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Carbohydrate Binding Protein 35: Characterization, expression, and localization of isoelectric variants in cultured cells

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CARBOHYDRATE BINDING PROTEIN 35: CHARACTERIZATION, EXPRESSION, AND LOCALIZATION OF ISOELECTRIC VARIANTS IN CULTURED CELLS

By

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ABSTRACT

CARBOHYDRATE BINDING PROTEIN 35: CHARACTERIZATION, EXPRESSION, AND LOCALIZATION OF ISOELECTRIC VARIANTS IN CULTURED CELLS

By

Elizabeth Ann Cowles

Purified Carbohydrate Binding Protein 35 (CBP35) and extracts of mouse cells containing CBP35 were analyzed by two-dimensional gel electrophoresis. Such an analysis on recombinant CBP35, obtained by expression of a cDNA clone in *Escherichia coli*, yielded a pI value of 8.7. When extracts of mouse 3T3 cells were subjected to two-dimensional gel electrophoresis and immunoblotting, two spots were observed, corresponding to pI values of 8.7 and 8.2. The pI 8.2 species represents post-translational modification of the CBP35 polypeptide (pI 8.7) by the addition of a single phosphate group. This conclusion was derived from the identification in purified CBP35 of a $^{32}PO_4$-labeled pI 8.2 spot that is sensitive to alkaline phosphatase. The pI 8.2 species was found in both the cytosol and nuclear fractions, while the pI 8.7 species was found exclusively in the nuclei. Quiescent populations of 3T3 fibroblasts (confluent monolayers or serum-starved sparse cultures) are characterized by the predominance of the pI 8.2 species. Stimulation of the same cells into the proliferative state resulted in an increase in the amount of the pI 8.2 species; more dramatic, however, is the elevation of the level of the pI 8.7 species, which is barely detectable in quiescent cells.

The level and localization of CBP35 was also compared in human fibroblasts of different replicative capacities by immunoblotting and immunofluorescence. Like 3T3 fibroblasts, early passage human skin fibroblasts displayed an increase of

CBP35 upon serum stimulation. Intermediate passage cells also showed an increase, but not as dramatic as that seen in younger counterparts. High passage human fibroblasts and those derived from a Werner's syndrome (WS) patient, a disease characterized by premature aging, had higher levels of CBP35 at quiescence than young cells, but this level was not altered upon serum-stimulation.

To Richard, for his love and support throughout the years,

to my parents, for their faith in my abilities,

and to Erin, who has changed our lives.

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ABBREVIATIONS

CBP35	Carbohydrate Binding Protein (Mr ~ 35,000)
WS	Werner's syndrome
CRD	carbohydrate recognition domain
hnRNP	heterogeneous ribonucleoprotein complex
RHL	rat hepatic lectin
CLL	chicken lactose lectin
hsp	heat shock protein
rCBP35	recombinant Carbohydrate Binding Protein 35
pI	isoelectric point
IEF	isoelectric focusing
NEPHGE	nonequilibrium pH gradient electrophoresis
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
DME	Dulbecco modified Eagle's medium
Tris	Tris (hydroxymethyl)aminoethane
EGTA	ethylene-bis(oxyethylenenitrilo) tetraacetic acid
PBS	phosphate buffered saline

Introduction

Carbohydrate Binding Protein 35 (CBP35, M_r ~ 35,000), originally purified from mouse 3T3 fibroblasts, has now been identified in a variety of tissues from a number of species. The protein consists of a single polypeptide with galactose-specific carbohydrate-binding activity. The amino acid sequence of CBP35, deduced from the nucleotide sequence of a cDNA clone, displayed two domains: the NH_2-terminal half was homologous to certain proteins of the heterogeneous nuclear ribonucleoprotein complex (hnRNP) and the COOH-terminal half was homologous to other b-galactoside specific lectins. Subcellular localization studies showed that the protein is predominantly intracellular, found in both the cytoplasm and nucleus of 3T3 fibroblasts.

When serum-starved, quiescent 3T3 cells were stimulated by the addition of serum, there was an increase in the expression of CBP35: (a) at the level of gene transcription as determined by nuclear run-off assays; (b) at the level of accumulation of mRNA as determined by Northern blotting; and (c) at the level of protein as assayed by immunoblotting and immunofluorescence. These latter experiments showed that mitogenic stimulation of 3T3 fibroblasts resulted in an elevated level of CBP35 in the cell, as well as accumulation of the protein in the nucleus. This raised interesting questions concerning the regulation of the nuclear versus cytoplasmic distribution of the protein.

One approach to this issue is to determine whether the protein exists in multiple isoelectric forms due to post-translational modification and whether an individual isoelectric variant can be identified with a particular subcellular compartment. To this end, studies were undertaken to identify the isoelectric point(s) of CBP35 and to correlate the isoelectric variants with: (a) state of proliferation; and (b) nuclear versus cytoplasmic localization. This thesis describes the results of our studies, which have provided insights on certain aspects of protein shuttling between intracellular compartments.

The following literature review emphasizes the key features of CBP35, namely the delineation of the structure into two distinct domains, and the dynamics of subcellular localization between the nucleus and the cytoplasm.

CHAPTER I

LITERATURE REVIEW

Introduction to Carbohydrate Binding Proteins

There are three classes of proteins involved with protein-carbohydrate interactions (1). First are the glycosyl-binding enzymes, such as the glycosidases and glycosyl transferases. Secondly, there are sugar-binding antibodies. Finally, some carbohydrate binding proteins have neither enzymatic activity nor immune function; this class, found in a variety of organisms, is termed the carbohydrate binding protein (CBP) group. These CBPs are referred to as lectins if they agglutinate cells and have specificity for particular sugar residues (2). In the present literature review the terms CBP and lectin will be used interchangeably.

Although lectins were originally identified in plant extracts, many animal lectins have been isolated and characterized. The following review concerns only animal lectins. Many excellent reviews of the plant lectins are available (3, 4, 5).

Animal Lectins

From structural studies on animal lectins, it is now apparent that many of these proteins arose form the fusion of two different domains (6, 7). It is this theme that I wish to develop in my review of the literature.

The first domain, if one exists, is termed the special effector domain; this confers unique properties on the particular lectin. The second domain is the carbohydrate recognition domain (CRD). Based upon the CRD and a few other

characteristics, Drickamer subdivided the animal lectins into two groups, the C-type and the S-type lectins (6).

a) C-type lectins

The C-type (Ca^{++} dependent or cysteine-containing) lectins are exemplified by the asialogycoprotein receptor (8). These lectins need Ca^{++} for proper carbohydrate-binding function, contain disulfide bridges, are extracellular in nature and display a variety of carbohydrate specificities (Table 1). The common feature of these lectins is the CRD (Figure 1). The CRD of C-type lectins contains 18 invariant amino acid residues located in a conserved motif (see Figure 1), despite the fact that these lectins show diversified carbohydrate binding specificities. In membrane-anchored C-type lectins, the CRD forms the COOH-terminal portion of the polypeptide. The same or a very similar CRD is found in the soluble C-type lectins.

The C-type lectin effector domain can exist in several forms (Figure 1). The asialoglycoprotein receptor (8), for instance, contains a membrane-spanning sequence and an NH_2-terminal cytoplasmic domain. The mannose binding proteins have a cysteine-rich NH_2-terminus, followed by collagenous tails of 18-20 repeats of the sequence Gly-X-Y with 4-hydroxyproline residues in several of the Y positions (28-30). The proteoglycan core lectins consist of a core protein with either attached glycosaminoglycan chains (26, 27) or areas of epidermal growth factor-like repeats (25) as the effector domains. The effector domain and CRD structure of these lectins suggest that the lectins have bifunctional properties; these will be discussed later. The two domain motifs of the C-type lectins yields a class of proteins with widely assorted properties.

Table 1. The molecular weights and specificities of C-type lectins from several animal sources.

Lectin	Species/Source	Subunit (M_r x 10^{-3})	Ligand[a]	Reference
fly lectin	flesh fly hemolymph	30, 32	Gal	9
moth lectin	Chinese oak silk moth	38	Gal	10
sea urchin lectin	sea urchin	17	Gal	11
barnacle lectin	acorn barnacle coelomic fluid	16	Gal	12
pancreatic stone lectin	human pancreas	15	Gal	13, 14
asialoglycoprotein receptor (rat hepatic lectin)	mammalian liver	42, 49, 54	Gal	8
chicken hepatic lectin	avian liver	26	GlcNAc	15, 16, 17
lymphocyte FcE receptor	human lymphocyte	36.3	Gal	18, 19
Kupffer cell receptor	rat liver	77, 88	Fuc	20
macrophage lectin	mouse tumerocidal macrophage	45-60 (35)	Gal	21
aveolar macrophage lectin	rat	180	Fuc	22, 23
lymphocyte homing receptor (MEL-14 antigen)	mouse spleen	90	M6P	24
fibroblast proteoglycan core	human fibroblast	160, 180, 340 360, 400	Gal	25
cartilage proteoglycan core	rat liver	300	Fuc	26, 27
mannose binding protein	rat liver	34	Man	28, 29, 30
	human liver	28, 31	Man	28, 29
	human serum	32	Man	31
	human serum	28	Man	31
rat core-specific lectin	rat plasma	26	Man	32
pulmonary surfactant (SP 28-36)	dog lung lavage	28-36	Man	33, 34

[a]Fuc, fucose; Gal, galactose; GlcNAc, N-acetyl glucosamine; Man, mannose; M6P, mannose-6-phosphate

Figure 1. **Summary of the structural features of C-type animal lectins.** The invariant residues found in the common carbohydrate-recognition domain of theC-type lectins are shown, flanked by schematic diagrams of the special effector domains (if any) found in individual members of the family. EGF, epidermal growth factor; GAG, glycosaminoglycan. (adapted from ref. 35).

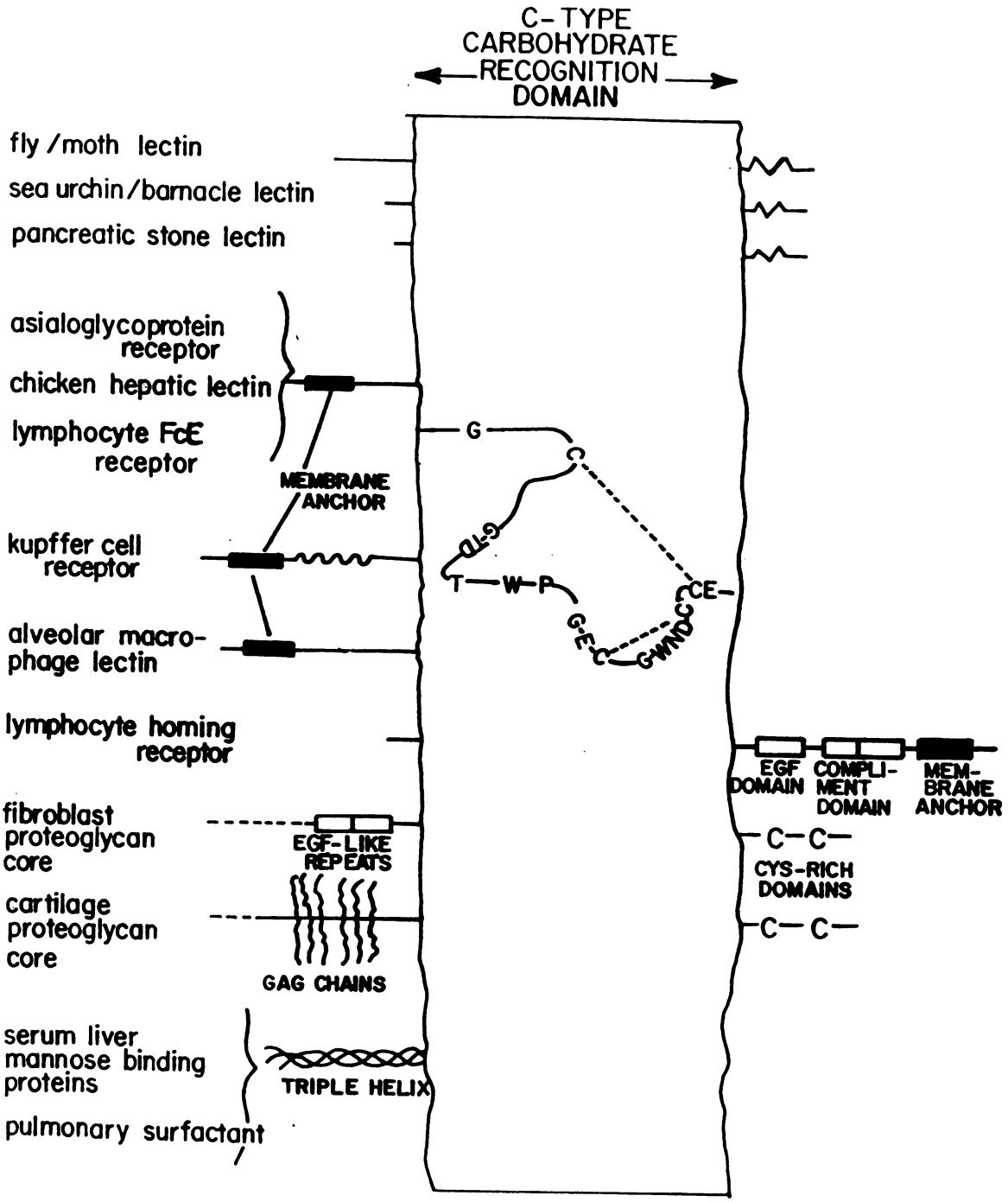
C-TYPE
CARBOHYDRATE
RECOGNITION
DOMAIN
fly /moth lectin
sea urchin/barnacle lectin
pancreatic stone lectin
asialoglycoprotein receptor
chicken hepatic lectin
lymphocyte FcE receptor
MEMBRANE ANCHOR
kupffer cell receptor
alveolar macrophage lectin
lymphocyte homing receptor
fibroblast proteoglycan core
EGF-LIKE REPEATS
cartilage proteoglycan core
GAG CHAINS
serum liver mannose binding proteins
TRIPLE HELIX
pulmonary surfactant
G
C
T—W—P
CE
EGF DOMAIN
COMPLI-MENT DOMAIN
MEM-BRANE ANCHOR
C—C
CYS-RICH DOMAINS
C—C

