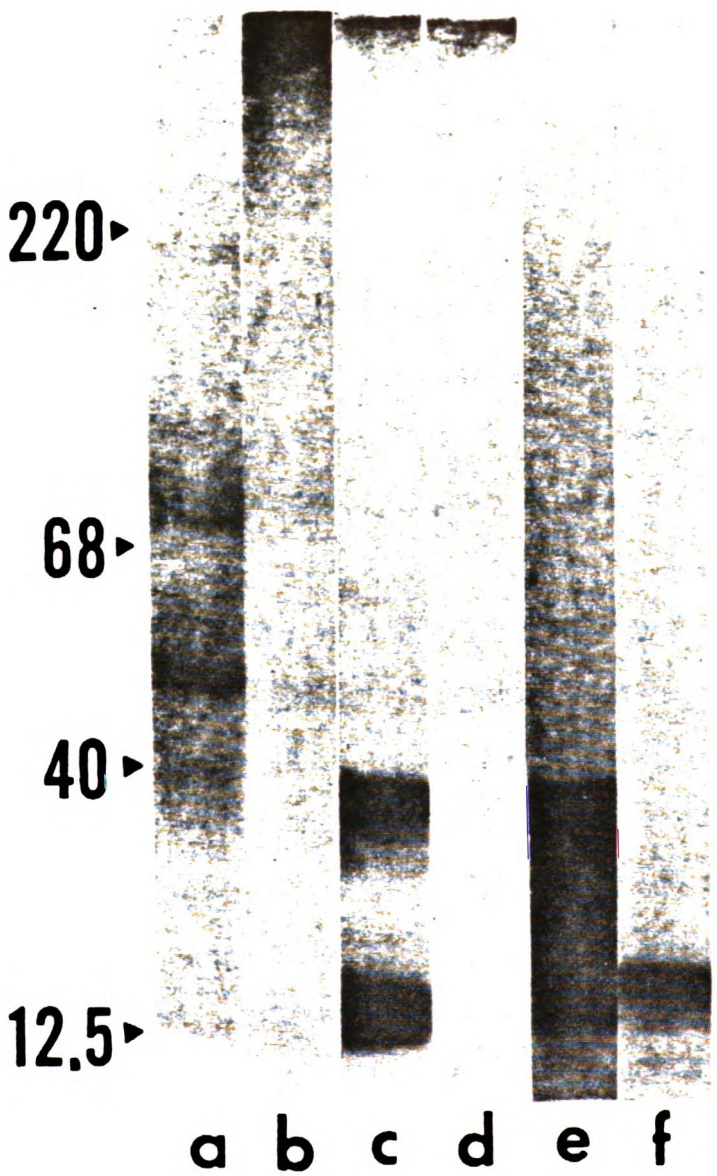


Figure 5

Figure 6. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of fractions derived from affinity chromatography of extracts of 3T3 cells on asialofetuin-Sepharose columns eluted with various saccharides (Fig. 5). Fractions from panel a, Fig. 5: lane a, component A; lane b, component B; and lane c, component C. Fractions from panel b, Fig. 5: lane d, component A; lane e, component B, and lane f, component C. Approximately 4,000 cpms were applied to each lane and the fluorograms were exposed for 30 days (lanes a-c) or 45 days (lanes d-f).


220
68
40
12.5
a b c d e f

polypeptides (CBP35, CBP16, and CBP13.5) were found in Component C (Fig. 7). It appears, therefore, that none of the CBPs require Ca^{2+} ions for saccharide-binding. This conclusion is further corroborated by experiments in which the same three CBPs were isolated from the ASF-Sepharose column when the extraction and affinity chromatography were carried out in calcium free buffer (Buffer A was replaced by 75 mM NaCl, 2 mM ethyleneglycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid, 2 mM NaN_3 and 75 mM Tris(hydroxymethyl)aminomethane, pH 7.0).

Fractionation of the Carbohydrate-Binding Proteins - Gel filtration of the CBPs purified by two cycles of affinity chromatography on Sephadex G-150 further fractionated the CBPs into two new components (Fig. 8). Component A (Fig. 8) chromatographed to a region corresponding to molecular weights of 30,000-35,000. Upon gel electrophoresis in SDS, it yielded only CBP35 (lanes b and d, Fig. 9). (In the sample used for Fig. 9, the gel also shows a minor band corresponding to a molecular weight of about 34,000; this represents the fourth band of the CBPs that occurs in some preparations.) Gel electrophoresis in SDS of Component B (Fig. 8) showed that it consisted of only CBP16 and CBP13.5 (lane c and e, Fig. 9). No CBP35 was observed in these fractions. Similar results were obtained both in the presence as well as in the absence of lactose (Fig. 8 and 9).

These results indicate that CBP35 does not interact with either CBP16 or CBP13.5. In addition, the position of migration of CBP35 (Component A, Fig. 8) suggests that the molecular weight of the protein in the absence of denaturants is 35,000 and therefore the polypeptide does not self-associate intodimers or oligomers. Similarly, the

Figure 7. Effect of EDTA on the elution of CBP35, CBP16 and CBP13.5 bound to asialofetuin-Sepharose. An extract of [^{35}S] methionine-labeled 3T3 cells was chromatographed on an ASF-Sepharose column (1.3 x 14 cm) in Buffer C. After nonbound material was collected, the column was eluted with three column volumes of calcium free buffer containing EDTA (75 mM Tris, pH 7.2, 10 mM EDTA, 60 mM NaCl, and 1mM NaN_3), followed by Buffer C containing 0.15 M lactose. The arrows indicate changes in developing buffer: 1, EDTA and 2, lactose. Fractions (1.5 ml) were collected and aliquots (0.4 ml) assayed for radioactivity.

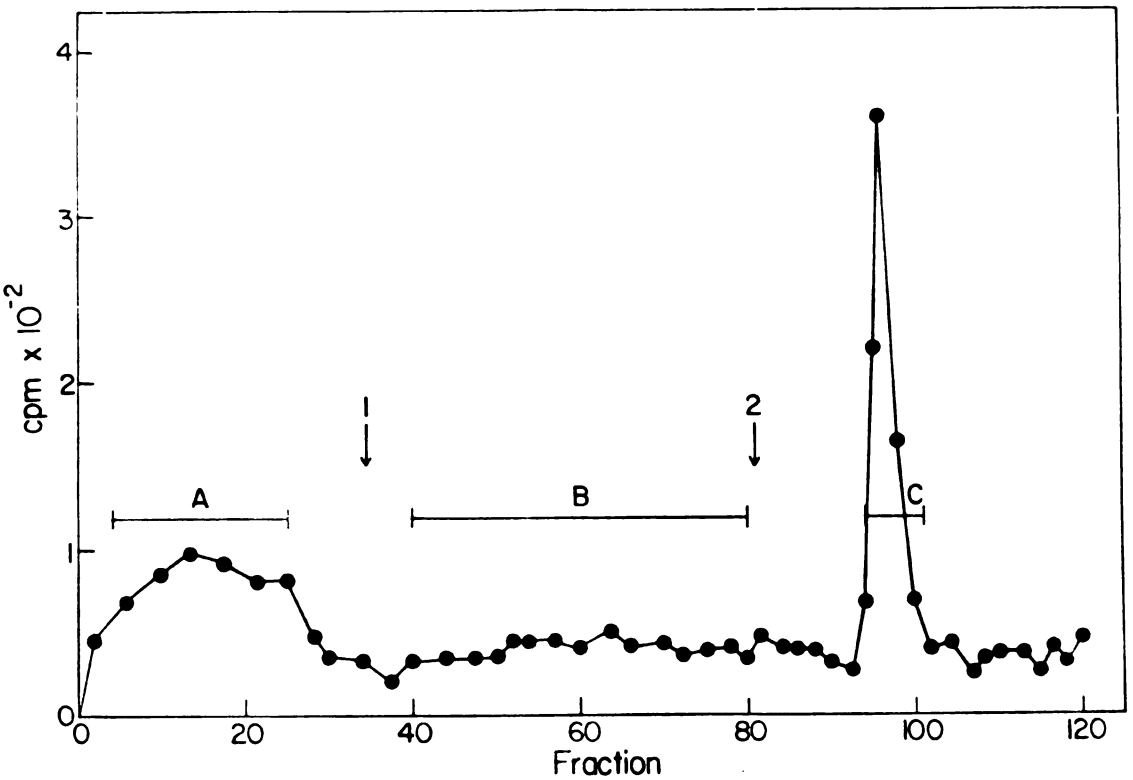


Figure 7

Figure 8. Sephadex G-150 chromatography of [^{35}S]methionine-labeled CBP35, CBP16, and CBP13.5. Purified material from the second ASF-Sepharose column was concentrated and chromatographed on a Sephadex G-150 column (0.9 x 120 cm) in Buffer C either in the presence (●—●) or absence (o--o) of 0.1 M lactose. Fractions (1.1 ml) were collected and aliquots (0.1 ml) were assayed for radioactivity. Molecular weight markers were: Ald, aldolase (158,000); BSA, bovine serum albumin (68,000); Chym, chymotrypsinogen A (25,000); and Cyt C, cytochrome C (12,500).

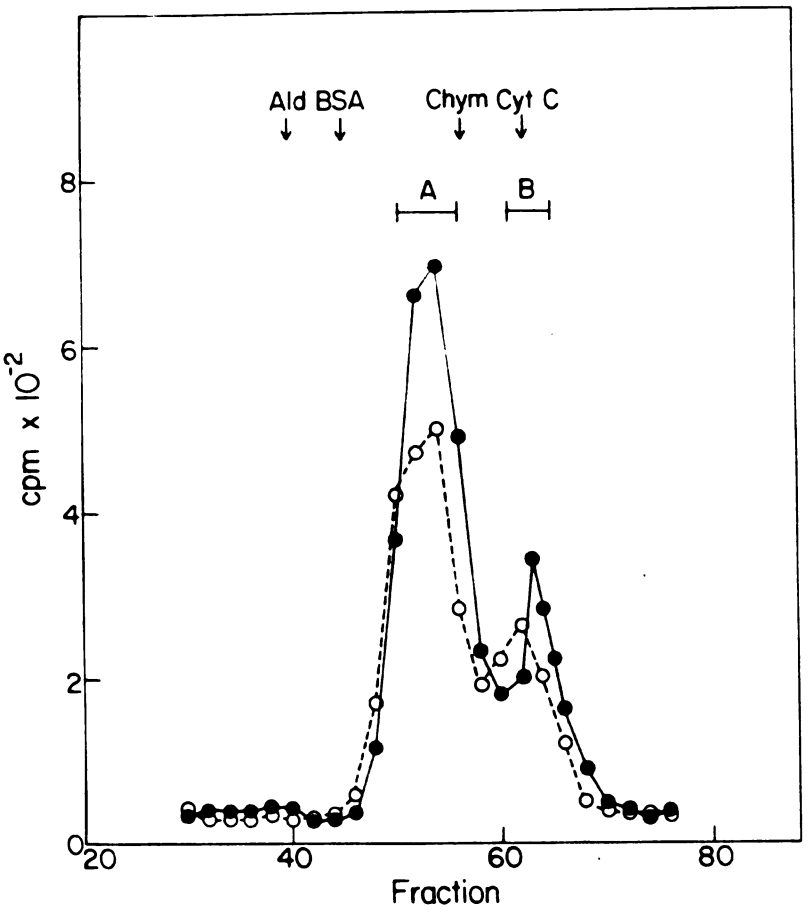
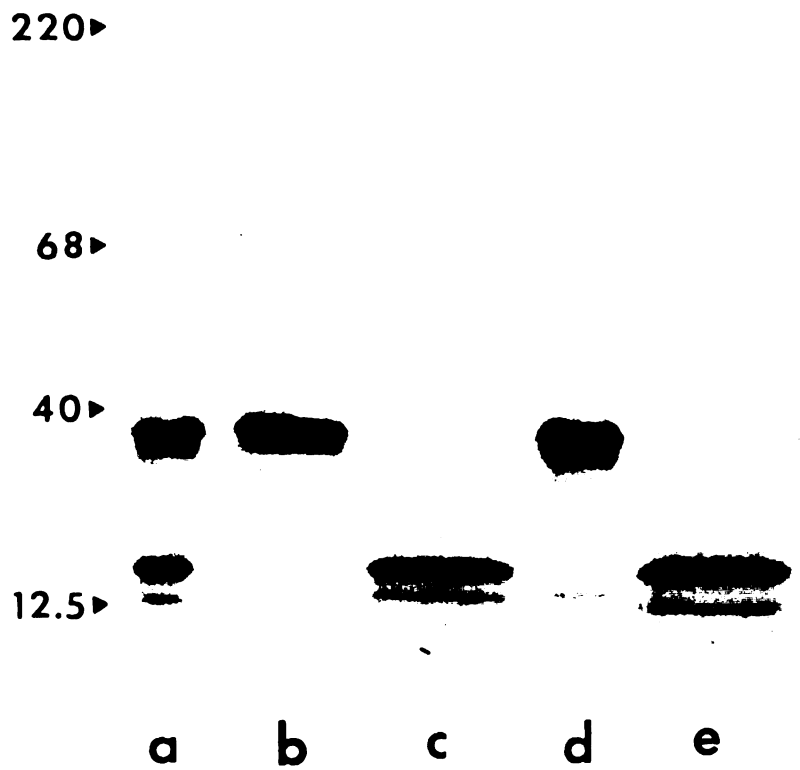


Figure 8

Figure 9. Polyacylamide gel electrophoresis in sodium dodecyl sulfate of highly purified CBP35, CBP16, and CBP13.5 after gel filtration on Sephadex G-150 (Fig. 8). Lane a is an aliquot of the sample which was applied to the column. Gel filtration in the absence of lactose: lane b, component A and lane c, component B, and in the presence of lactose: lane d, component A and lane e, component B. Aproximately 25,000 cpm were applied and the fluorograms were exposed for 48 hours.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d
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chromatographic position of Component B (Fig. 8) is consistent with polypeptides of molecular weight 13,000-16,000. Moreover, we have achieved a similar fractionation using columns of Sephadex G-50. Under this condition, the chromatographic position of CBP16 and CBP13.5 was well resolved from that of chymotrypsinogen (M_r = 25,000). Therefore, it appears that CBP16 and CBP13.5 do not bind to each other in non-denaturing solvents.

Intrinsic Binding Properties of the Carbohydrate-Binding Proteins - Component A (Fig. 8) was applied to an ASF-Sepharose column. More than 95% of the radioactivity applied was bound to the affinity column and could be eluted with lactose (Fig. 10). Polacrylamide gel electrophoresis in SDS of the recovered material showed that it was highly purified CBP35 (inset Fig. 10). This suggests that CBP35 can bind to carbohydrates independently of CBP16 and CBP13.5. Moreover, isolated CBP35 can agglutinate rabbit erythrocytes as well as rabbit erythrocytes previously treated with trypsin followed by glutaraldehyde fixation (Table I). This agglutination was inhibited by lactose (0.05 M).