Figure 1

b) S-type lectins

Unlike the C-type lectins, the carbohydrate-binding activities of the S-type (thiol-requiring or soluble) lectins is independent of divalent ions. Since oxidation impairs the binding ability, the presence of reducing agents is usually required. These lectins are located either intra- or extracellularly, but detergent is not necessary for their solubilization. Hence, these proteins are often referred to as the soluble tissue lectins. The molecular weights of the polypeptides of S-type lectins generally fall into two groups: 12-16 kD and 30-35 kD (Table 2). Several of the lectins exist as dimers of identical subunits.

Like the C-type lectins, some of the S-type lectins exhibit two domains. One domain varies among the lectins, while the second, the CRD, exhibits conservation of certain residues, implying that these residues are necessary for carbohydrate binding (see Figure 2). The 39 conserved amino acid pattern contains several charged residues. The CRD of S-type lectins is completely different from the C-type CRD. While the C-type CRD has highly conserved cysteine residues involved in disulfide bonds, there are no invariant cysteine residues in the S-type lectins. Most of the S-type lectins have b-galactose-binding specificity.

Some of the S-type lectins also have effector domains. The best example is CBP35, whose NH_2-terminal domain shows homology to proteins of the heterogeneous ribonucleoprotein complex (hnRNP) (51). This region exhibits a nine amino acid motif, PGAYPGXXX, which is repeated eight times. As a result of this repeated motif, the NH_2-terminal half has a high proportion of proline (27%) and glycine (24%) residues. This repeated sequence is reminiscent of that found in the DNA lampbrush chromosome loop protein. This nuclear protein has a proline-glycine rich 11 to 12 residue repeat near its NH_2 terminus: GR(P)RGDFPREME (52). RNA polymerase II (largest unit) has a tandem repeat of the sequence

Table 2. The molecular weights and specificities of S-type lectins from several animal sources.

Lectin	Species/Source	Subunit ($M_r \times 10^{-3}$)	Ligand[a]	Reference
CLL II	chicken	12	Gal	36, 37
CLL I	chicken	16	Gal	36, 38
Xenopus laevis lectin	skin	16	Gal	39
electrolectin	electric eel electric organ	16	Gal	40
chicken skin /bovine lectin	skin/heart, lung	14	Gal	41/42,43
HL-14/RL-14.5/CBP13.5	human, rat, mouse lungs	14	Gal	44/45/46
HL-22/RL-18/CBP16	human, rat, mouse lungs	~18	Gal	47/45/46
a) HL-29/RL-29/CBP35	human, rat, mouse lungs			47/45/46
b) Mac-2	mouse macrophage			55, 56
c) L-34	mouse fibrosarcoma	~31	Gal	48
d) IgE binding protein	rat basophilic leukemia cells			49
e) "17K"	rat bone marrow			50
elastin receptor (large component)	fetal bovine lung	67	Gal	54

[a]Gal, galactose

Figure 2. **Summary of the structural features of S-type animal lectins.** Conserved residues found in all of the family members so far sequenced are shown. In addition, the extra domain found in CBP35 is shown schematically. (adapted from ref. 6).

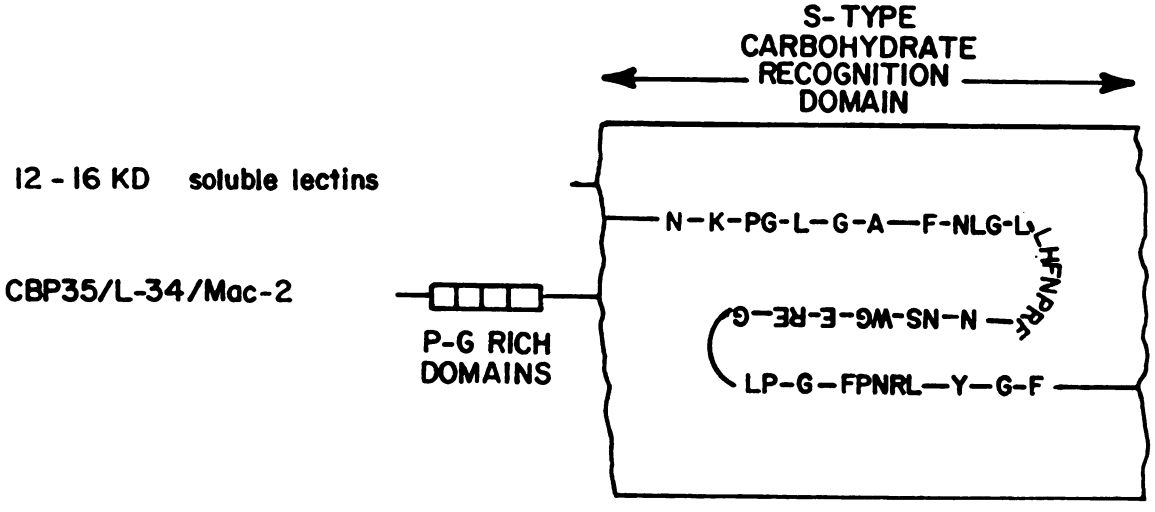
S-TYPE
CARBOHYDRATE
RECOGNITION
DOMAIN
12 - 16 KD soluble lectins
CBP35/L-34/Mac-2
P-G RICH
DOMAINS
N-K-PG-L-G-A-F-NLG-L
LHFNPRL
N-NS-WG-E-RE-G
LP-G-FPNRL-Y-G-F

Figure 2

SPTSPSY, a heptad containing two serine-proline sequences, which may be related to the SPXX motif found in gene regulatory sequences (53).

A metastasis-associated protein, L-34, which was isolated from murine fibrosarcomas (77), is identical to CBP35 (48). Raz and coworkers identified the proline-glycine rich half of L-34 to be homologous with a portion of the collagen a 1(II) chain NH_2-terminus. Five consensus repeat triplets were found of the Gly-X-Y motif. Eight repeats of nine amino acids were also located, as indicated for CBP35. This region is susceptible to collagenase cleavage, suggesting that L-34 (CBP35) has a collagen-like domain fused to a CRD.

A cell surface marker linked to mouse macrophage differentiation (55), Mac-2, is also identical to CBP35 (56). The macrophage Mac-2 expression is elevated in the inflammatory response but its function is unknown (56). The difference in the localization of Mac-2 (cell surface) and CBP35 (cell nucleus) is important and suggests two distinct forms of the same lectin. This issue will be discussed below.

CBP35 is homologous to the rat IgE-binding protein (49,57). This protein, isolated from rat basophilic cells, was also noted for its two domain structure. Comparing the amino acid sequences of 3T3 cell CBP35 with IgE-binding protein from basophilic cells indicates that they are the mouse and rat homologs of the same protein.

In summary, most animal lectins can be assigned to the C-type or S-type categories. Although each group shares common features, such as the CRD and ion requirements, differences in the effector domains ascribe a variety of functions to these lectins.

The Bifunctional Properties of Lectins

Some lectins, as noted in the previous section, have a two domain structure. The CRD's function is to bind to a specific carbohydrate structure. The second

domain may also be a binding site for a noncarbohydrate ligand (7). I shall review four lectins with an emphasis on their proposed bifunctional properties.

a) Asialoglycoprotein receptor

The asialoglycoprotein receptor (rat hepatic lectin, RHL) is found as three distinct species (see Table 1); RHL-1 (Mr 41,000) accounts for 80% of the total protein isolated (8). RHL-1 consists of NH_2-terminal hydrophobic domains followed by a long stretch of uncharged amino acids. RHL-1 is a transmembrane protein; the NH_2 terminus is cytoplasmic (58). The protein is oligomeric and exists as a hexamer in detergent solution.

The receptor has high affinity for serum asialoglycoprotein. Bound ligand is endocytosed via coated pits and vesicles; the asialoglycoproteins are then degraded within the lysosomes. Clearance of glycoproteins by the receptor occurs only after the removal of the terminal sialic acid moiety, exposing galactose residues.

RHL-1 can be divided into two domains, each with a specific function. The first domain of RHL-1 consists of the NH_2-terminal hydrophilic tail followed by a hydophobic region of about 25 amino acids (59). The hydrophobic portion is integrated into the cellular membrane. The membrane-spanning portion is thought to be the internal signal sequence, necessary for proper insertion in the lipid bilayer.

The COOH-terminal region, which is exposed on the extracellular side of the cell membrane, encompasses the second domain of RHL-1. This portion contains the CRD which binds to the serum asialoglycoproteins and a neck region which serves to project the CRD away from the cell surface. The CRD shares strong sequence homology with the chicken hepatic lectin, although they have different carbohydrate specificities (see Table 1). Both of the domains are required for proper ligand binding and endocytosis.

b) Pulmonary surfactant protein (SP 28-36)

The major surfactant-associated protein, SP 28-36, was originally isolated from dog lung lavage (60) and has a molecular weight of approximately 28,000 - 36,000. The surfactant lowers the surface tension at the air-fluid interface in the alveolar spaces. SP28-36 is water soluble but associates with phospholipids (PL), and causes PL aggregation in the presence of Ca+ + (61). This process, along with the association of other surfactant proteins, increases the rate of adsorption of lipids to the interface.

Examining the cDNA sequence of SP28-36 shows two domains (61). The first is the collagen-like NH_2-terminal region. This region has two functions (33). First, the collagenous portion of the polypeptide determines the oligomeric structure of SP28-36. Second, this sequence is also responsible for the PL-binding ability of the protein.

The COOH-terminal region is homologous to the amino acid sequence of other mannose-binding proteins. This portion of SP28-36 is a C-type CRD, and has mannose-binding capabilities (33). Possible functions of the CRD include regulation of surfactant metabolism through inhibition of surfactant secretion, and the coating of bacteria to facilitate phagocytosis by macrophages.

c) Lymphocyte homing receptor

The lymphocyte homing receptors (e.g. MEL-14 antigen) are adhesion molecules found on lymphocytes which mediate the cells' attachment to endothelial venules within secondary lymphoid organs (24). Accumulation of lymphocytes in the peripheral lymph nodes is inhibited by the monoclonal antibody, MEL-14 (62). Characterization of the cDNA clone for the MEL-14 antigen reveals several motifs (35, see Fig. 1).

Unlike most membrane-anchored C-type lectins, the MEL-14 antigen CRD is located near the NH_2-terminus. The homology between this region and other C-type CRDs may explain the lymphocyte's ability to bind to peripheral lymph nodes in a carbohydrate and calcium dependent manner. The MEL-14 antigen, therefore, might act as a specific cell adhesion molecule.

The next region of 33 amino acids has homology to epidermal growth factor (EGF) This motif orients the CRD for proper ligand binding or binds to EGF-like receptors on the endothelial cell surface. The larger complement-like region of MEL-14 antigen may bind complement proteins or simply may act as a spacer between the CRD and the cell surface. Another possible function of this region is to promote oligomerization of the MEL-14 antigen. Finally, as in the other C-type lectin receptors, there is a membrane-spanning region of 23 amino acids. These different domains of the MEL-14 antigen may play important roles in organ-specific lymphocyte homing.

d) Carbohydrate binding protein 35

CBP35 was originally isolated from murine 3T3 fibroblasts on the basis of its carbohydrate-binding ability (46). The protein is found in a variety of adult and embryonic tissues (64). Immunoblotting and immunofluorescence studies of 3T3 and human fibroblasts, using specific polyclonal antiserum directed against CBP35, demonstrate intracellular location and proliferation-dependent expression (64-66). The amino acid sequence of CBP35, deduced from the nucleotide sequence of the cDNA clone, suggests that CBP35 has two functional domains, an NH_2-terminal region homologous to certain proteins of the hnRNP and a COOH-terminal S-type CRD (51).

The hnRNP region is charcterized by a high percentage of proline(27%) and glycine (24%) residues; over 85% of both the proline and glycine residues are found

in the CBP35 NH_2 terminal region. In this respect, this domain is similar to that found in hnRNP proteins, such as human hnRNP protein C_1. All of the proline residues of human hnRNP C_1 are found in the NH_2-terminal half of the polypeptide (67). Similarly, the glycine-rich protein of brine shrimp hnRNP (GRP33) has a nonuniform distribution of proline and glycine residues; 63% of the proline residues and 77% of the glycine residues are found in the COOH-terminal region of GRP33 (68). CBP35 was identified as a component of the hnRNP complex by Laing and Wang (69). Triton X-100 permeabilized 3T3 cells released CBP35 upon treatment with ribonuclease A while deoxyribonuclease I had no effect. 3T3 nucleoplasm fractionated on cesium sulfate gradients localized CBP35 in samples with densities corresponding to those reported for the hnRNP complex. When 3T3 nucleoplasm was bound to a galactose affinity column and eluted, the fractions yielded RNA and polypeptides whose molecular weights corresponded to those of the hnRNP proteins.

The COOH-terminal sequence of CBP35 is the CRD element of the lectin. This region shows homology to other S-type lectins, such as bovine heart lectin and chicken skin lectin. CBP35 also has a sequence corresponding to the the ligand binding region of the electric eel lectin (40).

Localization of lectins

The cellular distribution of lectins may give clues for their endogenous functions. Oligosaccharides, such as glycoproteins, are widely distributed in many compartments, including the cell surface, extracellular matrix and intracellular organelles. Like glycoconjugates, lectins also are widely distributed. Their location may be transient or cell cycle dependent. I shall review examples of lectins found in various compartments, their ligands, if known, and thoughts on their possible functions.

a) Extracellular lectins

Barondes classifies the extracellular lectins as soluble proteins (70). These lectins, unlike the integral membrane proteins, do not require detergent for solubilization, but are free to bind to soluble or membrane bound glycoconjugates in aqueous compartments. Chicken lactose lectin I (CLL-I) is localized in the extracellular spaces between chicken pancreatic ascini and liver cells by immunofluorescence staining (71). In intestinal mucosa, CLL-I is found in the goblet cell secretory granuales and the extracellular matrix. In adult chicken kidney tubule cells, CLL-I is found in the cytoplasm, in some nuclei and on the luminal and basal surfaces (72). The level of CLL-I is modulated during *in vivo* chicken embryo development of muscles (38). In 8 day embryos, the myoplasts had low levels of intracellular (non-nuclear) CLL-I, and little extracellular lectin. Upon cell fusion to form myotubes (day 12), the CLL-I may act to organize glycoconjugates in the extracellular matrix.

Chicken lactose lectin II (CLL-II) was also localized in intestinal goblet cells (37), but at a greater concentration than CLL-I. CLL-II is found in the secretory granules and is secreted out onto the mucosal surface. CLL-II might act to organize the mucin or to crosslink the mucin molecules with other glycoproteins on the intestinal epithelial surface.

Other lectins undergo externalization as well. Chicken heparin lectin-2, which is released into the medium of cultured chick muscle cells, binds iduronic acid-containing glycosaminoglycans (GAGs); the lectin may mediate cell-cell interactions through GAG binding (73). RL-14.5 and RL-29 in lung aveolar and smooth muscle cells were concentrated in the elastic fibers, thus the lectins may organize the extracellular glycoconjugates (74).

Xenopus laevis skin lectin, concentrated in the cytoplasm of granular gland and mucous gland cells, is secreted upon injection of epinephrine. The lectin may serve to organize glycoconjugates in a cutaneous mucin (39).

b) Cell surface lectins

Barondes suggests integral membrane lectins bind glyconconjugates to membranes, allowing for the localization of the glycoconjugates or their transport to other cellular compartments (70). Two such lectins, the asialoglycoprotein receptor and the lymphocyte homing receptor, have already been discussed in the previous section. Other examples include the rat peritoneal macrophage lectin which bind desialated red blood cells leading to phagocytosis and lysis (75), and the mannose/N-acetylglucosamine specific macrophage lectin, which may mediate the binding and phagocytosis of microorganisms (76).

b-Galactoside-specific S-type lectins have been identified on the surface of various murine and human tumor cells. Raz and Lotan (77) noted that some tumor cell lines aggregate in the presence of asialofetuin, and that the aggregation could be inhibited by lactose. They hypothesized that tumor cell lectins may act to bind the cells to other cell surface carbohydrates, causing metastasis. A monoclonal antibody, 5D7, was isolated from mice injected with melanoma cell extracts enriched for lectin activity. Indirect immunofluorescence using 5D7, revealed that the lectin was localized on the cell surface and in the cytoplasm of melanoma and fibrosarcoma cells (78). A galactose/lactose specific lectin was isolated from the mouse fibrosarcoma cells and designated as L-34. This lectin is identical to CBP35 (51) and is homologous to rat IgE-binding protein (48). Surface L-34 appears to be localized in microclusters and may be rearranged by exogenous ligands. L-34 could be involved with the specific recognition and adhesion between tumor and host cells (79).

L-34 may affect anchorage-independent growth regulation and might promote tumor metastasis (80). Monoclonal antibody 5D7 inhibited the aggregation and adhesion of tumor cells to the substratum *in vitro* and suppressed the formation of lung tumor colonies in mice.

c) Intracellular lectins

As noted previously, some soluble, extracellular lectins, such as CLL-I, have also been identified in the intracellular compartment. Adult chicken kidney cells had endogenous CLL-I in the cytoplasm and nuclei (72). Antibodies to b-galactoside-specific bovine (12 kD) and chicken heart (13 kD) lectins were used for indirect immunofluorescence studies on a variety of tissues (42). The lectins were localized uniformly in the cytoplasm of calf thymocytes, calf kidney cells, and chicken fibroblasts. Some lectin crossreactivity was noted on the cell surface and extracellular face of the calf thymocytes.

Several lectins appear to have nuclear as well as cytoplasmic localization. b-Galactoside binding calf heart lectin is predominantly extracellular in muscle cells and capillary epithelial cells; but cytoplasmic and nuclear in pancreatic cells (74). A monoclonal antibody directed against this same protein indicate that this lectin is in the nuclei and cytoplasm of human lymphocytes; after stimulation or transformation, the level of lectin increase (81).

Nuclear lectins have also been implicated using neoglycoproteins, which are synthetic glycoproteins of bovine serum albumin coupled to saccharide residues (82). The fluoroscein-labeled neoglycoproteins were then incubated with permeabilized nuclei, which were then analyzed by fluorescence microscopy or flow microfluorometry. Lectins, detected by this method, fluctuate with the cell cycle; the intensity of nuclear staining is sharply reduced in contact inhibited cells, as compared with exponentially growing cells (83).

Hubert et al. (84) have suggested that nuclear lectin and nuclear glycoprotein interactions modulate nuclear functions. Many nuclear glycoproteins, such as high mobility group 14 and 17, and RNA polymerase II transcription factors, are localized either in territories known to be the sites of transcription and the maturation processes of RNA, or in the nuclear pores. Some lectins may have RNA and/or DNA binding capabilities and act to modulate transcription. Indeed, when transcription factor SP1 is incubated with WGA, the function of SP1 is repressed; SP1 needs to be glycosylated to be active (86).

Colocalization of nuclear glycoconjugates and plant lectins in animal nuclei suggests a functional role for possible endogenous lectins. For instance, wheat germ agglutinin (WGA) binds to nuclear pores and prevents protein import (85). An endogenous lectin may function in a similar manner or conversely, might aid in the import of proteins when complexed with them.(84). Nuclear lectins, such as CBP35, are found in hnRNP complexes (69). Due to the bifunctional nature of such lectins, the interactions could be protein-protein, leaving the CRD available for binding to a second ligand. These lectins may act as carriers, targetting ribonucleoprotein complexes to the nuclear pore glycoproteins.

Carbohydrate Binding Protein 35

In this discussion of the localization of lectins, particular note should be made of CBP35 for two reasons. First, although our own analyses have previously emphasized the intracellular localization of the lectin, recent studies by others have clearly identified CBP35 or a very close derivative of the polypeptide both outside and inside certain cells. Second, within the cell, CBP35 is found in both the nucleus and cytoplasm. I shall discuss these two different issues in turn.

a) Extracellular versus intracellular lectin

CBP35 was originally purified on the basis of its carbohydrate-binding activity (46). Immunochemical studies identified it as predominantly (>95%) an intracellular protein in 3T3 fibroblasts (64). Several laboratories have purified their proteins and, after structural analyses via cDNA sequencing have identified their proteins from other systems to be identical to CBP35. Thus, it appears that the same molecule or very closely related derivatives exist under several different names: a) CBP35; b) L-34 (identified as a cell surface lectin, 78); c) Mac-2 antigen (identified as an antigen reactive with a monoclonal antibody directed against the macrophage cell surface, 55); and d) IgE-binding protein (εBP) (identified on the basis of purification on IgE-affinity columns, 49).

In all of these studies, the immunochemical evidence indicates that the majority of the antigen is intracellular. For example, εBP was found in the cytoplasm of rat basophilic leukemia cells and in COS-1 monkey cells transfected with εBP cDNA (88). Similarly, much of the Mac-2 antigen was localized intracellularly in unstimulated macrophages (56). However, there is clear evidence that L-34 and Mac-2 can also be found on the outside of the cell. In fact, in macrophages stimulated by thioglycolate, the appreciable secretion of the protein cannot be accounted for by cell leakage (56).

This problem of finding CBP35 (under all its names) is also true for the 14 kD S-type lectins; the majority of the lectin is intracellular, but under certain conditions the lectin is localized extracellularly. Thus far, none of the cloned cDNAs for any of the lectins show a signal sequence for entry into the endoplasmic reticulum (78,90). Moreover, certain of the mature polypeptides contain potential N-glycosylation sites in the amino acid sequence, but no glycosylation has been found on any of the lectins (89). These observations suggest that the lectins do not

pass through the endoplasmic reticulum-golgi endomembrane pathway; thus, an intracellular localization would be most reasonable.

Recent studies on the Mac-2 antigens may shed light on the extracellular form of the molecule (56). Using the sequence information derived from the cDNA clone for Mac-2, polymerase chain reaction was carried out on the mRNA of thioglycolate-elicited macrophages. This revealed an alternatively spliced cDNA with the potential to encode an NH_2-terminally extended Mac-2 protein. The 14 amino acids upstream from the Met/start sequence contains a stretch of uncharged, predominantly hydrophobic residues and could conceivably serve as a signal sequence for cellular export. Thus, it is possible that the S-type lectins are coded by mRNA that result in intracellular proteins, but under "stimulated" conditions, a second mRNA species codes for the extracellular form of the protein.

b) Nuclear and cytoplasmic intracellular distribution

CBP35 was localized in both the nuclei and the cytoplasm of 3T3 cells, as well as the cell surface as shown by cell fractionation and indirect immunofluorescence (64-66). The expression and localization is proliferation-dependent (65,66). Quiescent cells, either confluent or serum-starved, show cytoplasmic CBP35. Upon stimulation or loss of density-dependent inhibition, the level of nuclear CBP35 increased. The increase of CBP35 polypeptide parallels the higher levels of mRNA and elevated transcription rates of the gene following serum stimulation (87).

The specific chemical form of CBP35 in its differential subcellular distribution is the subject of my experimental work to be detailed in Chapters II and Chapter III of this thesis.

Cytoplasmic/Nuclear Protein Shuttling

As indicated in the previous section, lectins are not found exclusively in one cellular compartment. Several lectins are both cytoplasmic and nuclear; the levels and localization of the endogenous lectins change with the cell cycle. Since CBP35 moves between the cytoplasm and the nucleus as a function of growth factor control, I have chosen to review protein shuttling between these two compartments.