Component B (Fig. 8), which consisted of CBP16 and CBP13.5, also exhibited lactose-inhibitable agglutination activity (Table I). Component B (Fig. 8) can be bound to ASF-Sepharose columns and eluted with specific saccharides. Elution with galactose yielded a fraction (Component A, Fig. 11) containing only CBP13.5 (see inset, Fig. 11). Subsequent elution with lactose yielded a fraction (Component C, Fig. 11) containing CBP16, although this protein can also be detected at the latter part (Component B, Fig. 11) of the galactose elution (0.15 M galactose). These results corroborate the previous demonstration that galactose can elute CBP13.5 and lactose can elute the bulk of the CBP16

TABLE I

Agglutination and Enzymatic Activities of CBP35, CBP16 and CBP13.5

Assay Sample	Agglutination	β-Galactosidase	Sialyl transferase
Component C Fig. 1	+	-	nt
Component B Fig. 3	+	-	-
CBP35 (Component A, Fig. 8)	+	nt	nt
CBP16 and CBP13.5 (Component B, Fig. 8)	+	nt	nt

+ activity detected
- no activity detected
nt not tested for activity

Figure 10. Binding of [^{35}S]methionine labeled CBP35 to an ASF-Sepharose column. CBP35, fractionated on a Sephadex G-150 column (component A, Fig. 8) was bound on an ASF-Sepharose column (1.5 x 8 cm). The arrow marks the addition of lactose (0.15 M) in the developing buffer. Fractions (0.9 ml) were collected and aliquots (0.2 ml) were assayed for radioactivity. The inset shows the fluorogram of Component A from this column after polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

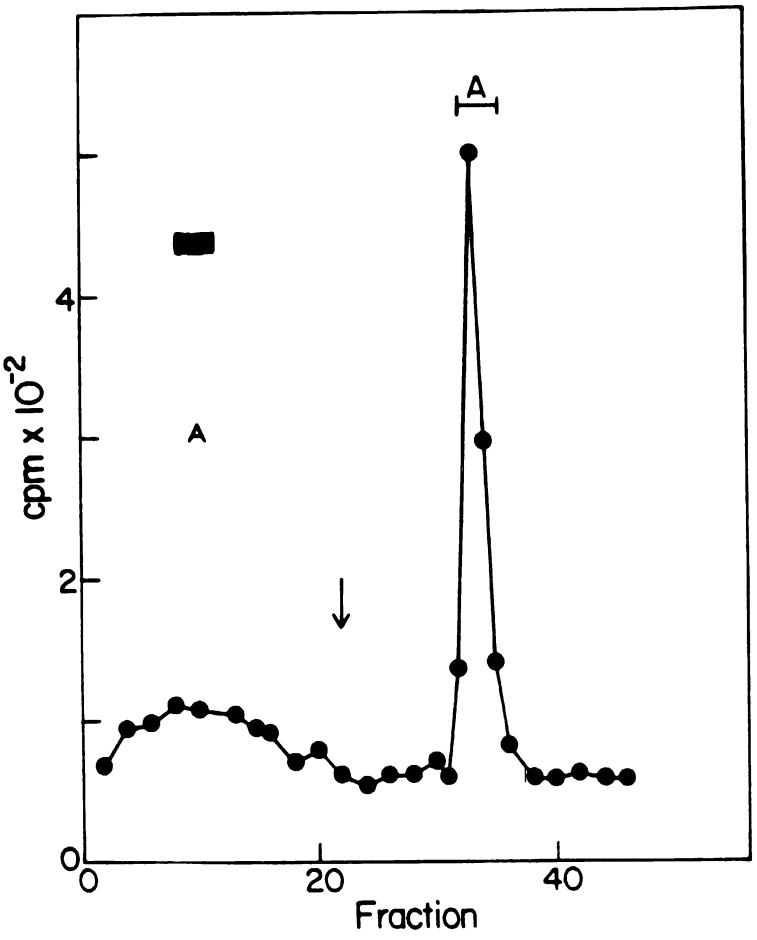
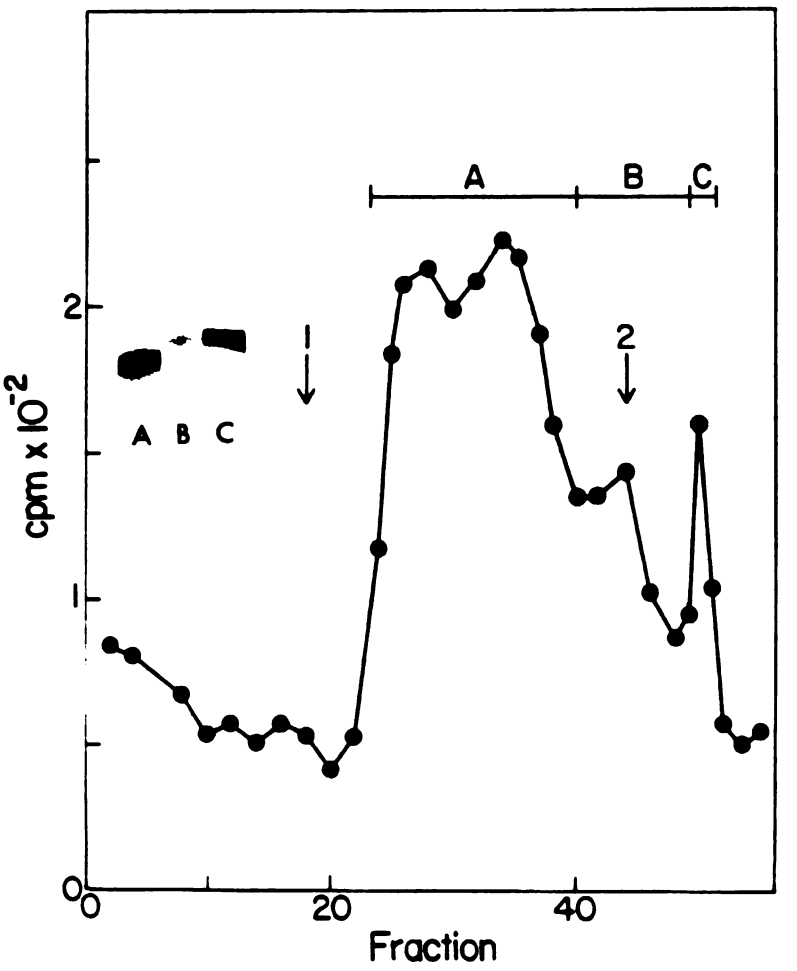

A
A
cpm x 10^{-2}
4
2
0
0
20
40
Fraction

Figure 11. Binding of [^{35}S]methionine labeled CBP16 and CBP13.5 to an ASF-Sepharose column. Component B (Fig. 8) was affinity chromatographed on an ASF-Sepharose column (1.5 x 8 cm). The arrows indicate changes in carbohydrate (0.15 M) included in the developing buffer: 1, galactose; 2, lactose. Fractions (0.9 ml) were collected and aliquots (0.2 ml) were assayed for radioactivity. The inset shows the fluorograms of pooled fractions corresponding to Components A-C from this column after polyacrylamide gel electrophoresis in sodium dodecyl sulfate.


A
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C
1
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A B C
cpm x 10⁻²
0
1
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40
Fraction

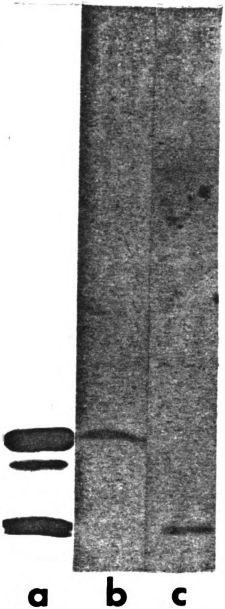
from ASF affinity columns (Fig. 5b). Together, they suggest that both CBP16 and CBP13.5 have intrinsic carbohydrate-binding capacities, independent of other associated polypeptides.

We have also tested the various fractions containing the CBPs for enzymatic activities such as glycosidases and transferases. Using 4-methylumbelliferyl- β-D-galactopyranoside as a substrate, we found no β-galactosidase activity associated with the CBPs (Component C, Fig. 1 and Component B, Fig. 3) at pH 4.6 and at pH 7.2. Moreover, all of the β-galactosidase activity observed in the original cell extract could be accounted for in the material not bound by the first ASF-Sepharose column (Component A, Fig. 1). Similarly, we found no transferase activity associated with the CBP fractions (Component B, Fig. 3) as assayed with ^{14}C-labeled CMP-sialic acid and ASF. This conclusion is based on experiments which showed that the radioactivity precipitated along with ASF by phosphotungstic acid was identical when the transferase assay was carried out in the presence and absence of the CBPs. Moreover, analysis of the assay reaction mixture by chromatography on Sepahdex G-25 showed that no radioactivity migrated in the void volume fractions, at a position corresponding to ASF.

Immunoprecipitation of the Carbohydrate-Binding Proteins - The availability of two antisera, one directed against CBP35 (anti-CBP35) and the other directed against CLL I (anti-CLL I), provided the necessary reagents to study the structural relationships between CBP35, CBP16, and CBP13.5, as well as to test for relatedness to lectins characterized in the chicken intestine and muscle systems. Component C (Fig. 1), which contained a mixture of CBP35, CBP16 and CBP13.5, was subjected to immunoprecipitation by anti-CBP35 and anti-CLL I.

Analysis of the immunoprecipitates by gel electrophoresis showed that anti-CBP35 reacted only with CBP35 but not with CBP16 and CBP13.5 (Fig. 12, lane b). In contrast, anti-CLL I precipitated only CBP 16 and no other CBP from the mixture (Fig. 12, lane c). These results suggest that CBP35 is not structurally related and therefore, is most probably not a higher molecular weight precursor of CBP16. In addition, the immune reactivity of CBP16 with anti-CLL I indicates that this CBP is probably a murine fibroblast counterpart of the lactose-specific lectins described in a number of other systems.

Figure 12. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of CBPs identified by specific antisera. (a) Partially purified CBPs (Component C, Fig. 1) subjected to immunoprecipitation; (b) Material immunoprecipitated by antibodies directed against CBP35; and (c) Material immunoprecipitated by antibodies directed against chicken lactose lectin I. Approximately 1000 cpms were applied to each lane and the fluorograms were exposed for 30 days.


a
b
c

DISCUSSION

The results obtained in the present study indicate that we have purified, from the 3T3 fibroblast system, three distinct CBPs: (a) CBP35 (M_r = 35,000); (b) CBP16 (M_r = 16,000); and (c) CBP13.5 (M_r = 13,500). These three proteins are similar in several key properties. First, they all appear to recognize specifically galactose-containing glycoconjugates. In the present paper, we have isolated them on the basis of their binding to columns containing the asialoglycoprotein, ASF and we have demonstrated differences between them on the basis of their elution from the affinity column using lactose or galactose. We have also obtained evidence that these proteins will bind polyacrylamide beads derivatized with disaccharide ligands of defined structure (DGalβ(1 4)βDGlcNAc) but not to beads containing only the monosaccharide βDGal (23). Moreover, fractions containing CBP 35 or CBP 16 and CBP 13.5 exhibited agglutination activity when assayed with rabbit erythrocytes. This agglutination can be inhibited by lactose. These binding and agglutinating results demonstrate unequivocally that the isolated proteins are carbohydrate-binding proteins.

Second, these CBPs have low isoelectric points (pIs 4.5-4.7), indicating that they are most probably acidic proteins. In our two dimensional gel elec- trophoretic analysis, CBP35 was actually resolved into two different spots with pIs of 4.5 and 4.7. The relationship of

these two polypeptides, of the same molecular weight but of different isoelectric points, has not been determined.

Third, the binding activity of all three of the CBPs was rapidly lost in the absence of β-mercaptoethanol. This suggests that functional groups, most likely free sulfhydryl or tryptophan residues (23), may be sensitive to air oxidation. Fourth, CBP35, CBP16, and CBP13.5 do not appear to require Ca^{2+} for activity.

Finally, gel filtration studies in non-denaturing solvents indicate that the CBPs do not interact with each other, both in the presence and absence of lactose. The chromatographic data also suggest that none of the three polypeptides appear to self-associate to form oligomers of identical subunits. Because these column separation experiments were carried out using minute amounts of radiolabeled proteins, however, the concentrations of CBPs used in our column experiments may be below the threshold required for aggregation. In any case, it should be emphasized that each of the fra tionated proteins can bind to ASF, independent of associated polypeptides.

Comparisons of the polypeptide molecular weights, the isoelectric points, agglutination activity, and the carbohydrate-binding specificity of CBP16 and CBP13.5 with the lactose-specific lectins isolated from electric eel organ (24), calf heart and lung (13), and chicken intestine (25) and muscle (26, 19) suggest that they may be related proteins. This conclusion is supported by the observation that antibodies raised against CLL I (M_r = 16,000) immuneprecipitated only CBP16 out of a mixture containing all three CBPs. In addition, these proteins all share the similar properties of being highly sensitive to air oxidation and of being calcium-independent. CBP16 and CBP13.5

differ from these lactose-specific lectins in one major respect. Whereas all but one of the previously identified lectins self-associated to form dimers and oligomers, CBP16 and CBP13.5 remain in monomeric form. If these proteins do indeed turn out to be analogous counterparts of each other in different species (27, 28), then the question concerning their postulated tissue-specific function(s) must be raised.

To the best of our knowledge, a protein analogous to CBP35 has not been previously identified and isolated in other species or from other cell types. It does not appear that CBP35 is a higher molecular weight precursor to CBP16 and/or CBP13.5. Antibodies directed against CBP35 did not show cross reactivity with either CBP16 or CBP13.5. Conversely, antibodies that recognized CBP16 failed to react with CBP35. Therefore, CBP35 most probably represents a new carbohydrate-binding protein, co-isolated with CBP16 and CBP13.5, which do have analogous counterparts.

At present, it does not appear that CBP35 is the fibroblast counterpart of the galactose-specific receptor on hepatocytes, originally identified by Ashwell and co-workers (29-33). The differences between the two proteins include: (a) molecular weight of the polypeptide chain; (b) aggregation properties of the polypeptides; (c) effect of EDTA on binding of carbohydrates; and (d) sensitivity to air oxidation.

We have recently purified a carbohydrate-binding protein from mouse lung tissue using the same procedures described for the isolation of CBP35. The molecular weight of this protein was 35,000, as determined by SDS polyacrylamide gel electrophoresis and Coomassie blue staining.

When the mouse lung protein on the polyacrylamide gel was transferred onto nitrocellulose paper and then immunoblotted with anti-CBP35, a single radioactive band (M_r = 35,000) was observed after autoradiography. These results suggest that we can isolate CBP35 in large amounts (microgram levels) from mouse lung. This in turn will allow us to carry out structural studies on the polypeptide, to study its cellular localization, and to search for its endogenous ligand in the cell.