Proteins redistribute between the cytoplasm and nucleus in response to certain stimuli, such as during embryonic development, hormonal stimulation or in response to heat shock (environmental stress)(91). I shall give an example of each type.

a) Redistribution during embryonic development

The *dorsal* gene is a maternally controlled, dorsal-ventral polarity gene (92,93). The *dorsal* protein is necessary for proper formation of dorsal-ventral asymmetry in *Drosophila* embryos. During early cleavage, the *dorsal* protein is uniformly distributed in the cytoplasm. As the nuclei migrate to the surface of the embryo, the protein enters the nucleus, but non-uniformly, so that a dorsal-ventral asymmetry gradient is formed; ventral nuclei stain strongly for *dorsal* protein, while the protein stays cytoplasmic in the dorsal cells. The *dorsal* protein is only active in the nuclear form; the protein is a transcriptional regulator and is referred to as the ventral morphogen.

Dorsal protein shuttling is controlled by a cytoplasmic factor. *Dorsal* overproduction led to nuclear localization, even in dorsally-located cells. This suggests that a saturable, cytoplasmic factor must be binding to the protein to prevent its movement.

b) Relocalization upon hormonal stimulation

Replitase is a complex of at least seven enzymes, including DNA polymerase, ribonucleotide diphosphate reductase, thymidylate synthase, dihydrofolate reductase, NDP kinase, topoisomerase, and dCMP kinase (94,95). The complex is found in the nucleus of synchronized CHO cells during S phase, but in the cytoplasm during quiescence and G1 phase.

When CHO cells were fractionated into karyotypes and cytoplasts, then analyzed for enzymatic activities. After sucrose gradient sedimentation, replitase was found as a complex in S phase but the enzymes sedimented as free entities during G1 phase. Novobiocin, an inhibitor of topoisomerase, caused replicase to fall apart; this inhibitor induces a conformational change (96). Perhaps an endogenous cytoplasmic inhibitor, with a similar effect, prevents the enzyme from complexing during G1 phase.

NF-κB is a protein that binds to the κ light chain enhancer, κB (97). It is found to be localized in B cells, but has also been found in non-B cells, in a covert cytoplasmic form. In these non-B cells, NF-κB will move to the nucleus and exhibit binding to various promoters and regulate DNA transcription (98). Induction of NF-κB by phorbol esters does not require new protein synthesis, but appears to be activated (99). While in the cytoplasm, NF-κB is complexed with an inhibitory protein (IκB), which prevents NF-κB activity. Recent experiments have shown that treatment of cytosolic proteins or purified IκB with protein kinase C releases NF-κB. Phosphorylation of the inhibitor, either by protein kinase C or through cyclic AMP dependent protein kinase A, therefore, releases NF-κB, allowing it to shuttle to the nucleus (100).

c) Translocation of proteins during heat shock

Drosophila heat shock protein 70 kd (hsp70) is produced when the organism undergoes an envirionmental stress, such as exposure to temperatures 5-10^{o}C above the growth optimum. Although hsp70 is not constitutive, once induced, the protein is stable. Upon heat or anoxia treatment of *Drosophila* larvae, hsp70 is rapidly translated and translocated to the nucleus (101). During the recovery period, hsp70 moves back to the cytoplasm. Reexposure to stress causes rapid movement into the nucleus (102).

Transport of hsp70 to the cytoplasm may be necessary for restoration of translation after the shock. Since hsp70 protects the open chromatin structure and hnRNA complexes from degradation during stress, removal of hsp70 may be required to restore proper nuclear function. hsp70 also binds to RNA and may aid in protecting mRNA, allowing repression of further hsp70 synthesis by auto-feedback inhibition. hsp70 undergoes similar movement in heat shocked chicken fibroblasts (103).

It is not clear how hsp70 partitioning works. Three possibilities have been suggested (102): 1) hsp70 is post-translationally modified, 2) binding sites in either the cytoplasm or nucleus have been unmasked, allowing hsp70 to bind, and 3) a possible hsp70 transport system could be activated. hsp70 is released from its nuclear binding sites in an ATP dependent fashion (103).

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Chapter II

Carbohydrate Binding Protein 35: Isoelectric Points of the Polypeptide and a Phosphorylated Derivative

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Footnotes

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1 The abbreviations are: CBP35, Carbohydrate Binding Protein (M_r ~ 35,000); rCBP35 recombinant Carbohydrate Binding Protein 35; hnRNP, heterogeneous nuclear ribonucleoprotein complex; IEF, isoelectric focusing; NEPHGE, nonequilibrium pH gradient electrophoresis; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; DME, Dulbecco modified Eagle's medium.

2 N. Agrwal and J.L. Wang, manuscript in preparation

Summary

Purified Carbohydrate Binding Protein 35 (CBP35) and extracts of mouse cells containing CBP35 were analyzed by two-dimensional gel electrophoresis. Such an analysis on recombinant CBP35, obtained by expression of a cDNA clone in *Escherichia coli*, yielded a pI value of 8.7. This is in agreement with the value of the isoelectric point, calculated from the amino acid composition. These results indicate that the pI of the unmodified CBP35 polypeptide chain is 8.7. When extracts of mouse 3T3 cells were subjected to two-dimensional gel electrophoresis and immunoblotting, two spots were observed, corresponding to pI values of 8.7 and 8.2. The pI 8.2 species represents post-translational modification of the CBP35 polypeptide (pI 8.7) by the addition of a single phosphate group. This conclusion is derived from the identification in purified CBP35 of a $^{32}PO_4$-labeled pI 8.2 spot that is sensitive to alkaline phosphatase. In the present study, no CBP35 species were detected on isoelectric-focusing gels; this result differs from the results of our previous studies in which pI 4.7 and pI 4.5 species were found. Experiments using ^{14}C-labeled cyanate suggest that these low pI species may be due to carbamylation of the pI 8.7 and 8.2 polypeptides by cyanate present when undeionized urea is used.

Introduction

Carbohydrate Binding Protein 35[1] (CBP35; M_r 35,000), a lectin endogenous to cultured 3T3 fibroblasts and certain mouse tissues, was isolated on the basis of its ability to bind to galactose-containing glycoconjugates (1-3). This protein can be found in the cytoplasm and nucleus of 3T3 cells (4,5). More recently, we have provided evidence to indicate that CBP35 is a component of the heterogeneous nuclear ribonucleoprotein complex (hnRNP) (6). Consistent with this notion, the amino acid sequence of CBP35, deduced from the nucleotide sequence of a cDNA clone (7), showed that the polypeptide consisted of two domains: The sequence of the amino terminal portion was homologous to proteins of the hnRNP and the sequence of the carboxyl terminal half was homologous to other β-galactoside specific lectins (8).

When extracts of 3T3 cells, derived from [^{35}S]methionine-labeled cultures, were fractionated by affinity chromatography, they yielded two species of CBP35 with pI ~ 4.5 and pI ~ 4.7, as revealed by two-dimensional gel electrophoresis (isoelectric focusing (IEF) in the first dimension and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) in the second dimension) (2). In contrast, however, the isoelectric point calculated from the deduced amino acid sequence (8) yielded a value of pI ~ 8.7. This has prompted us to identify the isoelectric variants of CBP35, paying particular attention to the methods of polypeptide separation on the basis of differing isoelectric points. The results of our studies are now reported in the present communication.

Experimental Procedures

Extracts of Cultured 3T3 Fibroblasts

Swiss 3T3 fibroblasts (CCL92, American Type Culture Collection) were cultured in Dulbecco modified Eagle's medium (DME) containing 10% (v/v) calf serum (5,6). Confluent monolayers and sparse cultures of these cells were obtained by seeding at a density of 5×10^4 cells/cm^2 and 0.7×10^4 cells/cm^2, respectively. The cells were allowed to attach overnight in growth media, followed by a 24-hour period in DME containing 0.2% (v/v) calf serum. For comparing serum-starved versus serum-stimulated cultures, cells were seeded at an initial density of 1.5×10^4 cells/cm^2, starved by deprivation of serum (0.2% calf serum for 48 hours), followed by addition of serum to 10% (v/v) (5,6).

For extract preparation, the cells were washed with phosphate-buffered saline, scraped off the culture dish, and isolated by centrifugation (1330 x g, 3 minutes). The cells were then resuspended in 10 mM Tris buffer (pH 7.5) containing 2 mM EGTA, 1 mM phenylmethyl sulfonyl fluoride, 1 μg/ml soybean trypsin inhibitor, and 1 U/ml aprotinin. After 20 minutes on ice, the cells were sonicated for 15 seconds four times. Aliquots of the cell lysate were subjected to the Bradford assay (9) for protein determination. Samples containing 200 μg total protein were concentrated and resuspended in lysis buffer for two-dimensional gel electrophoresis analysis (10) as described below.

Isolation of ^{32}P-Labeled CBP35 and Sensitivity to Alkaline Phosphatase

Confluent cultures of 3T3 fibroblasts were washed in phosphate-free DME. To each of ten 150-cm^2 flasks was added 10 ml of phosphate-free DME containing 50 μCi/ml $^{32}PO_4$ (New England Nuclear). An additional 10-20 flasks of the same cells were cultured in parallel in DME without radioactive phosphate. After 24 hours of labeling, the medium was decanted and the cells were washed with 75 mM Tris, 50 mM $CaCl_2$, pH 7.5. The cells were then scraped in a minimal volume of 10 mM Tris, 10 mM β-mercaptoethanol, 0.2% Triton X-100, pH 7.8. The cells from the radiolabeled flasks were combined with those from unlabeled flasks. They were homogenized with 20 strokes in a Dounce homogenizer, followed by sonication (four times, 15 seconds each). After centrifugation (3,000 x g, 15 minutes), the supernatant was subjected to affinity chromatography on a column (1.3 x 4 cm) containing asialofetuin-derivatized (11) Affi-gel 15 (BioRad). The bound material was eluted with 10 mM Tris (pH 7.8), 10 mM β-mercaptoethanol, 0.3 M lactose, pooled, and characterized by SDS-PAGE, as well as by two-dimensional gels.

For alkaline phosphatase and phosphodiesterase analyses, ^{32}P-labeled CBP35 was dialyzed against buffer containing 40 mM Tris, pH 8.0, 15 mM $MgCl_2$, 1 mM $ZnCl_2$, and the previously mentioned protease inhibitors. The samples were digested with either 0.2 U/ml phosphodiesterase I (Boehringer-Mannheim) (12) or with 2 U/ml alkaline phosphatase (calf intestine) (Boehringer Mannheim) for 2 hours at 37°C. Control samples were incubated in parallel. All reactions were stopped by lyophilizing the samples, and resuspension in lysis buffer (10).

Recombinant CBP35 and Derivatization with Cyanate

The cDNA clone for CBP35 was identified from a library derived from the mRNA of mouse 3T3 fibroblasts (7). This clone was shown to be an authentic clone for CBP35 and its nucleotide sequence was determined (8). The cDNA was engineered into the procaryotic expression vector pKK-233-2 (Pharmacia), which provides the trp-lac fusion promoters, the lac Z ribosome-binding site (13), followed by an ATG translation initiation codon plus an extra Ala residue in front of the polypeptide encoded by the cDNA sequence.[2] The expressed protein was purified by affinity chromatography on asialofetuin - Affi-gel 15 (11). The material bound and eluted with 0.3 M lactose was purified and characterized, the details of which will be reported elsewhere.[2] This material, referred to as recombinant CBP35 (rCBP35), was generously provided by N. Agrwal (Michigan State University).

rCBP35 was incubated with 0.1 M potassium [^{14}C]cyanate (specific activity 58 mCi/mmole; New England Nuclear) (14) for 2 hours at 37°C. The protein then was lyophilized and resuspended in lysis buffer (10) for two-dimensional gel analysis. Control rCBP35 was incubated as described above, but in water.

Calculation of the Isoelectric Point

The isoelectric point (pI) of CBP35 was calculated based on the deduced amino acid sequence from the cDNA (8). The theoretical pI of CBP35 and its derivatives was obtained by using the method of Sillero and Ribeiro (15), which was designed to calculate pIs between 4 and 11.4.

One-Dimensional and Two-Dimensional Gel Electrophoresis

For two-dimensional gel electrophoresis, the methods of O'Farrell (10,16) were used: a) isoelectric focusing (IEF) in the first dimension and SDS-PAGE in the second dimension, and b) non-equilibrium pH gradient electrophoresis

(NEPHGE) in the first dimension and SDS-PAGE in the second dimension, with the following modifications. The IEF gels were prepared using 0.8% pH 4-6.5, 0.8% pH 2-5, and 0.4% pH 3-10 ampholines (Pharmacia) and run for 16 hours at 400 V and 1 hour at 800 V. NEPHGE gels were made using only pH 3-10 ampholines and the electrophoresis was run at 400 V for 5 hours. The basic electrode solution in both methods contained 10 mM NaOH and 5 mM $Ca(OH)_2$. Standard proteins were used to track the pH gradient. The second dimension SDS-PAGE had 12 cm of separating gel containing 12.5% acrylamide.

All extracts were digested with ribonuclease A (50 μg/ml; Boehringer-Mannheim) and deoxyribonuclease I (50μg/ml; Boehringer-Mannheim) on ice for 30 minutes. They were then dissolved in lysis buffer (10) with a final urea concentration of 9.5 M. Purified proteins were dissolved in lysis buffer containing 9.5 M urea. In some cases, the lyophilized sample was dissolved in 8 M urea. The acrylamide mixture (10) was then added and the sample was incorporated directly in the polymerizing mixture within the first dimension tube.

Proteins were detected by Coomassie blue (17) and silver (18) staining of the gel. ^{32}P-Labeled samples were detected by autoradiography, using Kodak X-OMat film. Immunoblotting was performed as described previously (3,4) with the following modifications. Proteins were transferred to Immobilon-P (Millipore) and incubated in Tris (10 mM Tris, pH 7.5, 0.5 M NaCl, 0.05% thimerosal) containing 3% bovine serum albumin for at least 1 hour. The blots were incubated in rabbit anti-CBP35 antiserum (1:150) overnight at 4°C. Positive bands were revealed with the colored products of the horseradish peroxidase conjugated goat anti-rabbit immunoglobulin (BioRad). ^{14}C-Labeled samples were detected by fluorography after transfer to Immobilon; the membranes were immersed in 20% (w/v) 2.5-diphenyloxazole in toluene and then exposed to Kodak X-OMat film.

Results

Isoelectric Point of the CBP35 Polypeptide Calculated from the Amino Acid Sequence

We had previously reported the amino acid sequence of CBP35, deduced from the nucleotide sequence of a cDNA clone (8). This cDNA clone was derived from the mRNA of mouse 3T3 fibroblasts. It was an authentic clone for CBP35 on the basis of: (a) immunoreactivity of the fusion protein, encoded by the cDNA insert within the λgt11 expression vector, with affinity-purified, monospecific antibodies directed against CBP35; (b) carbohydrate-binding activity of the cDNA-encoded polypeptide, derived from the expressed fusion protein by V-8 protease digestion; and (c) comparison of amino acid sequence identities with homologs, as well as other galactose-specific lectins. From all of these studies and comparisons, it was concluded that the amino acid sequence as reported previously (8) accounted for the entire CBP35 polypeptide chain, except for the NH_2-terminal methionyl residue (19,20).

The amino acid composition of the CBP35 polypeptide chain was tabulated from the sequence. For the calculation of the pI value, based on the method of Sillero and Ribeiro (15), only the numbers of those amino acids with ionizable side chains were used (Table I). This calculation yielded a value of 8.7 for the isoelectric point of the unmodified CBP35 polypeptide.

Two-Dimensional Gel Electrophoresis of Recombinant CBP35

The cDNA clone for CBP35 (7) was engineered into the vector, pKK-233-2, which was then used to transform *E. coli*. This construct included the trp-lac fusion promoters, the lac Z ribosome-binding site, followed by a ATG translation initiation codon as well as an extra Ala residue before the first amino acid residue encoded by

TABLE I. **Isoelectric Points of CBP35 Calculated from the Amino Acid Composition**

		Calculated pI[a]	Arg	His	Lys	Tyr	Cys	Asp	Glu	PO_4
1)	Unmodified	8.7	10	5	7	14	2	6	9	0
2)	Singly Phosphorylated	8.2	10	5	7	14	2	6	9	1
3)	Partially Carbamylated	6.2	10	5	2	14	2	6	9	0
4)	Partially Carbamylated Singly Phosphorylated	5.8	10	5	2	14	2	6	9	1
5)	Fully Carbamylated	4.7	10	5	0	14	2	6	9	0
6)	Fully Carbamylated Singly Phosphorylated	4.5	10	5	0	14	2	6	9	1

[a] The amino acid composition was tabulated from the sequence of cDNA clone (8). The calculation of the pI value was carried out according to Sillero and Ribeiro (15).

the cDNA sequence. The additional Ala residue was inserted as a result of linkers used to engineer the initiation codon onto the cDNA.[2] The resultant expressed protein, rCBP35, can be purified on the basis of its carbohydrate-binding activity on asialofetuin affinity columns (2,11).

Two types of two-dimensional gel analysis were carried out on rCBP35: IEF and NEPHGE. rCBP35 yielded no observable spots on IEF and a single spot on NEPHGE analysis. This conclusion was derived from both the silver stained gel (Fig. 1, A and B) and the immunoblot with rabbit anti-CBP35 (Fig. 1, C and D). The pI value of the single spot observed on NEPHGE was ~ 8.7. Because the production of rCBP35 by the transformed *E. coli* most probably did not result in post-translational modification of the polypeptide chain, the pI 8.7 value is likely the isoelectric point of the CBP35 polypeptide. This value agrees with the pI of the polypeptide, calculated from its amino acid composition (Table I).

Two-Dimensional Gel Electrophoresis of 3T3 Cell Extracts

In previous studies, we had reported that when CBP35, purified from [^{35}S]-methionine-labeled extracts of mouse 3T3 fibroblasts, was subjected to IEF gels and fluorography, two radioactive spots were observed, at pI values of 4.7 and 4.5 (2). Our present demonstration that the pI value of the CBP35 polypeptide was ~ 8.7 raised two questions: (a) Why was the pI 8.7 species not observed in our previous experiments? and (b) How were the pI 4.7 and 4.5 species derived from the unmodified polypeptide chain, with a pI value of 8.7? To answer these questions, a more detailed analysis was carried out on the protocols of two types of two-dimensional gel electrophoresis.

Certain proteins do not focus at the high pH end of the isoelectric gradient under IEF analysis (10,16). To illustrate the relevance of this point to the present study, seven protein standards were subjected to IEF analysis. Coomassie blue

Figure 1: **Two-dimensional electrophoretic analysis of recombinant CBP35 (~ 30 ng) on IEF (A and C) and NEPHGE (B and D) gels.** In A and B, the protein was detected by silver staining the gels. In C and D, the protein was revealed by immunoblotting with rabbit anti-CBP35. The numbers at the top indicate the pH values of the ampholine gradient. The open arrows indicate positions of migration of authentic CBP35 (M_r ~ 35,000).

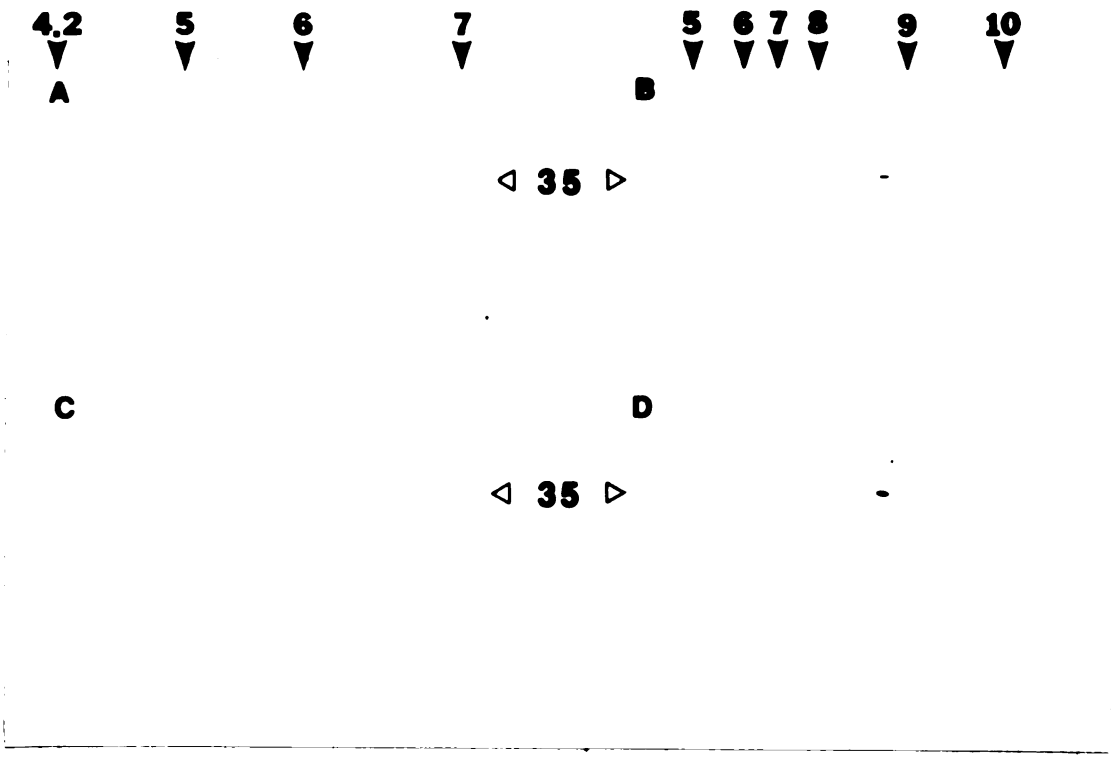

Figure 1

staining of the gel yielded only four identifiable spots consistent with the molecular weights and pI values of the protein standards (Fig. 2A). When the same mixture of seven proteins was subjected to NEPHGE analysis, seven identifiable spots corresponding to the molecular weights and pI values of the protein standards were observed (Fig. 2B). On the basis of comparing Fig. 2A with 2B, we find that proteins with a basic pI, such as carbonic anhydrase, phosphorylase a and phosphoglycerate kinase, do not focus on an IEF gel with its limited pH range (Fig. 2A). Although all seven protein standards were identified in the NEPHGE analysis, the resolution at the low pH end was poor (compare, for example, positions of spots 1, 2, and 3 in Fig. 2A and Fig. 2B). Therefore, both IEF and NEPHGE analyses were carried out in parallel on certain samples reported in the present study.

Extracts of mouse 3T3 fibroblasts were subjected to electrophoresis on IEF and NEPHGE gels, followed by immunoblotting with anti-CBP35. On IEF gels, no immunoreactive spots were observed (Fig. 2C). This result was identical to that obtained with rCBP35 (Fig. 1C); it was different, however, from the results of our previous studies (2), which showed two spots on IEF (pI 4.7 and pI 4.5). We had previously shown that the expression of CBP35, as well as its subcellular localization, were dependent on the proliferative state of the 3T3 cell culture (5). In the present study, we observed no CBP35 spots on IEF gels, irrespective of the source of the 3T3 cell extracts: (a) quiescent confluent cultures; (b) proliferating sparse cultures; (c) quiescent serum-starved cultures; and (d) proliferating serum-stimulated cultures. Therefore, the discrepancy of the results of the present study and those of our previous work (2) cannot be ascribed to differences in the expression of the CBP35 protein under proliferative versus quiescent culture conditions. We have also attempted to identify CBP35 on IEF gels using silver staining of purified preparations of the lectin or fluorography of purified

Figure 2: **Two-dimensional electrophoretic analysis of protein standards and CBP35 in extracts of mouse 3T3 fibroblasts on IEF (A and C) and NEPHGE (B and D) gels.** In A and B, protein standards were electrophoresed and were revealed by Coomassie blue staining. The markers 1-7 correspond to: 1) soybean trypsin inhibitor (8 μg); 2) rabbit muscle tropomyosin (10 μg); 3) rabbit muscle actin (10 μg); 4) bovine serum albumin (5 μg); 5) bovine erythrocyte carbonic anhydrase (10 μg); 6) rabbit muscle phosphorylase a (14 μg); 7) yeast phosphoglycerate kinase (8 μg). In C and D, extracts of 3T3 cells (200 μg total protein) were electrophoresed and the protein was revealed by immunoblotting with rabbit anti-CBP35. The numbers at the top indicate the pH values of the ampholine gradient. The numbers down the middle indicate positions of migration of molecular weight markers. The open arrows indicate the positions of migration of authentic CBP35 (M_r ~ 35,000).