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Chapter III

ENDOGENOUS LECTINS FROM CULTURED CELLS

II. Specific Affinity Columns for the Isolation of Carbohydrate-Binding Proteins*

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Running Title: Affinity Columns for Carbohydrate-Binding Proteins

SUMMARY

Treatment of polyacrylamide beads with hydrazine hydrate followed by nitrous acid converts the amide groups on the resin into acyl azides. These reactive acyl azides can be used to couple ligands such as glycosides of hexanolamine. Unreacted acyl azides are converted back into amide groups by treatment with ammonia. The conditions for this sequence of chemical reactions were investigated and optimized, with the following two particular objectives in mind: (a) efficient and stable coupling of ligands that are available in limited amounts (100 μmoles); and (b) minimizing the number of charged groups remaining on the polyacrylamide beads in the resulting product. Using these optimized conditions, we have synthesized polyacrylamide beads containing defined carbohydrate ligands. These saccharide-containing beads were used to demonstrate the carbohydrate-binding capacity of three proteins (M_rs 35,000, 16,000, and 13,500), isolated from cultured 3T3 mouse fibroblasts on the basis of their binding to asialofetuin-Sepharose. All three proteins bound to polyacrylamide beads containing the disaccharide DGalβ(1→4)βDGlcNAc but not to beads containing the monosaccharide βDGal. We have also purified, in a single step, a carbohydrate-binding protein from extracts of human foreskin fibroblasts using an affinity column of polyacrylamide beads derivatized with DGalβ(1→4)βDGlcNAc. This protein (M_r = 35,000) may represent the human counterpart of the mouse protein of similar

molecular weight and binding properties characterized in the 3T3 fibroblast system.

Affinity columns containing Sepharose covalently coupled to a glycoprotein such as fetuin have been used for the isolation of carbohydrate binding proteins (CBPs). This approach has been successfully applied in the purification of hemaglutinin receptor of influenza virus (1), carbohydrate-specific antibodies (2), as well as lectins from plant (3) and animal (4, 5) sources. Recently, we have isolated from 3T3 fibroblasts a fraction which binds to asialofetuin-Sepharose and is eluted with lactose. This fraction yielded three polypeptide chains on analysis by polyacrylamide gel electrophoresis.

In the course of these studies, it became apparent that an affinity support containing only carbohydrates (CHOs) of chemically defined structure would facilitate the analysis of the carbohydrate-binding capacity and specificity of these asialofetuin-binding proteins. Although the use of agarose supports derivatized with CHOs has been developed and used extensively in the purification of lectins (6) and enzymes (7), our observations as well as those reported by others (8-10) indicate that affinity supports derived from cyanogen bromide activation of polysaccharide resins are highly charged, resulting in appreciable non-specific binding, and are relatively unstable. Furthermore the use of polysaccharide resins and glycoproteins complicate interpretations of CHO binding since the saccharides are heterogenous. We have, therefore, optimized the procedure originally developed by Inman and Dintzis (11, 12) for the derivatization of polyacrylamide (PA) beads and have used CHO ligands of chemically defined structure.

In the present communication we report the coupling of defined CHO structures to inert PA beads. Our procedure incorporated three

important features: (a) the use of disaccharides synthesized by glycosyltransferases such that the CHO ligands are of chemically defined structure, (b) the coupling of limiting amounts of CHO in sufficient ligand density, and (c) synthesis of an affinity support which does not have a high capacity for ion exchange. Finally, we have used these PA supports derivatized with β-D-galactose (Gal-HA-PA) and D-galactose-β(1⟶4) βD-N-acetyglucosamine (Gal-GlcNAc-HA-PA) to demonstrate the CHO binding specificities of three CBPs isolated from 3T3 cells.

MATERIALS AND METHODS

(see supplemental material, p. 107)

RESULTS

Quantitation of Coupling Reactions - The coupling of sufficient quantities of ligand to PA beads when starting with a limited amount of the ligand was the main achievement of our chemical studies. Although the basic scheme (Fig. 1) is that of Inman and Dintzis (11, 12), there are features of the reactions that were particularly important for our isolation of CBPs and that needed to be modified. For example, it was of great importance to us that the derivatized PA beads do not carry a large number of positive charges because proteins bearing negatively charged residues may bind to them through ionic interactions rather than specific CHO interactions. Conversely, it was also important that the number of negative charges are sufficiently low so as not to interfere with specific carbohydrate recognition and binding.

The following variables were monitored using 1,2-[^{14}C]-2-aminoethanol to quantitate the amount of coupled ligand: (a) length of incubation in hydrazine hydrate; the effect of (b) pH, (c) time, (d) volume on the coupling of ligand (ii, iii, Fig. 1), (e) concentration of the ligand; and (f) conditions for the regeneration of amides from the acyl azides (iii iv, Fig. 1).

The results of our chemical studies indicate that the optimal conditions for the preparation of affinity PA beads using this method are (see supplemental material for details, p. 107):

Figure 1: Schematic representation of the method used to prepare CHO-PA affinity columns.

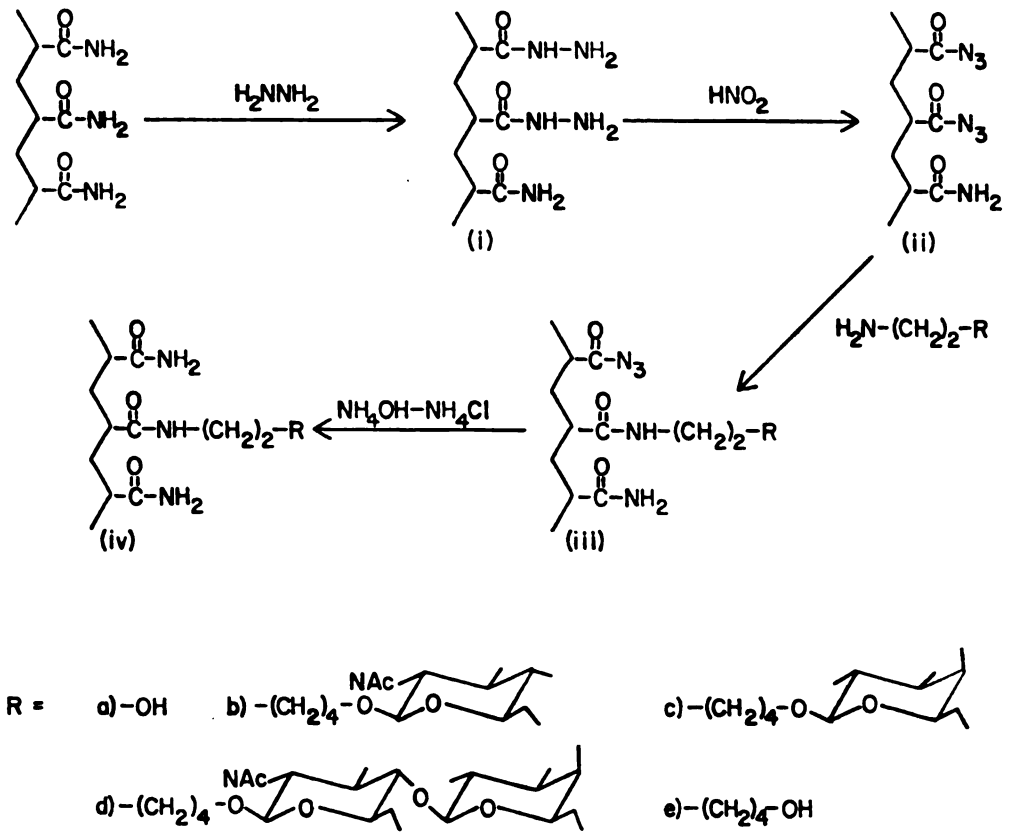
H2NNH2
HNO2
(i)
(ii)
H2N-(CH2)2-R
NH4OH-NH4Cl
(iii)
(iv)
R =
a) -OH
b) -(CH2)4-O
NAc
c) -(CH2)4-O
d) -(CH2)4-O
NAc
e) -(CH2)4-OH

Figure 1

(a) formation of the acyl hydrazide in 6.1 M hydrazine hydrate for 3 h at 50°;

(b) addition of the ligand in H_2O at pH 11.0;

(c) coupling reaction time of 4-5 h;

(d) a reaction volume of 1.5-2.5 times that of the resin;

(e) use of 5 M NH_4OH-NH_4Cl, pH 10.0, to regenerate amides from the acyl azides.

Binding Properties of Affinity Columns Containing CHO-PA - The binding properties of affinity columns containing CHO ligands coupled to PA beads were studied using various proteins with different CHO-binding as well as charge characteristics. Some representative results obtained with the GlcNAc-HA-PA column are presented in Figure 6. The ligand density of this column was approximately 8 μmol GlcNAc/ml of resin.

When a solution of wheat germ agglutinin (WGA), which has a high affinity for GlcNAc (20), is chromatographed on this column, the protein becomes bound to it (Fig. 6B). The bound material can be eluted from the column when soluble GlcNAc is introduced in the developing solvent. This binding of WGA is not due to properties of the PA backbone or the HA spacer arm since WGA is not retained by HA derivatized PA beads (HA-PA column; Fig. 6A). When soybean agglutinin (SBA) is applied to this column, an elution profile consistent with the chromatographic behavior of this protein on underivatized Bio-Gel P-150 was observed (Fig. 6C). This would be expected because SBA has been shown to be specific for Gal residues but not for GlcNAc (21).

The charge properties of the GlcNAc-HA-PA resin were investigated using the proteins pepsin and histones, which have low and high isoelectric points, respectively (22, 23). When a solution of pepsin

Figure 6: Elution profiles of GlcNAc-HA-PA affinity column. The GlcNAc-HA-PA (2.0 x 8.0 cm) and HA-PA (2.0 x 5.5 cm) columns were equilibrated with PBS. The proteins were applied in PBS, washed with PBS, 0.25 M GlcNAc in PBS (starting at arrow a) and 0.4 M NaCl in phosphate buffer (starting at arrow b). Fractions (3.4 ml) were collected and monitored for absorbance at 280 nm. The protein loaded, column used and % recovery were as follows: Panel A, 1.2 units of WGA on HA-PA column, 96% recovery; Panel B, 6.2 units of WGA on GlcNAc-HA-PA column, 87% recovery; Panel C, 23.2 units of SBA on GlcNAc-HA-PA column, 79% recovery; Panel D, 8.1 units of pepsin on GlcNAc-HA-PA, 99% recovery; Panel E, 5.1 units of histone II-A on GlcNAc-HA-PA column, 68% recovery. All units expressed are absorbances at 280 nm.

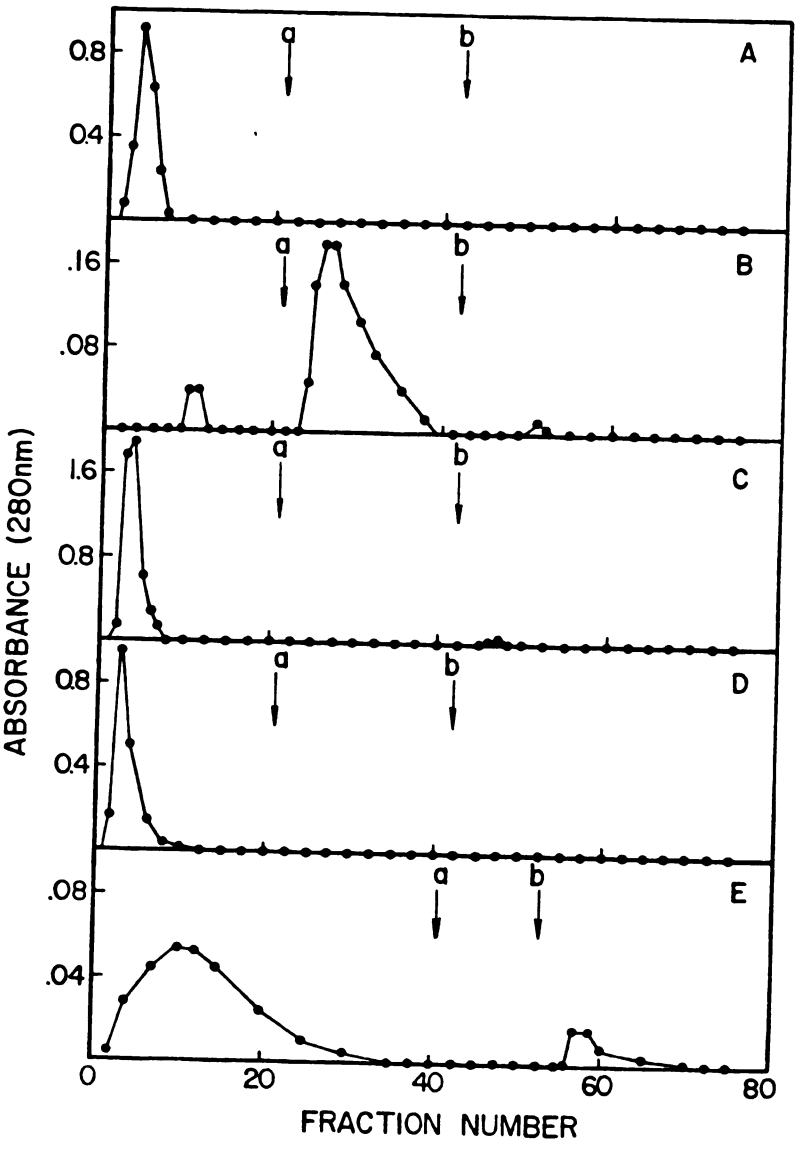
ABSORBANCE (280nm)
0.8
0.4
.16
.08
1.6
0.8
0.8
0.4
.08
.04
a
b
A
B
C
D
E
0
20
40
60
80
FRACTION NUMBER

Figure 6

was chromatographed on the column, there was no retention of the protein (Fig. 6D). This indicated that there was little or no net positive charges on the GlcNAc-HA-PA. The chromatographic profile obtained for the histones (Fig. 6E) indicated that there may be some negative charges because a small amount of the highly positively charged protein was bound. This represented less than 10% of the total protein applied to the column. Moreover, it is important to note that none of the bound protein could be eluted with GlcNAc (fractions between arrows a and b). Thus the bound material did not exhibit any saccharide specificity as was demonstrated when WGA was bound to the GlcNAc-HA-PA column (Fig. 6B). All these results strongly indicate that the GlcNAc-HA-PA resin has the requisite specificities to be used for the isolation of CBPs.

We have also tested, in a similar manner the specificities of the binding properties of other PA beads coupled with CHO ligands. For example, SBA, which interacts with Gal residues, binds to the Gal-HA-PA column. More importantly, the bound protein can be eluted with the competitive ligand, Gal. WGA did not bind to the Gal-HA-PA column.

Fractionation of Mouse and Human Fibroblast Components Using CHO-containing Affinity Columns - We have previously isolated from 3T3 mouse fibroblasts a fraction on the basis of its binding to Sepharose columns derivatized with asialofetuin and subsequent elution with a lactose gradient (Chapter II). Polyacrylamide gel electrophoresis analysis of this fraction yielded three polypeptide chains (CBP35, M_r = 35,000; CBP16, M_r = 16,000; and CBP13.5, M_r = 13,500). When this fraction was chromatographed on a column of Gal-GlcNAc-HA-PA,

approximately 80% of the radioactivity was bound by the column (Figure 7a). The bound material, which could be completely eluted with galactose, yielded all three polypeptides (Figure 7a, and Figure 8, lane b); no additional radioactivity was eluted with lactose. In contrast, no binding was observed when a column of Gal-HA-PA was used (Figure 7b and Figure 8, lane c). Inasmuch as the two affinity supports differ only in the saccharide portion of their chemical structures, these results demonstrate unequivocally that the three polypeptides isolated on the basis of their binding to asialofetuin- Sepharose are carbohydrate-specific proteins.

We have also used Gal-GlcNAc-HA-PA to isolate the CBPs directly from extracts of 3T3 fibroblasts. When [^{35}S]methionine-labeled 3T3 extracts were chromatographed on this affinity column, approximately 0.01% of the radioactive material was bound on the resin; this material could be eluted with lactose (Component A, Fig. 9). Polyacrylamide gel electrophoretic analysis of Component A (Fig. 9) yielded the three bands, corresponding to CBP35, CBP16, and CBP13.5 (Figure 8, lane d). These results strongly suggest that PA beads covalently derivatized with defined CHO structures provide a highly efficient method for the isolation of CBPs.

In a similar series of experiments, normal human foreskin fibroblasts (SL66) were labeled 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 resin and this could be eluted with lactose in a fashion similar to that observed with 3T3 cells (Figure 9). Polyacrylamide gel electrophoresis and fluorography of this material

Figure 7: Chromatography of [^{35}S]methionine-labeled CBP35, CBP16, and CBP13.5 on columns of (a) Gal-GlcNAc-HA-PA and (b) Gal-HA-PA. The radioactive CBP's were isolated by fractionating extracts of 3T3 fibroblasts on a column of asialofetuin Sepharose. The CBP-containing fractions were eluted with lactose, dialyzed in tubing impermeable to molecules of molecular weight greater than 3,500 against 50 mM $CaCl_2$, 1 mM NaN_3, 75 mM Tris (hydroxymethyl)aminoethane, 2 mM mercaptoethanol, pH 7.2. The material was then chromatographed on affinity columns (0.8 x 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 of 2 ml were collected and 0.4 ml aliquots were analyzed for radioactivity. The horizontal bars designated A and B represent fractions pooled for sodium dodecyl sulfate polyacrylamide gel electrophoresis.

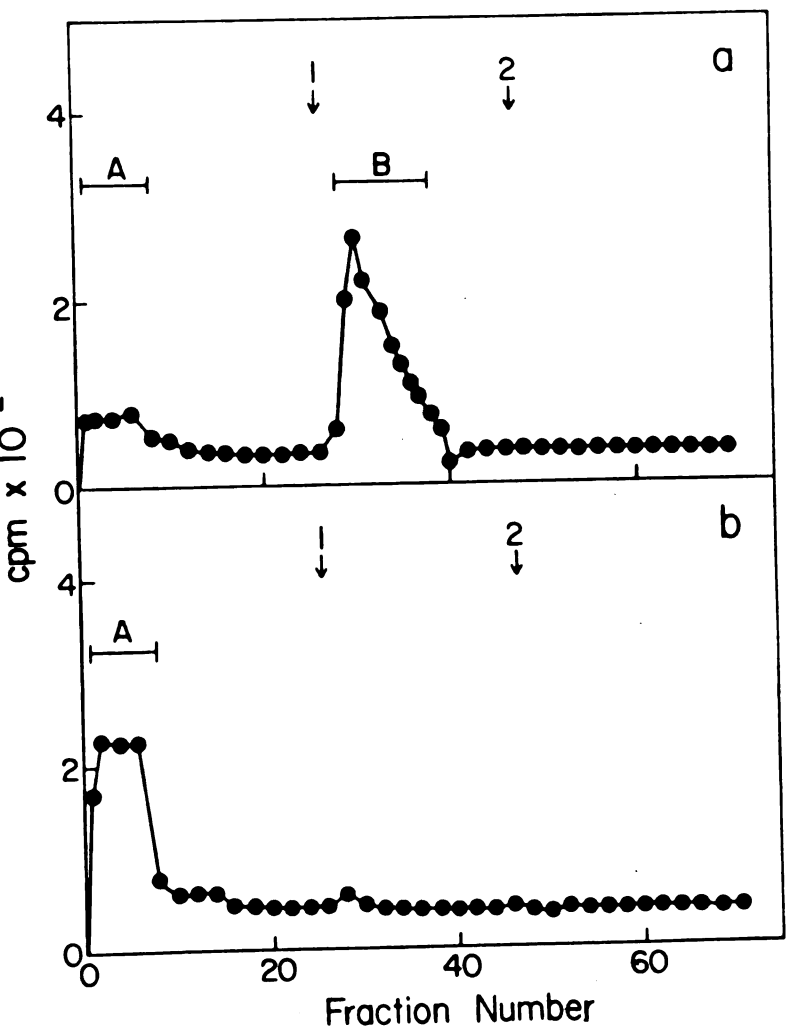

Figure 7

Figure 8: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of proteins derived from mouse 3T3 and human SL66 fibroblasts after affinity chromatography on CHO-HA-PA columns. Lanes a-c represent material isolated from 3T3 cells on the basis of binding to asialofetuin-Sepharose and to Gal- GlcNAc-HA-PA or Gal-HA-PA. (a) Component A, Figure 7a (3,200 cpm); (b) Component B, Figure 7a (3,200 cpm); (c) Component A, Figure 7b (5,000 cpm). Lanes d and e represent material isolated from mouse (3T3) and human (SL66) fibroblasts, respectively, by direct affinity chromatography of [^{35}S]methionine-labeled cell extracts on Gal-GlcNAc-HA- PA. (d) Component A, Figure 9 (13,000 cpm); (e) Protein from SL66 human fibroblasts corresponding to Component A, Figure 9 (3,000 cpm).

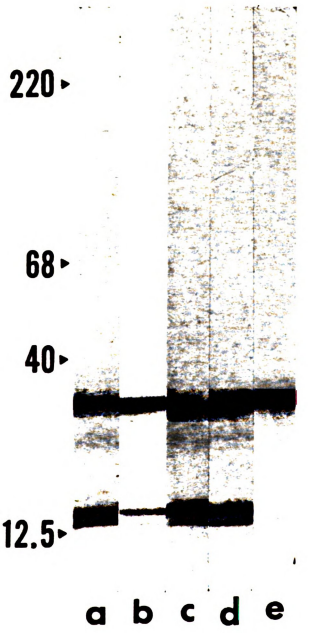
220
68
40
12.5
a b c d e

Figure 9: Affinity chromatography of [^{35}S]methionine-labeled, Triton X-100 solubilized, extracts of 3T3 fibroblasts on a column (0.8 x 10 cm) of Gal-GlcNAc-HA-PA. The column was equilibrated with 50 mM $CaCl_2$, 1 mM NaN_3, 75 mM Tris (hydroxymethyl) aminoethane, 1% Triton X-100, 1 mM phenyl methyl sulfonylfluoride, pH 7.2. The arrows mark the fraction at which the buffers were changed: 1, column buffer without Triton X-100; and 2, 0-0.08 M lactose gradient (----). Fractions of 4 ml were collected through the position of arrow 1; thereafter, fractions of 1.5 ml were collected. Aliquots of 0.2 ml were assayed for radioactivity. The horizontal bar designated A represents fractions pooled for analysis by polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

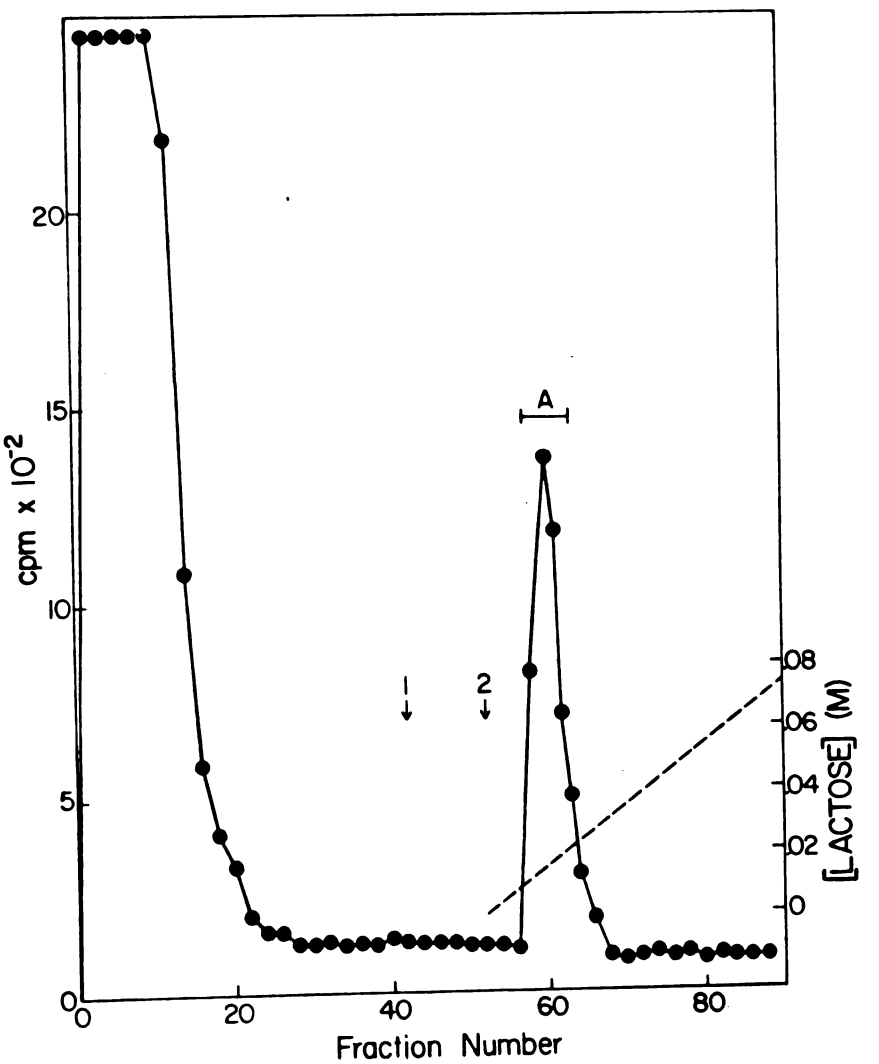
cpm x 10⁻²
20
15
10
5
0
0
20
40
60
80
Fraction Number
A
1
2
[LACTOSE] (M)
.08
.06
.04
.02
0

Figure 9

yielded one predominant band, migrating at the position corresponding to CBP35 (Figure 8, lane e). This band accounted for 99% of the radioactivity on the gel. 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 CBP35 of mouse 3T3 cells.

DISCUSSION

The general procedure for the derivatization of polyacrylamide beads with defined functional groups for the purpose of affinity chromatography was pioneered by the studies of Inman and Dintzis (11, 12). The studies described in the present paper provide new information on the optimization of this general scheme. In addition, the results indicate that we have made advances in the specific applicability of this method to the coupling of CHO ligands in the following major respects.

First, we have avoided the use of beads containing CHO backbones such as Sepharose or Sephadex. This allows us to derivatize specific CHOs of defined structure and specificity on inert supports. Second, we have maximized the efficiency of coupling using a minute amount of CHO ligands, which in this case is the chemical available in limiting quantity. Finally, we have taken particular care to minimize the number of charges, positive or negative. The formation of negative charges on the derivatized PA beads has been kept sufficiently low. More importantly, the derivatized beads were virtually free of a net positive charge, a problem which is particularly acute in derivatization schemes using cyanogen bromide activated Sepharose (9). Since most CBPs isolated from mammalian sources have low isoelectric points (5, 13, 14), this net positive charge may interfere with the purification of these CBPs.

Moreover, the syntheses described here were designed to generate CHO ligands with a side chain containing a terminal amino group suitable for direct condensation with the activated PA beads containing acyl azide moieties. It has been reported (24) that in some instances, ligands attached directly to the solid matrix are ineffective for affinity chromatography and that it is necessary to allow it to extend from the matrix through a linear side chain to enhance the binding of the protein. The use of the HA spacer arm allows for sufficient distance from the solid matrix to decrease any steric interference for binding when the affinity resins are used to bind soybean and wheat germ agglutinin.

We have also taken advantage of the recently developed procedure (18) of using partially purified glycosyltransferases and sugar nucleotides to synthesize disaccharides linked to the HA spacer arm, which in turn can be coupled to the PA bead. Direct coupling of a previously formed ligand of suitable structure assures that the PA beads possess a single kind of functional group. Synthesis of adsorbents by reaction of a ligand with a preformed arm on the PA bead may lead to mixed function adsorbents because of incomplete reactions. Obviously, this method can be extended to the synthesis of more complex oligosaccharides, such as the blood group substances (18).

Roseman and coworkers have recently reported an alternate approach to circumvent the problems associated with the use of carbohydrate containing supports and the formation of positive charges with the use of cyanogen bromide activation. They have copolymerized CHO-linked acrylic acid with acrylamide and bisacrylamide, resulting in PA gels containing specific CHO moieties (8). The present method has a

different applicability from that of Roseman et al. The use of beads instead of gel slabs allows us to carry out fractionation of mixtures in columns packed with derivatized beads. In addition, the use of beads with large and controlled pore size allows a variety of molecules of high molecular weight to penetrate within the resin to interact with internal CHO ligands.

We have tested the specificity of the binding of proteins to PA beads derivatized with various CHO ligands using lectins of known specificity and proteins of known isoelectric properties: (a) WGA (20) binds to GlcNAc-HA-PA but not to Gal-HA-PA; (b) Conversely, SBA (21) binds to Gal-HA-PA but not to GlcNAc-HA-PA; (c) Pepsin, which has an isoelectric point of less than 1 does not interact with GlcNAc-HA-PA, suggesting that the beads do not adsorb highly negatively-charged molecules; and (d) When histones are chromatographed on GlcNAc-HA-PA columns, less than 10% of the protein material is bound and this bound fraction cannot be eluted with the specific saccharide ligand.

In marked contrast to the specificity observed for the binding of WGA to GlcNAc-HA-PA and not to Gal-HA-PA, we have previously found that WGA binds to both GlcNAc-HA and Gal-HA derivatized Sepharose beads (15). The bound lectin could only be eluted from the Gal-HA Sepharose beads using acetic acid. These results represent a clear example of the increased specificity obtained using polyacrylamide as the support resin.

We have also used CHO-HA-PA columns to isolate carbohydrate-specific proteins, one from human fibroblasts and three from extracts of 3T3 mouse fibroblasts: CBP35, CBP16, and CBP13.5. All three proteins from 3T3 cells bound to PA beads containing the disaccharide

(Gal-GlcNAc-HA-PA) but not to beads containing the monosaccharide (Gal-HA-PA). Although the monosaccharide Gal is capable of eluting the polypeptides bound on either kind of affinity support (asialofetuin-Sepharose or Gal-GlcNAc-HA-PA), the mere presence of Gal residues does not appear to be sufficient for the binding of the CBPs to the columns. Since soybean agglutinin binds to Gal-HA-PA, the inability of the CBPs to bind to the resin does not appear to be due to total inaccessibility of the Gal residues. However the CBPs may have their binding sites in a cleft, such that the Gal-HA-PA is not of sufficient dimensions to reach the binding site. Alternatively the aglycone (GlcNAc for Gal-GlcNAc- HA-PA and HA for Gal-HA-PA) may be an important factor for these CBPs to bind.