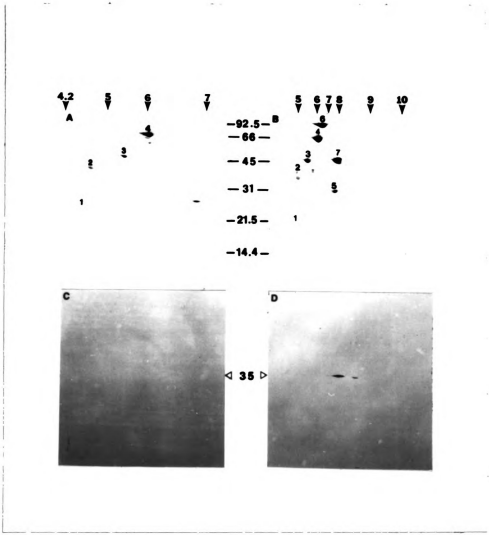

Figure 2

[^{35}S]methionine-labeled protein. In all of these cases, CBP35 could not be detected on IEF gels (see below, however).

Parallel analysis of the same extracts on NEPHGE gels revealed two spots with pI values of 8.7 and 8.2 (Fig. 2D). The pI 8.7 species corresponds to that observed on NEPHGE analysis of rCBP35 (Fig. 1D). It also matches the isoelectric point of the CBP35 polypeptide, calculated from its amino acid composition (Table I). The pI 8.2 spot was a new species; it was observed on immunoblots of cell extracts, as well as silver staining or fluorography of [^{35}S]methionine-labeled preparations of purified CBP35. These results indicate: (a) the pI 8.7 spot corresponds to the isoelectric point of the unmodified CBP35 polypeptide; (b) this pI 8.7 spot was missed in our previous two-dimensional gel analysis of purified CBP35 (2), most probably because only IEF but not NEPHGE was used; and (c) the pI 8.2 spot may represent a post-translationally modified product of the CBP35 polypeptide.

Isolation and Analysis of ^{32}P-Labeled CBP35 from 3T3 Cells

Extracts of 3T3 cells, cultured for 24 hours in ^{32}P-labeled phosphate, were fractionated on affinity columns containing asialofetuin. After extensive washing such that the level of radioactivity of the effluent reached a steady baseline, lactose was added to elute the bound material. A peak of ^{32}P radioactivity was observed upon the addition of lactose (Fig. 3). On one-dimensional SDS-PAGE, the material of this radioactive peak yielded a single polypeptide with a M_r ~ 35,000; this conclusion was obtained both with immunoblotting using rabbit anti-CBP35 (Fig. 3, inset, lane a) and with ^{32}P autoradiography (Fig. 3, inset, lane b). These results suggest that at least some of the CBP35 molecules, purified on the basis of carbohydrate-binding activity, contained radioactive phosphate.

Figure 3: **Affinity chromatography of a ^{32}P-labeled extract of mouse 3T3 cells on a column (1.3 x 4 cm) containing asialofetuin-derivatized Affi-gel 15. Fractions of 2 ml were collected and aliquots (50 µl) were monitored for ^{32}P radioactivity. The flow-through fractions (fraction numbers 1-9) contained large amounts of radioactivity and are not shown here. At the point indicated by the arrow, 0.3 M lactose was used to elute the bound material. Inset: The bound fraction was dialyzed, concentrated, and subjected to SDS-PAGE (5 x 10^4 cpm per lane). Lane a: The protein was revealed by immunoblotting with rabbit anti-CBP35. Lane b: The protein was revealed by autoradiography of the ^{32}P-labeled material.**

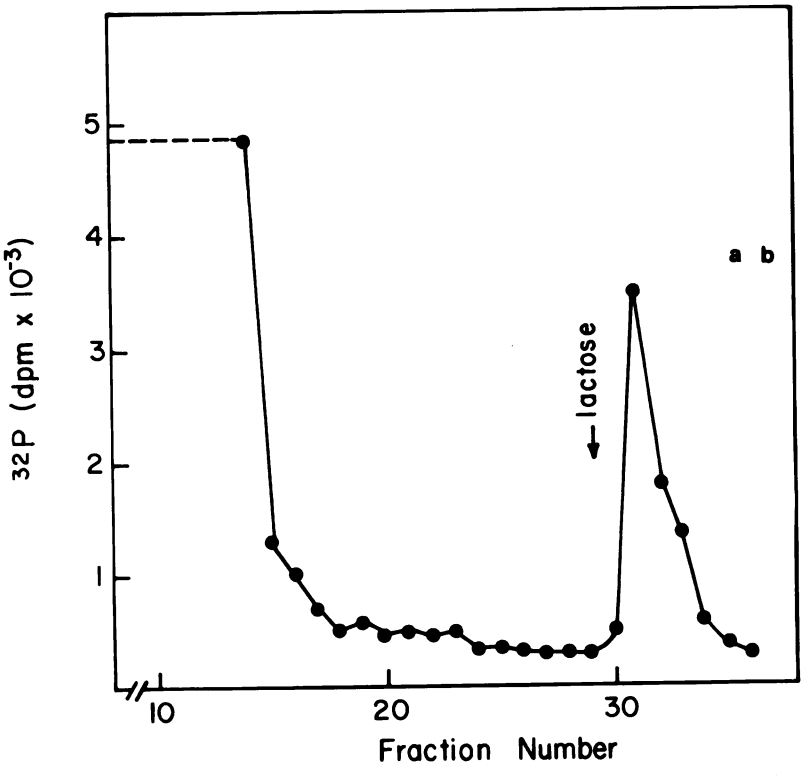

Figure 3

This ^{32}P-labeled sample of CBP35 was subjected to NEPHGE analysis, followed by immunoblotting with rabbit anti-CBP35 (Fig. 4A), as well as autoradiography of the nitro-cellulose membrane (Fig. 4B). The immunoblot yielded two protein spots, pI 8.7 and pI 8.2 (Fig. 4A), as was observed in Fig. 2D. Only the pI 8.2 species, however, was radioactive (Fig. 4B). The same ^{32}P-labeled sample was first treated with alkaline phosphatase prior to NEPHGE analysis. Alkaline phosphatase treatment reduced the immunoblot to a single spot (pI ~ 8.7; Fig. 4A); there was no radioactive spot in the autoradiogram (Fig. 4B). Parallel treatment with phosphodiesterase did not have any effect on the ^{32}P-labeled sample.

We interpret these results to indicate that the pI 8.2 spot is a post-translationally modified product of the pI 8.7 polypeptide chain by the addition of a single phosphate. The sensitivity of this ^{32}P-labeling to alkaline phosphatase suggests that it is an O-linked phosphate group. The introduction of one phosphate group to the amino acid composition of CBP35 converts the calculated pI from a value of 8.7 to 8.2 (Table I).

Carbamylation of CBP35

The identification of the pI 8.7 species as the isoelectric point of the unmodified CBP35 polypeptide chain and of the pI 8.2 species as a phosphorylated derivative still could not explain the original observation (2) that purified CBP35 yielded pIs of 4.7 and 4.5 on IEF analysis. Using CBP35 purified from 3T3 cells (2) or from mouse lung (3) and using different detection methods including silver staining, immunoblotting, and fluorography of [^{35}S]methionine-labeled samples, IEF analysis consistently yielded no detectable spots, whereas NEPHGE analysis reproducibly yielded the pI 8.7 and 8.2 spots, as shown in Fig. 2C and Fig. 2D, respectively. The origin of the pI 4.7 and 4.5 spots on IEF gels became apparent

Figure 4: **Two-dimensional NEPHGE gel analysis of purified ^{32}P-labeled CBP35 (5×10^5 cpm).** In A, the protein was revealed by immunoblotting with rabbit anti-CBP35; in B, the protein was detected by autoradiography of the ^{32}P label. Prior to electrophoresis, the samples were incubated for 2 hours at 37°C in 40 mM Tris, 15 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 8.0 (control) or in the same buffer containing 2 U/ml of calf intestine alkaline phosphatase (AP) or 0.2 U/ml of phosphodiesterase I (PDE). The numbers at the top indicate the pH values of the ampholine gradient.

Figure 4

when we used urea that had not been deionized to remove cyanate in the preparation of the IEF loading buffers.

CBP35, purified from mouse lung (3), was subjected to IEF analysis using two different protocols. In the first method, the protein sample was dissolved in buffer prepared using deionized urea and then loaded onto IEF gels. This yielded no spots detectable by immunoblotting (Fig. 5A). In the second method, the same protein preparation was dissolved in buffer prepared using undeionized urea. This sample was mixed with the acrylamide solution which was then polymerized into a gel containing the protein. The results after IEF showed two pairs of spots: (a) 4.7 and 4.5 as one predominant pair; and (b) 6.1 and 5.8 as a minor pair (Fig. 5B). Polymerizing the protein directly in the gel is known to subject the sample to heat produced during the polymerization reaction (21). Inasmuch as undeionized urea may contain cyanate, the results of Figure 5 suggest that the two pairs of spots observed on IEF gels were due to carbamylation of the CBP35 polypeptide. Consistent with this notion, derivatization of the lysyl residues of CBP35 by carbamylation would yield pI species (as unphosphorylated/phosphorylated pairs) of ~ 6.2/5.8 and 4.7/4.5, as calculated from the amino acid composition (Table I).

The final line of evidence for carbamylation is derived from [^{14}C]cyanate labeling of rCBP35. rCBP35 was incubated with ^{14}C-labeled cyanate at 37°C for 2 hours. The sample was then subjected to IEF analysis. Underivatized rCBP35 yielded no detectable spots on IEF gels, as shown in Fig. 1A and 1C. In contrast, incubation in cyanate resulted in spots with pI values of 4.7 and 6.1 as detected by silver staining and immunoblotting (Fig. 6A and 6B); parallel fluorographic analysis revealed that these spots contained radioactive proteins (Fig. 6C), indicative of the incorporation of ^{14}C-labeled cyanate. There were no 4.5 and 5.8 species because rCBP35 does not contain the phosphorylated group, as indicated by the absence of the pI 8.2 species on NEPHGE gels (Fig. 1B and 1D). All of these results strongly

Figure 5: **Two-dimensional electrophoretic analysis of CBP35 purified from mouse lung (50 ng) on IEF gels.** In A, the sample was dissolved in deionized urea and then loaded on an IEF gel that had already been polymerized. In B, the sample was dissolved in 8 M urea that had not been specifically deionized. Acrylamide solution was then added and the sample was incorporated directly in the polymerizing mixture in the first dimension tube. The protein was revealed by immunoblotting with rabbit anti-CBP35. The numbers at the top indicate the pH values of the ampholine gradient.

Figure 5

Figure 6: **Two-dimensional IEF gel analysis of recombinant CBP35 that had been incubated at 37°C for 2 hours with ^{14}C-labeled cyanate.** In A and B, the protein was detected by silver staining and by immunoblotting with rabbit anti-CBP35, respectively. In C, the protein was revealed by fluorography of the ^{14}C-labeled sample. The numbers at the top indicate the pH values of the ampholine gradient.

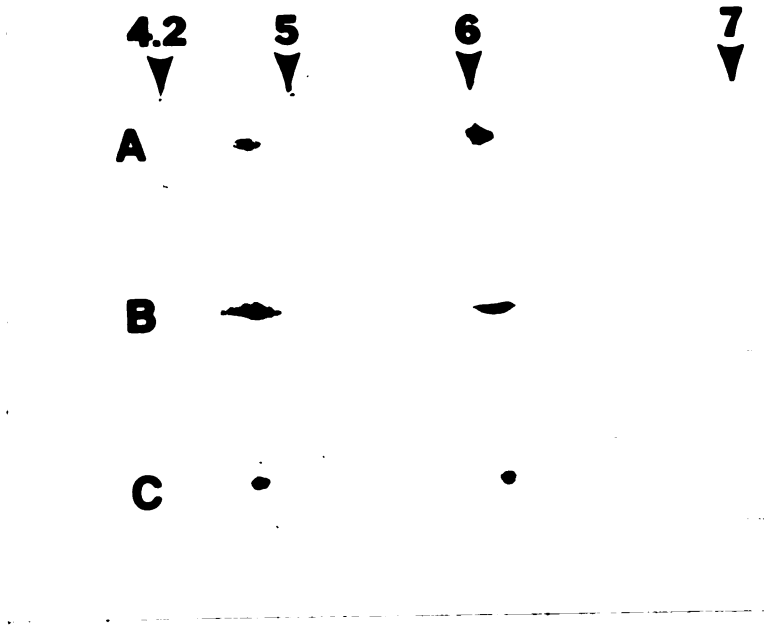

Figure 6

suggest that our previous observation (2) of the pI 4.7 and 4.5 species in IEF gels of 3T3 cell CBP35 was due to an artifact of carbamylation.