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SUPPLEMENTAL MATERIAL TO

ENDOGENOUS LECTINS FROM CULTURED CELLS
II. Specific Affinity Columns for the Isolation of Carbohydrate-Binding Proteins

Calvin F. Roff, Paul R. Rosevear, John L. Wang and Robert Barker

MATERIALS AND METHODS

Materials - Pepsin, histone II-A, soybean agglutinin (SBA) and 2-aminoethanol were purchased from Sigma Chemical Co. Hydrazine hydrate was obtained from Aldrich Chemical Co., wheat germ agglutinin (WGA) from Miles Laboratories, DME from K.C. Biologicals, calf serum from Microbiological Associates and Swiss 3T3 cells from American Type Culture Collection (CCL 92). [^{35}S] Methionine (1014 Ci/mmol) was from New England Nuclear and 1,2-[^{14}C]-2- aminoethanol was purchased from ICN Chemical and Radioisotope Division. Bio-Gel P-150 was a product of Bio-Rad Laboratories.

The scintillation cocktail was made of 667 ml toluene, 333 ml of Triton X-100 and 7.0 g of 2,5-diphenyloxazole (PPO).

Synthesis of Carbohydrate Ligands Containing Hexanolamine - GlcNAc-HA (6-amino-1-hexyl)-2-acetamido-2-deoxy-β-D-glucopyranoside) was synthesized by the method of Barker et al. (7) and Gal-HA ((6-amino-1-hexyl)-β-D- galactopyranoside) was synthesized by the procedure of Chiang et al. (15). The hexanolamine-containing disaccharide,

βDGal(1→4)βDGlcNAc-HA, was synthesized using UDPGal and GlcNAc-HA acceptor catalyzed by a partially purified preparation of N-acetylglucosaminide β(1→4)galactosyltransferase. The details of this preparation have been previously described (16). Assays of hexanolamine-containing compounds having a primary amino group were performed with fluorescamine (17).

Preparation of Carbohydrate Affinity Resins - The method of Inman and Dintzis (11,12) was used. Conditions were optimized so as to increase the coupling efficiency and to minimize the charge formation. The reaction scheme is shown in Figure 1. Bio-Gel P-150 was converted to the acyl hydrazide (i) by incubation in 6.1 M hydrazine hydrate at 50° for 3 h. After thorough washing, the acyl azide (ii) was formed by nitrous acid treatment. The acyl azide resin was then reacted with glycosides of hexanolamine to covalently couple the ligands (iii). The unreacted acyl azides were converted back to the amides (iv) by displacement of azide with ammonia.

Bio-Gel P-150 Hydrazide (i) - To 120 ml of 6.1 M hydrazine hydrate at 50° in a 125 ml ground glass stoppered Erlenmeyer flask was added, with stirring, 1 g (dry weight) of Bio-Gel P-150 beads. The flask was kept in a 50° constant temperature H_2O bath and stirred at 15 minute intervals. After 3 h the resin was filter washed on a Buchner funnel with 4 l of 0.2 M NaCl.

Bio-Gel P-150 Acyl Azide (ii) - the hydrazide derivative was transferred to a beaker containing 100 ml of 0.3 M HCl at 0-4° (all

subsequent steps in the synthesis of affinity resins were at 0-4° unless stated otherwise). The mixture was stirred for 10 minutes, after which 10 ml of 1.0 M $NaNO_2$ was added. After 20 minutes of gentle stirring, the beads were filter washed on a Buchner funnel with 2-3 l of H_2O. The resin was then filtered for 10 minutes to remove excess H_2O.

Quantitation of 2-Aminoethanol Covalently Coupled to Bio-Gel P-150 (iv a) - 1,2-[^{14}C]-2-Aminoethanol was diluted with unlabeled 2-aminoethanol and the same stock solution (2.6×10^4 cpm/100 µmol) was used in all reaction studies. To quantitate the amount coupled after the indicated reaction times, the resin was filtered on a 2 cm plastic Buchner funnel; washed with H_2O (40 ml), methanol (40 ml), H_2O (40 ml) and methanol (60 ml); transferred to a scintillation vial; dried for 5-10 minutes in a 100° oven; and counted in 18 ml of scintillation cocktail.

Coupling of GlcNAc-HA to Bio-Gel P-150 Acyl Azide (GlcNAc-HA-PA) (iv b) - To 75% of the acyl azide resin made from 1 g (dry weight) of Bio-Gel P-150 in a 30 ml plastic bottle was added 12 ml of an aqueous solution of GlcNAc-HA (380 µmol) which was adjusted to pH 10.8 immediately before addition. The clumped resin was suspended with a glass rod and the mixture was vortexed at 30 minute intervals throughout the entire reaction period. After 1.8 h, an additional 3 ml of aqueous GlcNAc-HA (95 µmol, pH 10.8) was added. The total reaction time was 4 h, after which an equal volume of 5 M NH_4OH (adjusted to pH 10.0 by the addition of concentrated HCl) was added and the mixture was kept at

0-4° for 20 h. The mixture was poured into a glass column at room temperature and washed sequentially with 200 ml portions of the NH_4OH- NH_4Cl solution, 0.04 M NaCl and PBS. This procedure resulted in the derivatization of 160 μmol (34%) of GlcNAc-HA to 20 ml (bed volume in PBS) of resin.

Coupling of Gal-HA to Bio-Gel P-150 Acyl Azide (Gal-HA-PA) (iv c) - To 75% of the acyl azide resin made from 1 g (dry weight) of Bio-Gel P-150 in a 30 ml plastic bottle, was added 8 ml of an aqueous solution of Gal-HA (462 μmol) which was adjusted to pH 11.5 immediately prior to addition. The mixture was vortexed at 30 minute intervals throughout the entire reaction period. After 1 h an additional 5 ml of H_2O (pH 11.5) was added. The total reaction time was 4 h. The remaining steps were identical to those used for the synthesis of GlcNAc-HA-PA.

Coupling of Gal-GlcNAc-HA to Bio-Gel P-150 Acyl Azide (Gal-GlcNAc-HA-PA) (iv d) - To 50% of the acyl azide made from 1 g (dry weight) of Bio-Gel P-150, was added 4 ml of an aqueous solution of Gal-GlcNAc-HA (80 μmol) which was adjusted to pH 11.5 immediately prior to addition. One h later an additional 2 ml of of H_2O (pH 11.5) was added. The remaining steps were identical to those employed for the synthesis of GlcNAc-HA-PA. Approximately 28 μmol (35%) of Gal-GlcNAc-HA was coupled to 10 ml (bed volume in PBS) of resin.

Coupling of 6-Aminohexanol (HA) to Bio-Gel P-150 Acyl Azide (HA-PA) (iv e) - The method was the same as that used to couple Gal-HA except that

8 ml of aqueous HA (854 μmol, pH 11.5) was added initially and 8 ml of H_2O (pH 11.5) was added after 1 h.

Isolation of CBPs from Mouse and Human Fibroblasts - Swiss 3T3 fibroblasts were obtained from American type Culture Collection (CCL92) and were cultured as previously described (18). Confluent monolayers of these cells were metabolically labeled with [^{35}S]methionine (19). They were then washed with buffer containing 50 mM $CaCl_2$, 2mM NaN_3, 2 mM phenylmethyl sulfonylfluoride and 75 mM Tris (hydroxymethyl) aminoethone, pH 7.2 (buffer A). The cell were scraped into buffer A containing 1% Triton X-100 (1 ml/150 cm^2 flask) and homogenized with 5 strokes/ml in a Potter homogenizer. Insoluble material was removed by centrifugation at 3,000 x g for 15 min. The supernatant was applied to the affinity columns and washed sequentially with a) buffer A containing 1% Triton X-100, b) buffer A and c) buffer A containing galactose or lactose. All column buffers contained 2 mM β-mercaptoethanol. The efluents were monitored for radioactivity. Fractions were pooled, dialyzed against water, lyophilized and anlayzed by polyacrylamide gel electrophoresis (Chapter II).

Human foreskin fibroblasts (SL66) were gifts of Drs. V. Maher and J. McCormick (Michigan State University). They were cultured in Eagle's Minimum Essential Medium containing 20% fetal bovine serum, 100 μg/ml streptomycin and 15 g IU/ml penicillin. The isolation of carbohydrate-binding proteins from these cells was carried out as described for 3T3 fibroblasts (Chapter II).

RESULTS

Incubation of Bio-Gel P-150 in 6.1 M hydrazine hydrate at 50° for 3 h resulted in the partial conversion of the amide groups into the hydrazide moieties. This treatment did not affect the morphology of the beads as examined by light microscopy. In addition, this treatment had no effect on the flow properties of columns packed with the derivatized beads. At longer incubation times, the beads began to crosslink into clumps of 4-8 beads and tended to collapse under normal use in columns.

The effect of pH on the coupling efficiency of ligands is shown in Figure 2. The two approaches that we investigated were to buffer the reactions with 0.2 M borate or to adjust the pH of an aqueous solution of the ligand with NaOH immediately prior to its addition into the acyl azide. The optimal pH for the addition in H_2O was 11.0 and that for the buffered reaction was 10.0. When the reaction was carried out in H_2O without any buffer, the pH of the solution dropped from an initial value of 11.0 to 7.7 after 5 h. By contrast, in a buffered solution the pH dropped less than 0.5 units during the entire reaction time. To avoid prolonged incubation in a basic solution, a condition which could lead to the base hydrolysis of the unstable acyl azide, the method chosen for preparative work was the addition of ligand in H_2O at pH 11.0

Figure 2: The effect of pH on the coupling of 2-aminoethanol to Bio-Gel P-150 acyl azide. Each reaction mixture contained 100 μmol of 2-aminoethanol, 1 g (wet weight) of Bio-Gel P-150 acyl azide (7 g wet weight azide/1 g dry weight Bio-Gel P-150) and either 8 ml of borate buffer (pH 7,8,9,10,11) or 8 ml of H_2O (pH 7,8,9,10 and 11 adjusted to the proper pH immediately prior to the addition to the resin). The reaction time was 26 h.

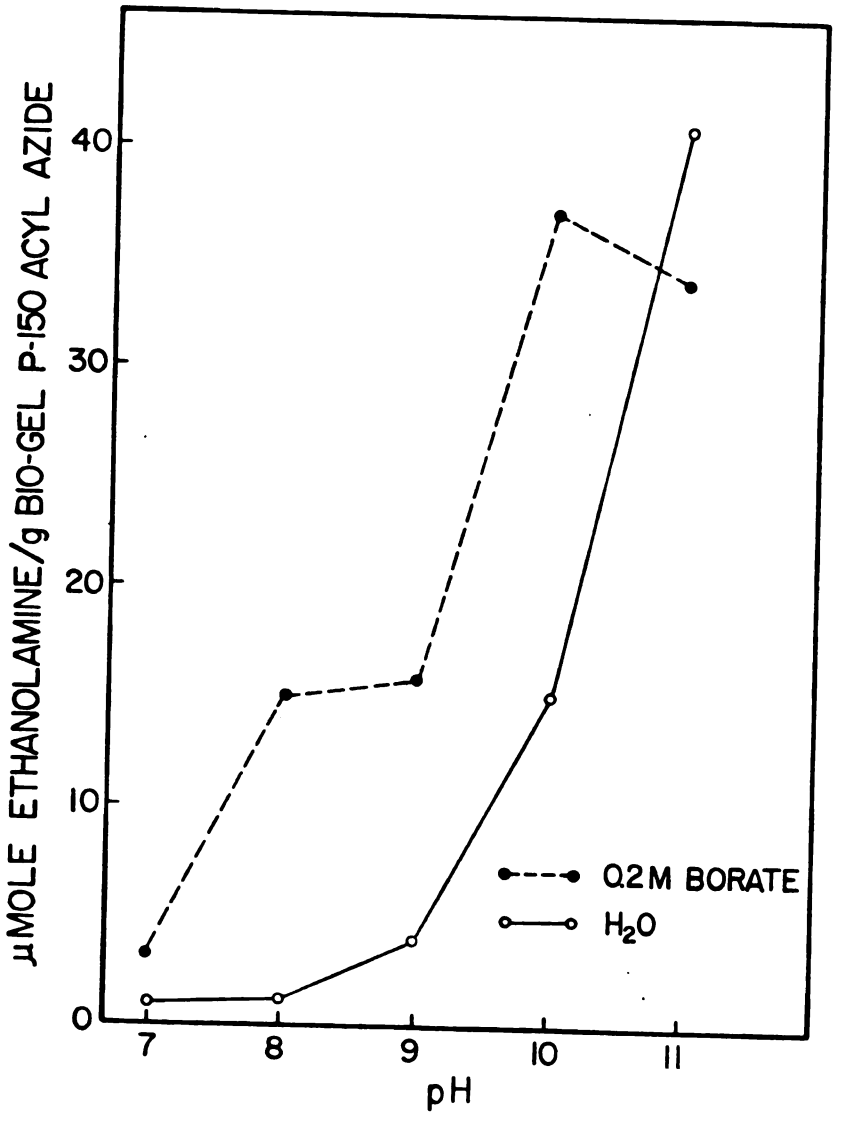
μMOLE ETHANOLAMINE/g BIO-GEL P-150 ACYL AZIDE
40
30
20
10
0
7
8
9
10
11
pH
0.2M BORATE
H2O

Figure 2

It has also been observed that slightly more ligand is coupled to the resin in H_2O, pH 11.0, than in the buffered reactions. Presumably this is due to the decreased swelling of the beads caused by salt effects in the buffered reaction.

The rate and extent of coupling was greater in reactions carried out in H_2O at pH 11.0 than those in H_2O, pH 9.5 (Figure 3). This coupling was also faster than those carried out in the buffered solutions (pH 8.5, 10.0). Although in this set of experiments the reaction in H_2O, pH 11.0, appears to be complete after 6 h, it was found that the final quantity of coupled ligand could be increased by 20-30% if the reaction was allowed to proceed for 24 h under optimal conditions. Again, in order to avoid possible free carboxyl formation, the method used for preparative work was addition of the ligand in H_2O, pH 11.0, and allowing the reaction to proceed for 5-6 h.

The effect of reaction volume on the coupling efficiency is shown in Figure 4. The optimal volume used for our reaction is 8-12 ml. Since the final wet weight of the acyl azide varies, depending on the length of time used to remove the excess H_2O from the resin, a more useful parameter for preparative work is the ratio of the total reaction volume to the resin volume after 1 h reaction time. The resin is fully swollen at this time. Expressed in these terms (reaction volume/resin volume), the optimal ratio is 1.5-2.5.

A key consideration in optimizing our reaction conditions is the limited amount of starting material, particularly the disaccharide ligands to be coupled (usually 100 μmol or less). The applicability of our reaction conditions to couple sufficient ligand to PA beads is

Figure 3: The rate of coupling 2-aminoethanol to Bio-Gel P-150 acyl azide. Each reaction mixture contained 100 μmol of 2-aminoethanol, 0.6 g of Bio-Gel P-150 acyl azide (8 g wet weight/1 g dry weight Bio-Gel P-150) and either H_2O or 0.2 M borate buffer. At each time point, the reaction was terminated by filter washing as described in Methods.

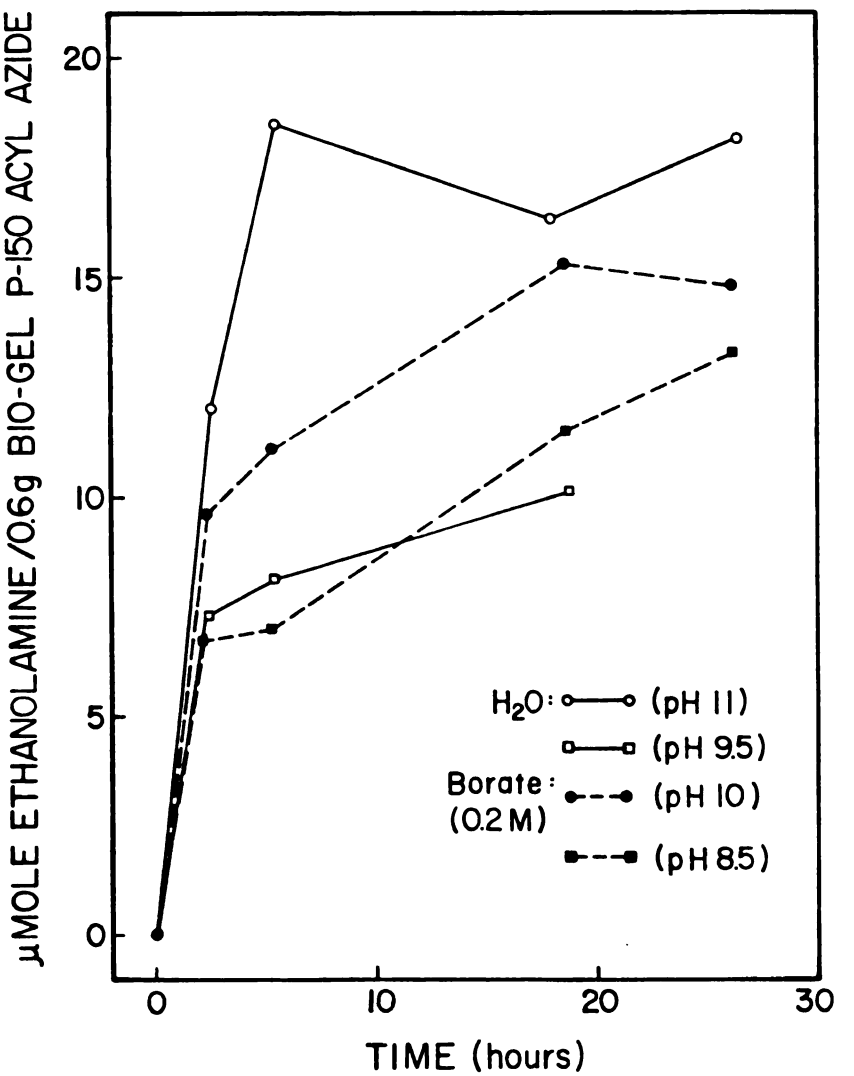
µMOLE ETHANOLAMINE/0.6g BIO-GEL P-150 ACYL AZIDE
20
15
10
5
0
0
10
20
30
TIME (hours)
H2O: (pH 11)
(pH 9.5)
Borate: (0.2 M)
(pH 10)
(pH 8.5)

Figure 3

Figure 4: The effect of reaction volume on the efficiency of coupling 2-aminoethanol to Bio-Gel P-150 acyl azide. Each reaction mixture contained 100 μmol of 2-aminoethanol in the indicated volume of H_2O (initial pH of 11.0) and 1 g of Bio-Gel P-150 acyl azide (8 g wet weight azide/1 g dry weight Bio-Gel P-150). The reaction time was 30 h. Under these conditions the ratios of the reaction volume to the resin bed volume were <1, 1, 2, 2.6, and 4, respectively, for reaction volumes of 2, 4, 8, 12, and 20 ml.

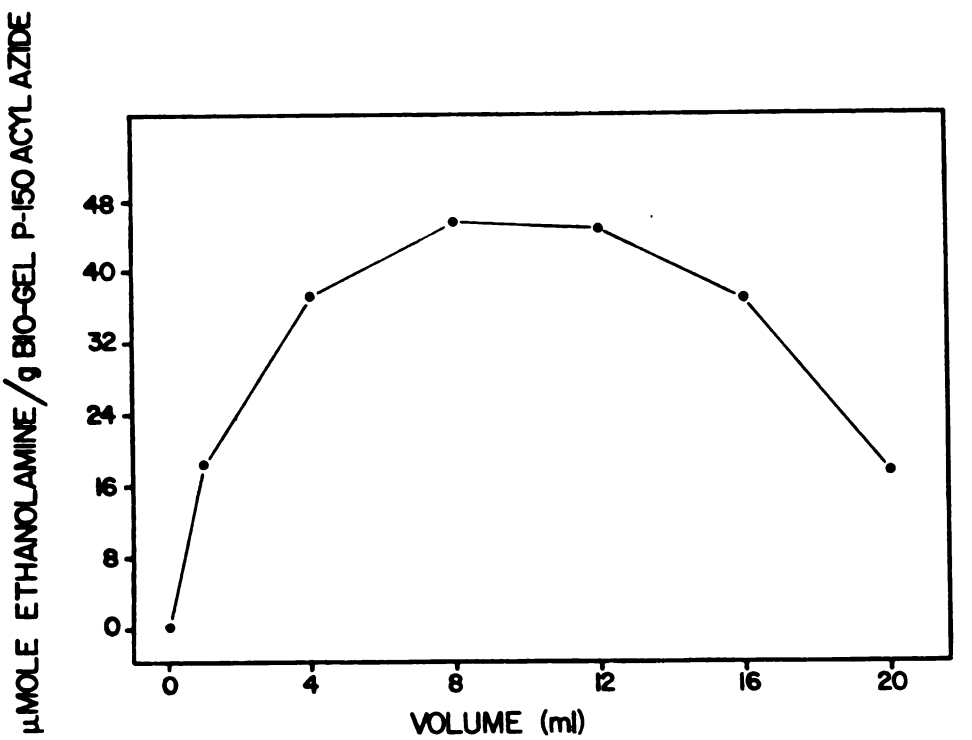
µMOLE ETHANOLAMINE/g BIO-GEL P-150 ACYL AZIDE
0
8
16
24
32
40
48
0
4
8
12
16
20
VOLUME (ml)

Figure 4

shown in Figure 5. After a reaction time of 30 h, a coupling efficiency of approximately 40% is obtained.

It was important for our purposes that the unreacted acyl azides be converted into a neutral amide rather than be hydrolyzed into negatively charged carboxyl groups. Regeneration of the amide moiety from the acyl azide was best accomplished with 5 M NH_4OH which had been adjusted to pH 10.0 with concentrated HCl (5 M NH_4OH-NH_4Cl, pH 10.0). The use of this reagent instead of NH_4OH drastically reduced the formation of free carboxyls, determined by titration studies (12). This conclusion was verified by chromatography of histones on columns containing beads prepared by each method. When the histones were chromatographed on beads which were converted to the amide form by treatment with NH_4OH, all the histones bound and were eluted with 0.4 M NaCl. In contrast, 90% of the histones did not bind when applied to a column containing beads which were converted back to the amides with 5 M NH_4OH-NH_4Cl, pH 10.0 (as demonstrated in Figure 6E).

Figure 5: Effect of increasing 2-aminoethanol added on the final amount of 2-aminoethanol coupled to Bio-Gel P-150. Each reaction contained 1 g of Bio-Gel acyl azide (8 g wet weight/1 g dry weight Bio-Gel P-150), 8 ml of H_2O (initial pH of 11) and the indicated amount of 2-aminoethanol. Reaction times were 30 h.

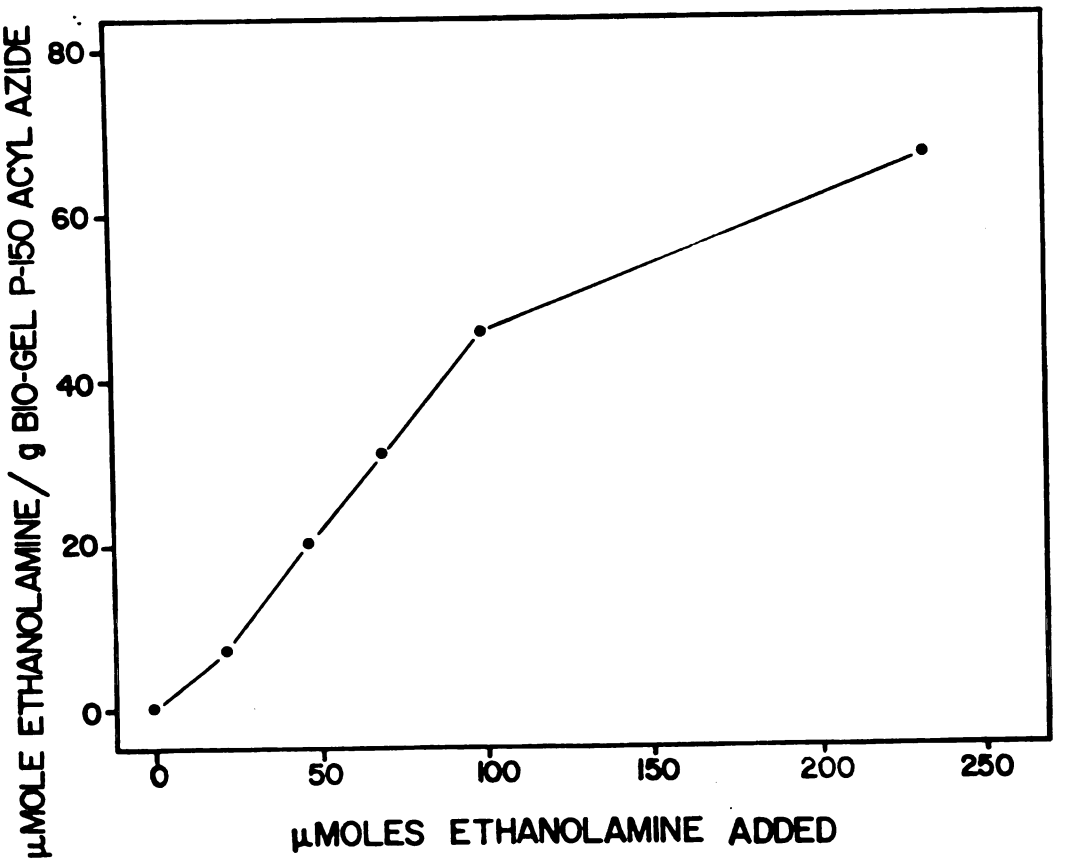
µMOLE ETHANOLAMINE / g BIO-GEL P-150 ACYL AZIDE
80
60
40
20
0
0
50
100
150
200
250
µMOLES ETHANOLAMINE ADDED

Figure 5

Chapter IV

Isolation and Binding Properties of a Lectin from Mouse Lung

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Running Title: Mouse Lung Lectin

SUMMARY

In previous studies, a lectin designated as Carbohydrate-Binding Protein 35 (CBP35) has been isolated from cultured mouse 3T3 fibroblasts. Antibodies directed against CBP35 were used to screen for cross-reactive proteins in various organs and tissues of the mouse. Cross-reactive proteins of the same molecular weight (M_r = 35,000) were found in lung, thymus, spleen and arteries. Fractionation of extracts of mouse lung on affinity columns of asialofetuin-Sepharose yielded a protein whose molecular weight, carbohydrate-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 glycoconjugates containing galactose were inhibitors of the binding; the disaccharide lactose was 100-fold more potent as an inhibitor than the monosaccharide galactose.

In previous studies, we have purified three galactose-specific carbohydrate-binding proteins (CBPs) from cultured mouse 3T3 fibroblasts (1, 2). 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 the 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, 4), embryonic eel (5), and bovine (6,7) 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 CBP35 in some detail. In the course of these studies, it became apparent that the purification of CBP35 from cultured fibroblasts yielded too little material (less than 1 μg per 10^8 cells) for the determination of the chemical structure and for the characterization of the carbohydrate-binding specificity. Therefore, we have screened for an analogous protein from mouse tissue so that a large scale isolation of CBP35 could be accomplished. In the present paper, we report the isolation and binding properties of CBP35 from mouse lung.

METHODS AND MATERIALS

Materials

Mice (A/J) were obtained from Charles River. Carrier free $Na^{125}I$ (100 mCi/ml) was purchased from Amersham. Sepharose and Sephadex were from Pharmacia, Aquacide III from Calbiochem, fetuin from Gibco, nitrocellulose paper from Schleicher and Schell and goat anti-rabbit IgG from Sigma. 1,3,4,6-Tetrachloro-3a, 6a-diphenylglycoluril was a generous gift from Dr. J.C. Speck Jr., Michigan State University.

Preparation of tissue samples

Tissues were removed from a freshly sacrificed adult male mouse and washed with buffer containing 0.9% NaCl, 0.1% (ethylenedinitrilo)-tetraacetic acid, 0.05% iodoacetamide, 2mM phenyl methyl sulfonylfluoride (PMSF), 20 mM citrate and 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (Hepes) pH 6.1 at 4°. The tissues were minced and homogenzied (10 strokes/ml) with a Potter homogenizer (102-152 μm clearance) in 0.9% NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecylsulfate (SDS), 0.05% iodoacetamide, 2 mM PMSF and 10 mM Tris (hydroxymethyl)aminoethane (Tris), pH 7.2 (3-4 volumes/tissue volume). Bone extracts were prepared by removing the femurs, breaking them into small pieces and extracting with the detegent 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 Lis and Sharon (8). The homogenates were centrifuged at 1500 x 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° for 20 min., insoluble material was removed by centrifugation at 1500 x g for 10 min. and the resulting supernatants diluted to 1 mg protein/ml with electrophoresis sample buffer.

Polyacrylamide gel electrophoresis and analysis by immunoblotting

Polyacrylamide gel electrophoresis in SDS was performed according to the method of Laemmli (9) on 1 mm thick, 9 cm long 5-16% acrylamide gradient slabs (0.21-0.67% bisacrylamide) with 1 cm long 4% stacking gels. Samples were dissolved (diluted for tissue distribution) in 1% SDS, 2% β-mercaptoethanol and 0.06 M 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 gel was dried and radioactive bands detected by autoradiography using Kodak X-Omat RP (XRP-5) film.

The procedure for transfer of proteins from polyacrylamide gels to nitrocellulose paper, for staining with Amido black and for blotting with antisera were those described by Tobin et al. (10) with minor modifications. After electrophoresis, the proteins were transferred at 400 mA for 4 hr. The nitrocelluose paper was then placed in saturating buffer overnight, followed by incubation with anti-CBP35 (3T3), (antiserum raised against CBP35 derived from 3T3 cells (2)), at a dilution of 1:250 for 5 h. After washing, the paper was incubated with 1.6 x

10^6 cpms/10 ml of ^{125}I-goat anti-rabbit IgG (1.9 x 10^8 cpm/mg) for 5 h. The papers were washed, dried and subjected to autoradiography.