Discussion

The results documented in the present study provide the basis for three main conclusions. First, the isoelectric point of the CBP35 polypeptide chain is 8.7. This conclusion is based on the experimentally determined pI value for rCBP35, derived from expression of the cDNA clone for the lectin (7) in *E. coli*. This purified rCBP35 sample yielded a single spot at pI 8.7 on NEPHGE gels. It yielded no observable spot on IEF gels, most probably because polypeptides with high pI values, such as 8.7, fail to focus on IEF gels (10,16). The experimentally determined isoelectric point is consistent with the predicted pI, calculated on the basis of the amino acid composition deduced from the nucleotide sequence of the cDNA clone.

Both the predicted and the experimentally determined pI value depended on the cDNA clone for CBP35, previously characterized (7) and sequenced (8). Thus, a key consideration in the interpretation of our results is the authenticity of the cDNA clone for CBP35. The evidence that is brought to bear on this issue includes: (a) The cDNA clone was immuno-selected during the initial screening of the λgt11 expression library on the basis of reactivity of the expressed fusion protein with affinity purified, monospecific antibodies against CBP35 (7). This fusion protein can be immunoblotted by both anti-β-galactosidase, as well as anti-CBP35 and its molecular weight was consistent with the summation of the molecular weights of both fusion partners. (b) The fusion protein, upon V-8 protease digestion, yielded a polypeptide component (M_r ~ 30,000) that exhibited galactose-specific binding activity, as well as reactivity with anti-CBP35. Thus, the polypeptide encoded by the cDNA contained sufficient structural information for carbohydrate-specific binding (7). (c) The amino acid sequence, deduced from the nucleotide sequence of the cDNA clone (8), showed sequence identities with homologs of CBP35 in other species, as well as other galactose-specific lectins of lower molecular weight

(8,19,20). Thus, we conclude that the pI value obtained for rCBP35 represents the isoelectric point of the unmodified CBP35 polypeptide chain.

The second conclusion is that the CBP35 polypeptide can be post-translationally modified by the addition of a single phosphate group. This converts the pI from 8.7 to 8.2. We have purified CBP35 from 3T3 fibroblasts cultured in $^{32}PO_4$. Parallel analysis was carried out on the isolated ^{32}P-labeled CBP35, using immunoblotting to detect the polypeptide and autoradiography to detect the ^{32}P label. Immunoblotting yielded two spots, pIs 8.7 and 8.2, on NEPHGE gels; autoradiography showed that only the pI 8.2 spot was ^{32}P-labeled. The presence of the radiolabel in the pI 8.2 spot was alkaline phosphatase sensitive, while parallel treatment with phosphodiesterase failed to yield the same effect. These results strongly indicate that the addition of $^{32}PO_4$ occurred on a Ser, Thr, or Tyr residue inasmuch as O-linked phosphorylation is alkaline phosphatase sensitive (22). The consensus sequence for Ser/Thr phosphorylation by cyclic AMP-dependent protein kinases has been deduced: -Arg-Arg-Xxx-Ser/Thr-Xxx (23). The sequence in CBP35, $_{199}$Glu-Arg-Gln-Ser-Ala$_{203}$, may correspond to this consensus sequence with sufficient similarity to be a substrate for phosphorylation. This notion is currently being tested by site-directed mutagenesis studies.

On the basis of both immunological localization studies (6) and amino acid sequence homology (8), it has been proposed that CBP35 is one of the proteins of the core particle of hnRNP. Core proteins isolated from hnRNP complexes of HeLa cells and rat liver nuclei have a range of pIs (pI 5.7 - 9.3) (24-26). Moreover, some of the hnRNP proteins are mitogen stimulated and/or cell cycle regulated (27). They undergo alterations during the cell cycle (28), with some isoelectric variants only expressed during mitosis. There is direct chemical evidence to indicate that many of these proteins are phosphorylated and/or contain dimethylarginine (24), both of which represent post-translational modifications that lower the pI of

the polypeptide. In this context, phosphorylation of CBP35 may be important in the physiological function of CBP35. We had previously documented that the level of expression of CBP35 and its subcellular localization was dependent on the proliferation state of the cell (5,29,30): quiescent cultures have low levels of CBP35, mostly in the cytoplasm, whereas serum-stimulated cultures of the same cells resulted in increased amounts of the protein and nuclear translocation. We have now obtained evidence correlating the relative abundance of the pI 8.7 and 8.2 spots with the proliferation state of 3T3 fibroblasts, as well as the nuclear localization of the protein. These results are documented in the accompanying manuscript (31).

The final conclusion is that because only IEF gels were carried out in our previous two-dimensional electrophoretic analysis of purified CBP35 (2), the isoelectric variants of pI 8.7 and pI 8.2 simply failed to enter into the gel and were missed. Moreover, the acidic components observed previously, pI 4.7 and pI 4.5 (2), are most probably due to artifacts of carbamylation. In the present study, we have reproduced these two isoelectric spots only when cyanate-containing undeionized urea was used for IEF analysis and the samples were loaded by incorporating directly in the polymerizing acrylamide mixture within the first dimensional tube. Under these conditions, minor variants, pI 6.1 and pI 5.8, were also observed.

The rat homolog to mouse CBP35 has been identified and sequenced. This protein has been designated as rat lung lectin (RL-29) (32) or IgE-binding protein (19,33). The amino acid sequence predicts a pI of ~ 9. Consistent with this calculation, RL-29 yielded pI 9 on one-dimensional isoelectric focusing gel analysis (32). Thus, the mouse and rat homologs of CBP35 both have isoelectric points in the basic pH range. This should be contrasted to the galactose-specific lectin isolated from human lung. HL-29, which is most probably the human homolog of CBP35, has at least five acidic isoelectric variants (34). It should be noted, however, that, like our initial determination of the isoelectric points of CBP35 (2), the

analysis of HL-29 has been made using IEF alone. Therefore, other isoelectric variants at basic pH may have been missed.

Acknowledgments

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Chapter III

Carbohydrate Binding Protein 35: Identification of Isoelectric Variants in Quiescent and Proliferating Cells

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Running Title: Isoelectric Variants of Carbohydrate Binding Protein 35

Footnotes

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1 The abbreviations are: CBP35, Carbohydrate Binding Protein (M_r ~ 35,000); NEPHGE, nonequilibrium pH gradient electrophoresis; DME, Dulbecco modified Eagle's medium.

Summary

The isoelectric forms of CBP35 were analyzed in cultures of mouse and human fibroblasts representing quiescent and proliferating states: (a) confluent monolayers versus sparse cultures of mouse 3T3 cells; (b) serum-starved versus serum-stimulated 3T3 cells; and (c) "young" (passage 11) versus "old" (passage 33) human SL66 cells. In all cases studied, quiescent cell populations are characterized by the predominance of the pI 8.2 species, representing a phosphorylated form of the CBP35 polypeptide chain. Cells that are induced into the proliferative state express higher levels of CBP35 overall, relative to quiescent cultures. This elevated level of CBP35 is accounted for, in part, by an increase in the amount of the pI 8.2 species; more dramatic, however, is the elevation of the level of the 8.7 species, which is barely detectable in quiescent cells. Analysis of the cytosol fraction of the various cultures showed that it consists almost exclusively of the pI 8.2 species of CBP35. In contrast, both the pI 8.2 and 8.7 species were found in the nuclei. Thus, the pI 8.2 species distributes between the cytosol and nuclear fractions, while the pI 8.7 species is found only in nuclei. These results suggest that stimulation of cells leads to the increased production of the CBP35 polypeptide, some of which accounts for an increase in the phosphorylated cytoplasmic form, but some of which are translocated into the nucleus.

Introduction

In the accompanying paper (1), we identified the isoelectric point of the polypeptide chain of Carbohydrate Binding Protein 35 (CBP35)[1] to be pI 8.7. We also documented that CBP35 isolated from mouse 3T3 cells and mouse tissues consists of two isoelectric variants with pI values of 8.7 and 8.2. The former value represents the pI of the unmodified polypeptide chain, while the latter is a post-translationally modified product, due to the addition of a single phosphate.

When quiescent 3T3 fibroblasts were stimulated by the addition of serum, there was an increase in the overall level of CBP35, as well as a dramatic rise in the amount of the polypeptide in the nucleus (2,3). Together, these previous results, along with the more recent identification of the isoelectric variants, raise the question of whether we can identify which of the isoelectric species is stimulated by serum growth factors and/or is associated with the cell nucleus. In the present communication, we report the results of our studies directed at this question.

Experimental Procedures

Cell Culture

Swiss 3T3 fibroblasts (CCL92, American Type Culture Collection) were cultured in Dulbecco modified Eagle's medium (DME) containing 10% (v/v) calf serum (1,2). Sparse, proliferating cultures of 3T3 cells were seeded at an initial density of 0.7×10^4 cells/cm^2 in DME containing 10% serum. These cultures were synchronized by incubation in DME containing 0.2% serum for 48 hours and were stimulated to proceed through the cell cycle by the addition of 10% serum. Extracts of serum-stimulated cultures were prepared 16 hours after serum addition. Confluent monolayers of the same cells were seeded at an initial density of 5×10^4 cells/cm^2 in DME-10% serum. These cultures were also serum-starved (0.2% serum in DME for 48 hours) and serum-stimulated (readdition of serum to 10% in DME) following the same protocol.

Human fibroblasts (SL66) were a gift from Drs. J. J. McCormick and V. M. Maher (4) and were used at passages 11 and 33. The cells were cultured in Eagle's minimal essential medium containing 20% fetal calf serum (5). These cells were synchronized by serum starvation in Eagle's minimum essential medium containing 0.2% fetal calf serum for 48 hours, followed by readdition of fetal calf serum (20%) for 16 hours (3).

Preparation of Cell Extracts and Subcellular Fractions

For extract preparation, the cells were washed with phosphate-buffered saline, scraped off the culture dish, and centrifuged (1330 x g, 3 minutes). The cells were resuspended in 10 mM Tris (pH 7.5) containing 2 mM EGTA, 1 mM phenylmethylsulfonyl chloride, 1 μg/ml soybean trypsin inhibitor, and 1 U/ml aprotinin. The cells were incubated on ice for 20 minutes, followed by sonication

(four times, 15 seconds each). For subcellular fractionation, homogenates were prepared in hypotonic buffer, 0.02 M Tris (pH 7.2), 5 mM KCl, 105 mU/ml aprotinin (6-8). The homogenate was subjected to low speed centrifugation (375 x g) to yield a nuclear pellet and a post-nuclear supernatant fraction. The pellet fraction, containing mostly nuclei and some intact cells, was resuspended in the same Tris buffer and then aspirated through a 25-gauge needle. Resedimentation yielded the final nuclear pellet. The post-nuclear supernatant was separated into a high speed (150,000 x g) pellet and a soluble fraction (supernatant). The latter constituted the cytosol fraction.

Protein was measured by the Bradford assay (9). The procedures for assaying for the cytoplasmic marker enzyme, lactate dehydrogenase, has been described (10). The binding of the DNA-specific dye, Hoechst 33258 (10 μg/ml; Sigma), was used as a marker for nuclei (11). Aliquots (~ 100 μg total protein) of whole cell lysate, cytosol, and nuclear pellet fractions were digested with ribonuclease A (50 μg/ml; Boehringer-Mannheim) and deoxyribonuclease I (50 μg/ml; Boehringer-Mannheim) on ice for 30 minutes. They were then dissolved in lysis buffer (12) with a final concentration of urea at 9.5 M.

Two-Dimensional Gel Electrophoresis

For two-dimensional gel electrophoresis in this study, the methods of O'Farrell (12,13), using non-equilibrium pH gradient electrophoresis (NEPHGE) in the first dimension and sodium dodecyl sulfate polyacrylamide gel electrophoresis in the second dimension, were used. NEPHGE gels were prepared using pH 3-10 ampholines and the electrophoresis was carried out at 400 V for 5 hours. The electrode solution contained 10 mM NaOH and 5 mM $Ca(OH)_2$. Standard proteins were used to track the pH gradient. The second dimension polyacrylamide gel had 8 cm of separating gel containing 12.5% acrylamide. Proteins were detected after two-dimensional gel electrophoresis and transfer to Immobilon (Millipore) by immunoblotting with rabbit anti-CBP35 antiserum (1:150) overnight at 4°C, followed by reaction with horseradish peroxidase conjugated goat anti-rabbit immunoglobulin (BioRad). The details of these procedures have been described (1,8,14).