Isolation of CBP35 from murine lung

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 and 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 approximately 1 ml/lung. The slurry was homogenzied with one stroke on a Potter-Elvehjem homogenizer at 1,200 rpm and the resulting homogenate was stirred for 30 min. The homogenate was centrifuged at 12,000 x 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 the CBPs from 3T3 cells (2). The CBPs were further purified by a second round of affinity chromatography and fractionated by molecular sieve chromatography as previously described (2). All procedures were performed at 4°. Column eluants were assayed for protein by the method of Bradford (11).

Iodination procedures

CBP35 was labeled with ^{125}I by the method of Fraker and Speck (12). After two rounds of affinity chromatography, 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 µg of chloroglycoluril. To this was added 800 µCi 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-15-120 (1.4 x 27 cm). Material eluted in the void volume was pooled, concentrated by reverse dialysis and chromatographed on a Sephadex G-150 column (0.8 x 115 cm). Material chromatographing to a region corresponding to a M_r = 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 (13). 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. (3). To 175 µl of a 20% (v/v) suspension of erythrocytes was added 100 µl of ^{125}I-labeled CBP35, 100 µl sugar containing solution and 25 µl Buffer A. All reagents were in Buffer A containing 2 mM β-mercaptoethanol. The suspension was incubated for 20 min. at 4°, centrifuged at 1,000 x g and the supernatant discarded. The erythrocyte pellet was resuspended in 0.4 ml Buffer A containing 2 mM β-mercaptoethanol, repelleted at 1,000 x g and the supernatant discarded. Radioactive CBP35 bound to the erythrocytes was determined by counting the washed pellet on a LKB 1271 Riagamma counter.

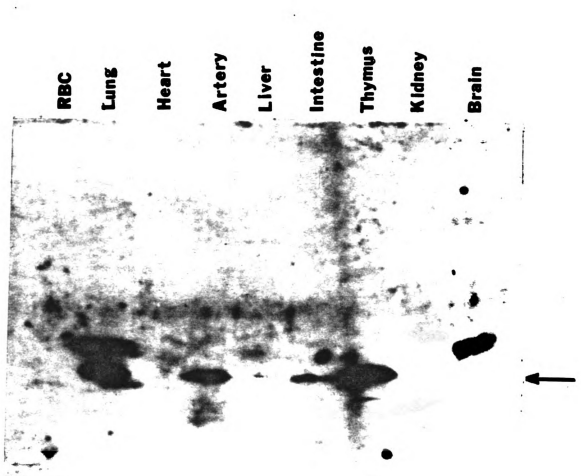
RESULTS

Survey of the Distribution of CBP35 in Tissues of the Mouse

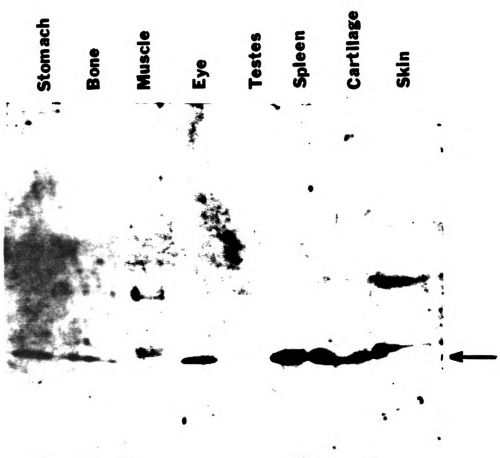
To determine the tissue distribution of CBP35, an immunoblotting technique was used. Equal amounts of protein (100 µg) from various mouse tissue extracts were subjected to SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose paper and blotted with antisera directed against CBP35 (Fig. 1). Since equal amounts of protein were loaded, the intensity of the band corresponding to a polypeptide of molecular weight 35,000 detected by autoradiography most probably reflects the relative abundance of CBP35 in the tissues.

Autoradiography of the blotted nitrocellulose paper revealed relatively large amounts of reactive material with M_r = 35,000 in lung, artery, thymus and spleen (Fig. 1). The reactive material is also present to a lesser extent in small intestine, stomach, bone, eye, cartilage and skin. Finally, it was not detected in erythrocytes, heart, liver, kidney, muscle and testes. An additional band was observed in lung and brain. This material migrated to a position of M_r = 42,000. No other reactive polypeptides were detected. In particular, there was no reactive material corresponding to CBP16 and CBP13.5 in any of the tissues tested with anti-CBP35.

Figure 1. Survey of the tissue distribution of CBP35 in adult male mouse. Tissue extracts 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 Materials and Methods. The gels were elctrophoresed from top to bottom. The arrow marks the position of migration of CBP35 from 3T3 cells.



Panel A



Panel B

Purification of CBP35, CBP16 and CBP13.5 from Mouse Lung

Because the lung showed a relatively large amount of material reactive with anti-CBP35 (Fig. 1, panel A, lane 2), we tested whether this tissue could serve as a source for large scale preparation of CBP35. Homogenates of lung were fractionated by two cycles of affinity chromatography on asialofetuin-Sepharose. Upon elution with lactose, a peak of protein was eluted from the columns (Fig. 2). A polypeptide (M_r = 35,000) was present in this peak as determined by gel electrophoresis and Coomassie Blue staining (Fig. 3, lane a). This polypeptide was the only stained protein observed.

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 stained with Amido black; this revealed a polypeptide with M_r = 35,000 (Fig. 3, lane b). In parallel, another strip of nitrocellulose paper was blotted with anti-CBP35 (3T3) followed by ^{125}I-labeled goat anti-rabbit IgG. Autoradiography revealed a reactive polypeptide of M_r = 35,000 (Fig. 3, lane c). This polypeptide migrated to a position identical with that of the Amido black stained polypeptide. The CBP35 isolated and identified from lung tissue will be hereafter designated CBP35 (lung).

Although CBP16 and CBP13.5 were not detected by staining with Coomassie Blue, they were present in the material purified by two cycles of affinity chromatography (Fig. 2). When this material was labeled with ^{125}I (see below), subjected to gel electrophoresis and autoradiography, three major bands are seen. These corresponded to CBP35, CBP16 and CBP13.5 (data not shown).

Figure 2. Representative column profile showing the affinity chromatography of mouse lung extracts on an asialofetuin-Sepharose column (1.2 x 15 cm). Lung extracts from 100 mice were subjected to affinity chromatography on asialofetuin-Sepharose as described in Materials and Methods. The lactose eluted material from the first cycle of affinity chromatography was dialyzed in the presence of asialofetuin-Sepharose and poured into a column. Fractions (4 ml) were collected and 0.4 ml aliquots were assayed for protein by the method of Bradford (11). The arrow marks the beginning of the lactose gradient (0-150 mM lactose, 100 ml total volume). The horizontal bar indicates fractions which were pooled for gel electrophoresis analysis, for gel filtration on Sephadex G-150, and for iodination.

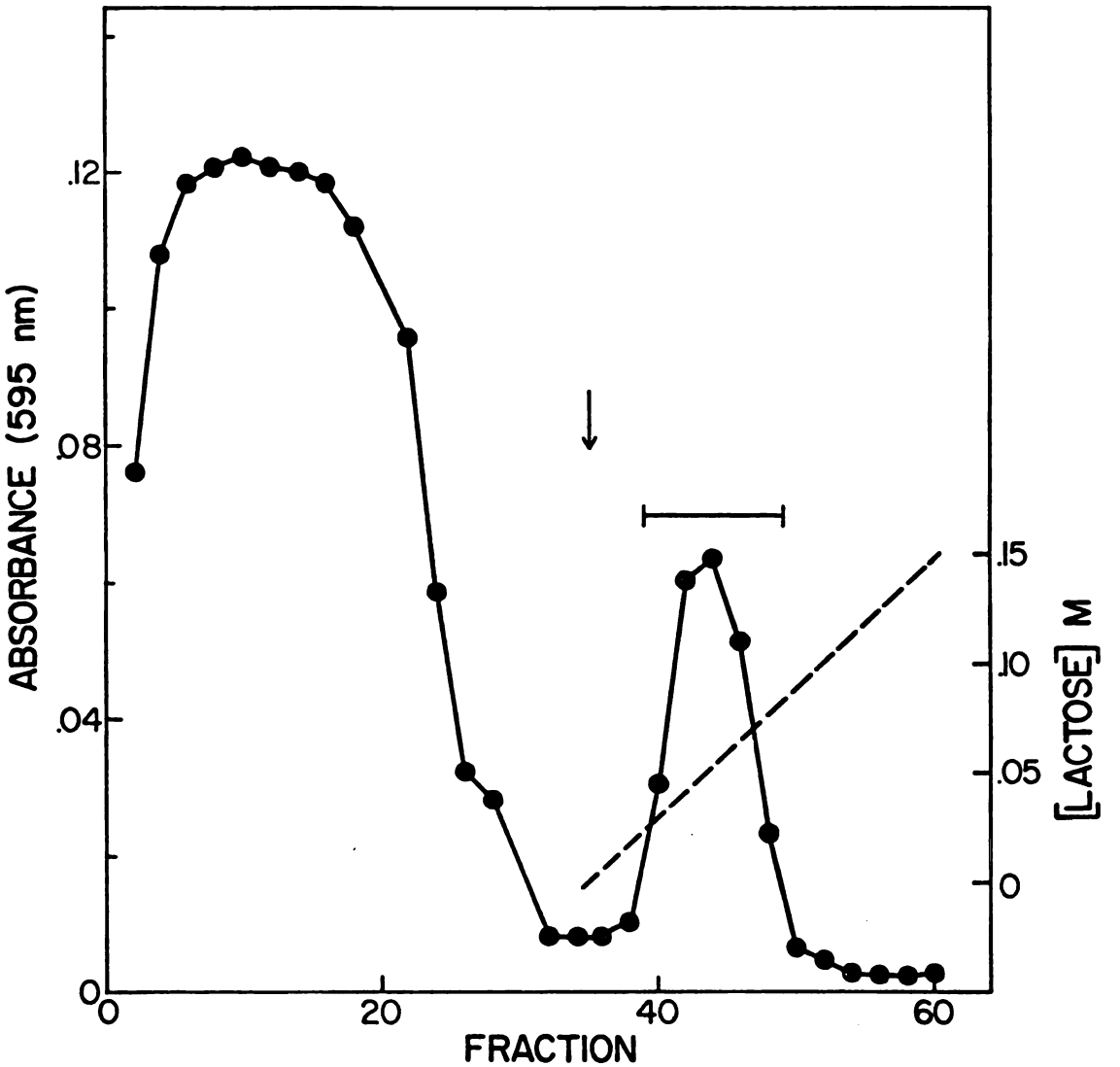
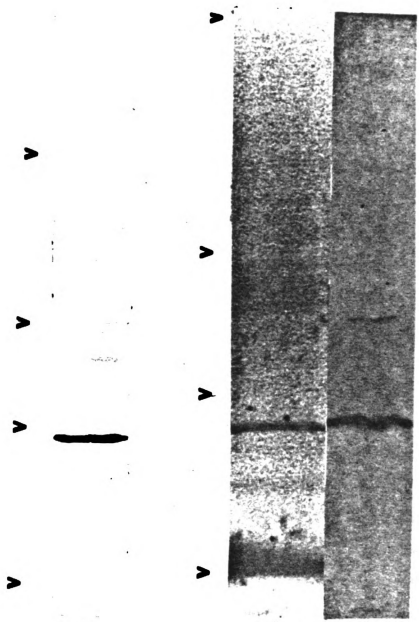

ABSORBANCE (595 nm)
.12
.08
.04
0
0
20
40
60
FRACTION
.15
.10
.05
0
[LACTOSE] M

Figure 2

Figure 3. Polyacrylamide slab gel electrophoresis of CBP35 purified from mouse lung. Lane a: Coomassie Blue stained gel; Lane b: Amido black stained nitrocellulose paper after transfer from a polyacrylamide gel; Lane c: 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, Lanes b and c are from an 11 cm long slab gel. The arrows mark the positions of migration of molecular weight standards which were run in parallel lanes. 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).

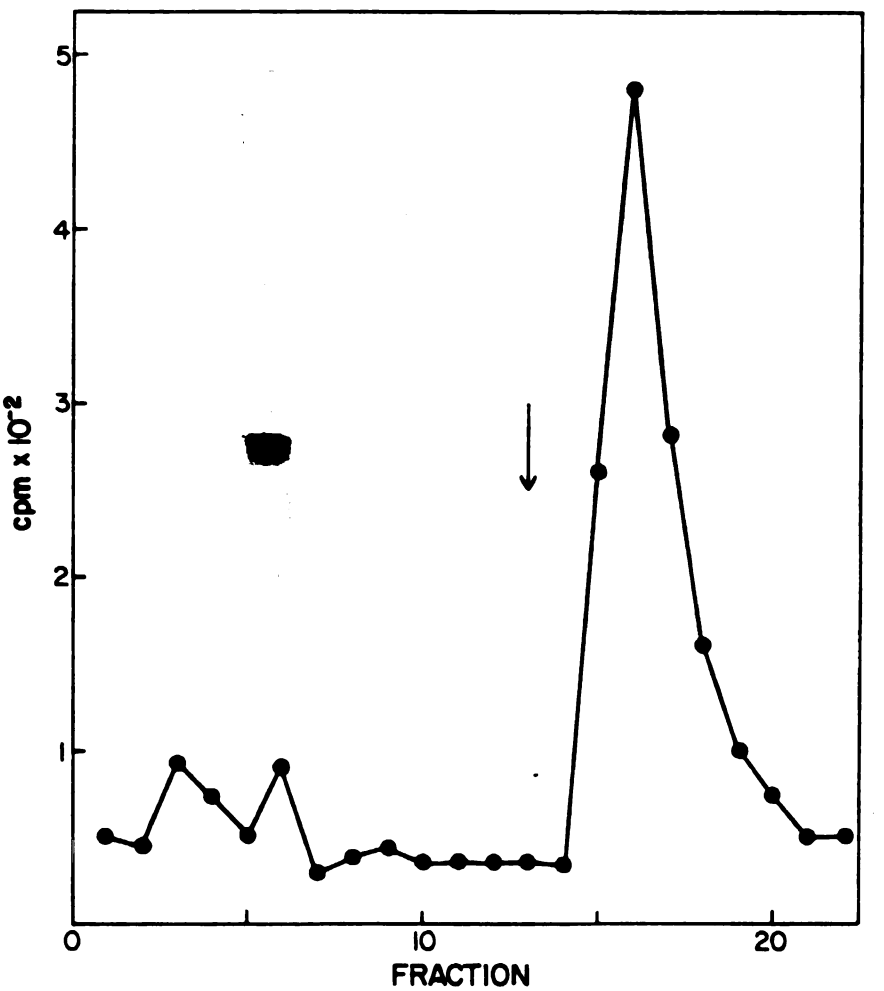

a
b
c

Iodination of CBPs with Retention of Carbohydrate-Binding Activity

We have previously noted that all three fibroblast CBPs are sensitive to air oxidation (2). This oxidation is most probably the cause of loss of binding activities of the CBPs upon iodination by numerous methods. We have found, however, that one method, employing glycoluril, does not destroy their activity. Mouse lung CBPs purified by two cycles of affinity chromatography were iodinated by the method of Fraker and Speck (12). 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 over Sephadex G-150 (2). The material which migrated to a position corresponding to molecular weight of 35,000 was rechromatographed on an asialofetuin-Sepharose column (Fig. 4). More than 90% of the material loaded was bound. Furthermore this bound material could be eluted with lactose. Polyacrylamide electrophoresis of the material loaded onto this column revealed CBP35 (lung) and a trace of a polypeptide of M_r = 34,000 (Fig. 4, inset). The 34,000 dalton polypeptide has also been seen in some preparations of CBP35 from 3T3 fibroblasts (2).

The iodinated material has also been dialyzed in tubing, containing asialofetuin-Sepharose, to remove unincorporated ^{125}I. When the resin was poured into a column and eluted with lactose, only CBP35, CBP16 and CBP13.5 were present in the resulting peak. Relatively small amounts were present in the flow through fractions. Therefore this iodination procedure can be used to label all three CBPs without destroying their binding activities.

Figure 4. 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 determined by scintillation 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.


5
4
3
2
1
cpm x 10⁻²
0
10
20
FRACTION

Binding of CBP35 (lung) to Erythrocytes and Inhibition by Specific Saccharides

CBP35 (lung) labeled with ^{125}I bound to trypsinized glutaraldehyde-fixed rabbit erythrocytes. 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-red blood cell interaction. For these studies, the amount of ^{125}I-labeled CBP35 (lung) bound to the erythrocytes saturated at a concentration of 5% (v/v) for the erythrocytes. Further increases in the number of red blood cells 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 haptene inhibitor.

At a concentration of 0.1 M, all haptenes which contained galactose or derivatives of galactose exhibited between 63-79% inhibition as compared to controls (Table I). 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 effect of galactose and lactose on the binding of CBP35 were analyzed in greater detail (Fig. 5). 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 0.01 mM and above, with 50% of the maximal inhibition at

Table I

Effect of Saccharides on the Binding of CBP35(lung) to Erythrocytes

HAPTENE*	CPM Bound	% Inhibition
No Additions	754	0
Lactose	161	79
N-Acetyllactosamine	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

*All monosaccharides were of the D-configuration except for L-fucose. The final concentrations of haptenes in the binding assay were 0.1 M except N-acetyllactosamine which was 0.006 M.

Figure 5. Effect of galactose, o——o, and lactose, ●——●, on the binding of ^{125}I-labeled CBP35 (lung) to trypsinized, glutaraldehyde fixed rabbit erythrocytes. ^{125}I-Labeled CBP35 (1552 cpms) was incubated with erythrocytes for 30 min. at 4° in the presence of various sugar haptenes. Nonbound CBP35 was removed by a centrifugation washing procedure as described in Materials and Methods. In the absence of inhibitors 48% of the labeled CBP35 bound.

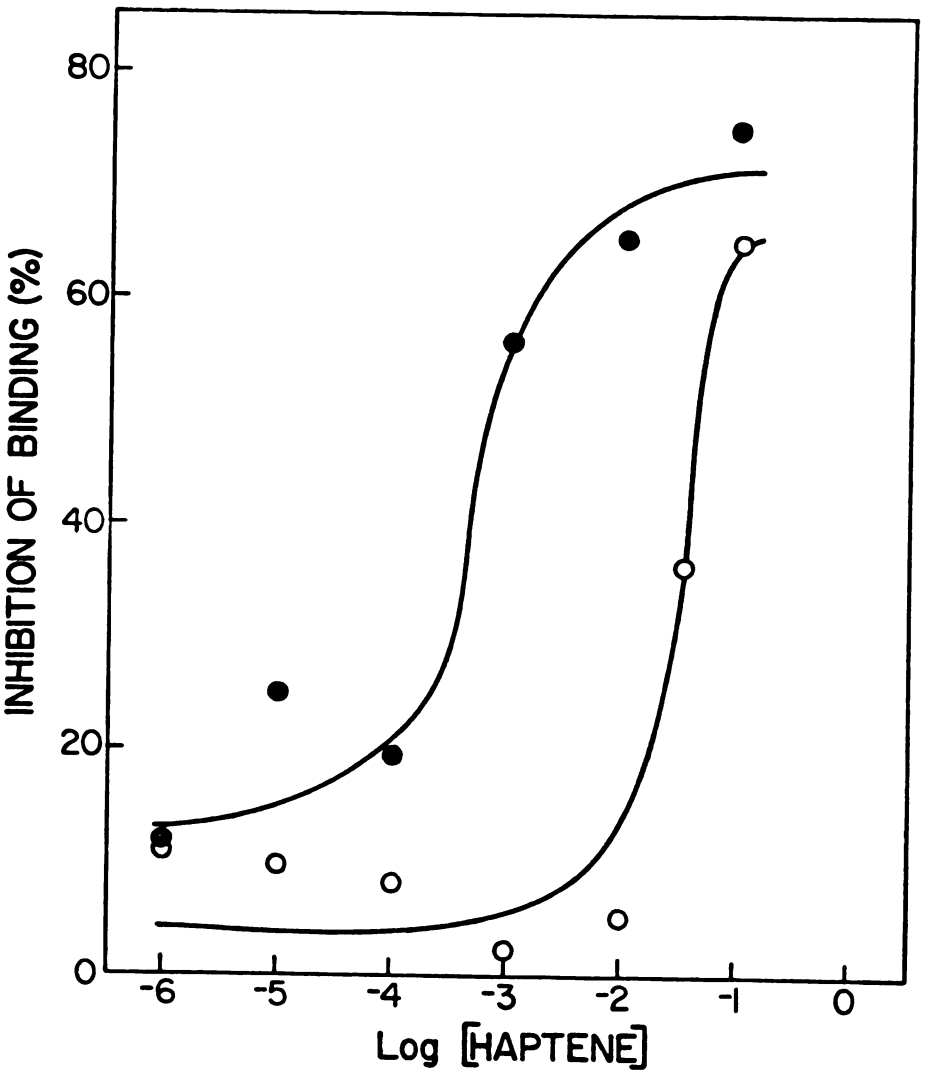


Figure 5

.25 mM. 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 (Chapter II), and to bind to polyacrylamide beads derivatized with β-gal (1⟶4)βglcNAc (Chapter III), all in a lactose inhibitable manner, these proteins appear to be fibroblast lectins. In addition, an antisera to chicken lactose lectin I (3) precipitated CBP16.

In order to isolate CBP35 on a larger scale than previous experiments, we have searched various mouse tissues for a source of this protein. The distribution of CBP35 in various mouse tissues correlated with the fibroblast content of these tissues. CBP35 is abundant in lung, artery, thymus and spleen, tissues which are rich in fibroblasts. It was also present to a lesser extent in other tissues. Bone, skin, eye and cartilage contained lower amounts of CBP35. These tissues also contain fibroblasts. However, due to the nature of these tissues, extracts were difficult to obtain and therefore these tissues may contain more CBP35 which was not extracted. In contrast, CBP35 was not detected in liver, muscle, heart and testes. These tissues share a feature of containing a preponderance of single, highly differentiated cell types. Therefore, we conclude that CBP35 is absent or is present in very diminished amounts in hepatocytes, Kupffer cells, muscle cells

and sperm cells. Although these results suggest that CBP35 is a fibroblast specfic lectin, it remains to be determined on a more defined level (i.e. homogeneous cell population). Furthermore, it has been shown that in the case of chicken lactose lectin I and II, the quantities of these lectins were developmentally regulated (15). Therefore, for the highly differentiated cells, CBP35 may have been present only during one stage of development.

The absence of any detectable CBP35 in liver, heart and muscle, all metabolically active tissues, argue against the possibility that CBP35 is an enzyme involved in glycolysis. We have previously shown that CBP35 has neither β-galactosidase nor sialyltransferase activity. Therefore the only activity which CBP35 has been shown to possess is carbohydrate binding activity.

A crossreactive polypeptide (M_r = 42,000) was also detected in lung and brain (Fig. 1, panel a). Examination of the Coomassie Blue stained gel patterns of these tissues did not reveal any additional or more intensely stained bands at this position, when compared to 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 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.

There were no other detectable crossreactive polypeptides present in these extracts. Based on this result and results from similar immunoblotting experiments of extracts of Swiss 3T3 cells (unpublished)

observation), as well as the purification of the lectin from cultured fibroblasts and from mouse lung, it appears that CBP35 is not a cleavage product of a higher molecular weight protein.

Since CBP35 was found to be most abundant in lung tissue, we have used it as a source for large scale preparation of CBP35. The purification scheme worked out for the isolation of the CBPs from cultured cells (2) was found to be applicable to the isolation of corresponding proteins from lung. The protein from lung was transferred to nitrocellulose paper and blotted with antisera raised against CBP35 isolated from Swiss 3T3 fibroblasts (Fig. 3, lane c). The presence of a radioactive band corresponding to M_r = 35,000 indicates that this protein is indeed CBP35. This result corraborates the results obtained from the immunoblotting of tissue extracts.

The quantity of CBP35 per mouse lung has been estimated in the following manner. The peak fractions from the second asialofetuin column (Fig. 2) contains $\sim$140 µg of protein as determined by the method of Bradford using bovine serum albumin as a standard. Using a ratio of 3:1:1 for CBP35:CBP16:CBP13.5 (as determined for fibroblasts) (2), then approximately 84 µg of CBP35 was isolated from the lungs of 100 mice. Therefore, the lungs of each adult mouse contain approximately 0.84 µg of CBP35. This estimate is most probably high since after dialysis and lyophilization, substantially less material was obtained.

During the course of our studies on CBP35, we have attempted to iodinate it using chloramine T (13) and lactoperoxidase (14). In both cases, we have failed to obtain labeled CBP35 which will bind to asialofetuin. One method which results in the retention of binding

activity is that which employs the sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (12). The use of this reagent results in destruction of less than 10% of the binding activity (Fig. 4). Therefore, this iodination technique was useful in labeling the purified CBPs. Furthermore, the combination of antibodies specific for CBP and an iodination procedure amenable to the labeling of cell surface molecules will permit the subcellular localization of CBP35.

The availability of an iodination procedure which results in labeled CBP35 with binding activity permitted the determination of the effect of sugar haptenes on the binding of CBP35 to erythrocytes. All terminal galactose-containing haptenes inhibited the binding. At the concentration used (0.1 M), the anomeric or glycosidic linkage did not alter their inhibitory ability. All other mono- and disaccharides were noninhibitory. This is in good agreement with our previous reports of the inability of sucrose, mannose and N-acetyglucosamine to elute the CBPs from the affinity columns. Therefore, CBP35 binds specifically to galactose-containing glycoconjugates.

The ability of galactose to inhibit the binding of CBP35 was compared to that of lactose. Lactose was 100 fold more potent in this assay. These results are very similar to the differences seen in the ability of the same sugars to inhibit binding of the lectins from chicken (3, 4) and bovine (6, 7) tissues to erythrocytes.

We have also used this iodination procedure to label a cell membrane preparation from human fibroblasts. This labeled membrane preparation was passed over columns of asialoorosmucoid. Material eluted with lactose contained CBP35, CBP16, CBP13.5 and an additional

band of M_r = 15,000 (unpublished data). This polypeptide may represent a fourth galactose specific CBP found in fibroblasts.

CBP35, CBP16 and CBP13.5 show striking physiochemical similarities to the chicken lactose lectins. These include: a) sensitivity to air oxidation, b) lack of divalent cation requirement for binding, c) sugar specificity and haptene inhibition, and d) low isoelectric points. We have shown that, based on immunological crossreactivity, CBP16 is the murine analogue of chicken lactose lectin I. However, anti-chicken lactose lectin I does not crossreact with CBP35. We have also shown that antibodies directed at CBP35 do not precipitate CBP16 or CBP13.5 when these proteins are in the native form. The studies reported here confirm these results with one major addition; anti-CBP35 does not crossreact with CBP16 and CBP13.5 when they are in the denatured from. Therefore CBP35 has distinct antigenic sites.

Furthermore, in the adult animal, CBP35 shows drastically different tissue distribution from that reported for chicken lactose lectin I and II (15). CBP35 is abundant in lung and spleen (Fig. 1); the chicken lactose lectins are not. Whereas chicken lactose lectins are found in adult kidney, CBP35 is absent. Finally, chicken lactose lectin I is quite abundant in chicken liver, but CBP35 was not detected in mouse liver. When the immunoreactivities and tissue distribution are taken together, CBP35 appears to be an unique lectin. Of course, this remains to be detemrined on a more refined biochemical level such as amino acid composition and peptide mapping. The purification of the CBPs in sufficient quantities from tissues will permit this type of investigation.

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CLOSING STATEMENT

Although carbohydrate recognition had been implicated in a number of cellular processes, including the regulation of cell proliferation in fibroblasts, very little detail concerning the molecular nature of the interacting components (recognizer: CBP; recognizee: glycoconjugates) has been elucidated. In fact, when this project was initiated, CBPs had not even been demonstrated to be present in 3T3 fibroblasts. It is now known that these cells contain four CBPs: (a) the mannose 6-phosphate receptor; (b) CBP35; (c) CBP16; and (d) CBP13.5.

Whereas the function of the mannose 6-phosphate receptor has been established, that of the galactose-specific lectins are still unknown. Comparison of the properties of these galactose-specific CBPs with those of the chicken lactose lectins (Table I) suggest that at least some of them are related. Therefore, the data obtained from the present studies may complement the information accumulated in the chicken system. Together, they may provide important knowledge in the search for the function which may be common to some of the lectins.

Moreover, because CBPs have been identified in cultured fibroblasts, a system is now available for the analysis of their possible function(s) under specific and well-defined conditions (e.g. growth arrest versus proliferation; sparse versus confluent densities etc.). The availability of purified lectins as well as antibodies directed

against the lectins form the foundation upon which the functional studies will be based.

	CBP 35	CBP 16	CBP 13.5	HEPATIC LECTIN	CLL I	CLL II
SUBUNIT M_r	35K	16K	13.5K	52K	16K	12K
OLIGOMER*	MONOMER	MONOMER	MONOMER	AGGREGATE DIMER	DIMER	MONOMER
pI	4.5, 4.7	4.5	4.5	4.5-5.0	4.1	6.3
DIVALENT CATION REQUIREMENT	NO	NO	NO	YES	NO	NO
REDUCING CONDITIONS REQUIRED	YES	YES	YES	NO	YES	YES
REACTIVE WITH ANTI-CBP 35	YES	NO	NO	—	—	—
REACTIVE WITH ANTI-CLL I	NO	YES	NO	—	YES	—

Table I: Comparison of the Properties of Galactose-Specific Lectins.

* The states of aggregation of the various galactose-specific lectins were compared on the basis of data available. However, some of the conditions (e.g. concentration of protein) used for the determinations were not identical.