Results

Expression of CBP35 Isoelectric Variants in Unsynchronized 3T3 Cell Cultures

Mouse 3T3 fibroblasts exhibit the phenomenon of density-dependent inhibition of growth (15,16). In sparse cultures (cell density of $< 5 \times 10^3$ cells/cm^2, for example), the cells undergo DNA synthesis and cell division. Unless specifically synchronized by experimental manipulation, the cells are found distributed in different phases of the cell cycle. In confluent monolayers (cell density 5×10^4 cells/cm^2), there is little or no DNA synthesis and cell division. These cells are reversibly arrested in a quiescent state; they can be reactivated by dilution to low cell density (15) or by stimulation with serum growth factors (17).

In previous immunofluorescence studies using anti-CBP35 (2), we found intense staining, predominantly in the nucleus, of the 3T3 cells in sparse cultures. By contrast, there was faint staining of the cytoplasm, with little or no staining of the nucleus, in confluent monolayers of the same cells. Extracts were prepared from unsynchronized sparse cultures and confluent monolayers of 3T3 cells. These extracts of whole cells were subjected to two-dimensional NEPHGE analysis (Fig. 1). The extract of quiescent cultures at high cell density yielded a single spot at pI 8.2. The extract of proliferating cultures, at low cell density, yielded a spot at pI 8.7, in addition to the pI 8.2 spot.

Equal amounts of protein from both extracts were subjected to NEPHGE analysis. Therefore, the intensities of the immunoblot staining reflect the relative levels of the protein in the two cell extracts. Such a comparison indicated that the sparse culture showed an increase in the expression of CBP35 protein, consistent with previous documentation of elevated levels of CBP35 in proliferating cultures (2,3). This increase is reflected in both the intensity of the pI 8.2 spot of sparse

Figure 1: **Comparison by NEPHGE gels of the isoelectric variants of CBP35 in quiescent confluent monolayers of 3T3 cells (C) and in proliferating sparse cultures of the same cells (S).** Ext, whole cell extract; Cyt, cytosol fraction; Nuc, nuclear pellet. Approximately 100 μg of each sample were electrophoresed and the protein was detected by immuno-blotting with rabbit anti-CBP35. The positions of migration of the pI 8.2 and pI 8.7 species are indicated at the top.

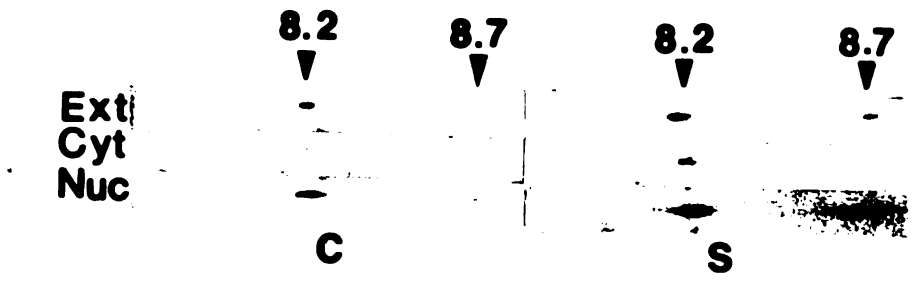

Figure 1

cultures (relative to the corresponding spot in confluent cells), as well as the appearance of the pI 8.7 spot.

Analysis of CBP35 Isoelectric Variants in Nuclear Versus Cytoplasmic Fractions

The cells of confluent and sparse cultures were also subjected to subcellular fractionation to yield a cytosol fraction (150,000 x g supernatant) and a nuclear pellet. These two subcellular fractions were assayed for marker molecules: lactate dehydrogenase for cytosol and DNA- specific binding of the Hoechst 33258 dye for nuclei (Table I). The cytosol, which accounted for ~ 50% of the total cellular protein, carried 97% of the lactate dehydrogenase enzyme activity and showed about 5% of the DNA-binding Hoechst dye. Approximately 20% of the cellular protein was found in the nuclear pellet; the remainder of the protein was in membrane components that did not fractionate into the 150,000 x g supernatant or the nuclear pellet. The nuclei contained < 2% of the cellular lactate dehydrogenase activity and accounted for 70% of the DNA-binding Hoechst dye. Thus, the results showed little or no contamination of the nuclear fraction by cytoplasmic components.

The cytosol fraction from both the confluent and sparse cultures yielded only the pI 8.2 spot (Fig. 1). The intensity of the spot in the cytosol fraction of sparse cultures was much greater than that of the corresponding spot from confluent cultures. This reflects the increase in the expression of the CBP35 polypeptide in proliferating cells.

The nuclei fraction of confluent cells showed both the pI 8.7 and the pI 8.2 spots (Fig. 1). The intensity of the pI 8.2 spot was greater than that of the same spot in whole cell extract. Both the increase in intensity of the pI 8.2 spot and the appearance of the pI 8.7 spot are probably due to the enrichment in the specific activity of CBP35 (amount of CBP35 per unit amount of total protein) in the

Table I. **Nuclear versus Cytoplasmic Fractions of 3T3 Fibroblasts**

Fraction	Protein[a]		Lactate Dehydrogenase		DNA[b]	
	mg	% recovered	units	% recovered	μg	% recovered
Whole cell extract	4.1	100	60	100	170	100
Cytosol (150,000 x g supernatant)	1.9	46	58	97	9.3	5.4
Nuclear pellet	0.8	20	< 1[c]	< 2[c]	119	70

[a] Protein was estimated by the Bradford assay. Approximately 30% of the protein fractionated with membrane-containing material in the 150,000 x g pellet.

[b] DNA was estimated by the binding of the Hoechst 33258 dye.

[c] Only an upper limit was estimated because the absorbance readings of the enzyme assay for the nuclear pellet was barely above background and could not be determined accurately.

nucleus. In the nuclear pellet of sparse cells, both the pI 8.7 and pI 8.2 spots showed increases, relative to whole cell extract as well as relative to the nuclear fraction of confluent cells (Fig. 1). Thus, the subcellular fractionation studies substantiate the conclusion obtained with whole cell extracts. In proliferating cultures, there is more CBP35 expressed, with some increase in the amount of pI 8.2 species and a dramatic increase in the amount of the pI 8.7 species.

CBP35 Isoelectric Variants in Synchronized Sparse Cells

An alternative method was used to obtain population of quiescent and proliferative 3T3 cells for comparison. Sparse cultures of 3T3 cells were deprived of serum (0.2% serum for 48 hours) to arrest the cells. Readdition of serum resulted in a wave of synchronous DNA synthesis 20 hours later (2,3). When extracts of serum-starved quiescent 3T3 cells were subjected to NEPHGE analysis, a single spot of pI 8.2 was observed (Fig. 2). In contrast, serum-stimulated proliferating 3T3 cells yielded both the pI 8.2 and pI 8.7 spots.

The cytosol fraction from both serum-starved and serum-stimulated cultures yielded only the pI 8.2 spot (Fig. 2). This is what was observed when the cytosol fractions of unsynchronized confluent and sparse 3T3 cultures were compared (Fig. 1). Similarly, the nuclei fraction of both serum-starved and serum-stimulated cultures yielded pI 8.7, as well as pI 8.2 spots (Fig. 2). In this case, however, there was a dramatic increase in intensity of both the pI 8.7 and the pI 8.2 spots in the serum-stimulated nuclei sample, compared to whole cell extracts or compared to the serum-starved sample. Thus, it again appears that in proliferating 3T3 cell cultures, the increased CBP35 expression is manifested most clearly in the nuclear fraction, with increases in both the pI 8.7 as well as in the pI 8.2 species of CBP35 isoelectric variants.

Figure 2: **Comparison by NEPHGE gels of the isoelectric variants of CBP35 in sparse cultures of 3T3 fibroblasts synchronized by serum starvation (0.2% calf serum for 48 hours) (Q) and by serum stimulation (10% calf serum for 16 hours) (P).** Ext, Whole cell extract; Cyt, cytosol fraction; Nuc, nuclear pellet. Approximately 100 μg of each sample were electrophoresed and the protein was detected by immunoblotting with rabbit anti-CBP35. The positions of migration of the pI 8.2 and pI 8.7 species are indicated at the top.

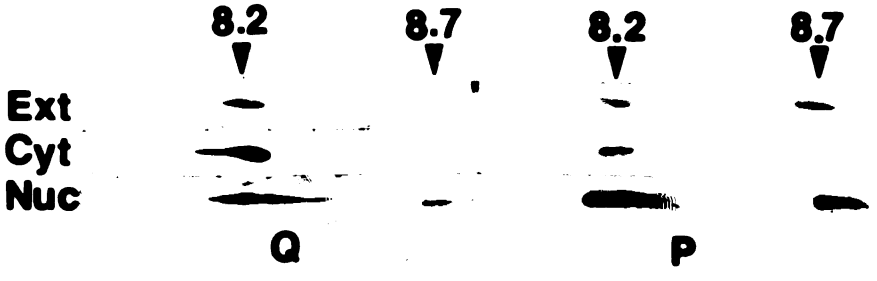

Figure 2

CBP35 Isoelectric Variants in Stimulated Confluent Monolayers

Addition of serum growth factors to density-arrested monolayers of 3T3 cells results in one round of DNA synthesis and cell division (17). The extract of quiescent confluent monolayers yielded a spot at pI 8.2, along with a faint minor spot at pI 8.7. The extract of serum-stimulated confluent cultures showed both the pI 8.2 and the pI 8.7 spots, with the increase of pI 8.7 spot, relative to unstimulated monolayers, most noticeable (Fig. 3). This increase in the pI 8.7 species is also observed in the nuclei fractions of serum-stimulated monolayers.

CBP35 Isoelectric Variants in Human Fibroblasts of Different Replicative Capacities

In previous studies (3), we had compared the expression and localization of CBP35 in human SL66 fibroblasts of "young" (passage 11) and "old" (passage 33) age. Our results indicated that in human fibroblasts of high passage, the level of CBP35 was not modulated by serum or growth state of the cell. Thus, the level of CBP35 did not diminish upon serum starvation; nor was the level increased upon serum readdition to cultures held in 0.2% serum for 48 hours. In contrast, human fibroblasts of low passage behaved identically to 3T3 cells in terms of serum/growth regulated expression of CBP35. Thus, it was of interest to compare the isoelectric variants of CBP35 expressed in "young" versus "old" SL66 cells.

Serum-starved SL66 fibroblasts of low passage (passage 11) yielded a single isoelectric species upon NEPHGE analysis, pI 8.2 (Fig. 4). Upon serum stimulation, the intensity of this pI 8.2 spot increased dramatically. In addition, the pI 8.7 species also appeared (Fig. 4). This result is qualitatively similar to that obtained in comparing serum-stimulated versus serum-starved sparse cultures of 3T3 cells (Fig. 2). There were quantitative differences, however, between mouse and human fibroblasts. In human cells, the increase in the level of CBP35 upon serum

Figure 3: **Comparison by NEPHGE gels of the isoelectric variants of CBP35 in confluent monolayers of 3T3 fibroblasts serum-starved (0.2% calf serum for 48 hours) (Q) and serum-stimulated (10% calf serum for 16 hours) (P).** Ext, whole cell extract; Cyt, cytosol fraction; Nuc, nuclear pellet. Approximately 100 μg of each sample were electrophoresed and the protein was detected by immunoblotting with rabbit anti-CBP35. The positions of migration of the pI 8.2 and pI 8.7 species are indicated at the top.

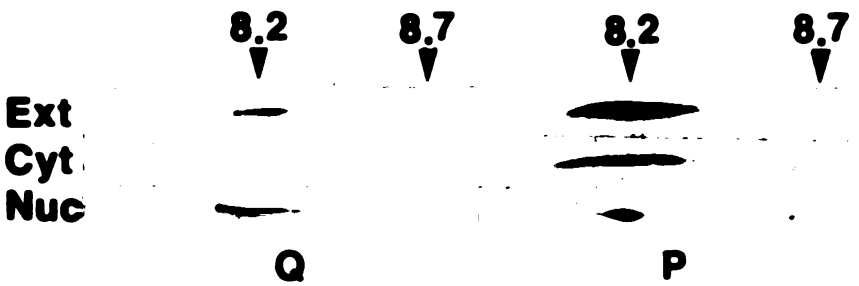

Figure 3

Figure 4: **Comparison by NEPHGE gels of the isoelectric variants of CBP35 in sparse cultures of human SL66 fibroblasts synchronized by serum starvation (0.2% fetal calf serum for 48 hours) (Q) and by serum stimulation (20% fetal calf serum for 16 hours) (P).** p11, SL66 cells at passage 11; p33, SL66 cells at passage 33. Approximately 100 μg of each sample were electrophoresed and the protein was detected by immunoblotting with rabbit anti-CBP35. The positions of migration of the pI 8.2 and pI 8.7 species are indicated at the top.

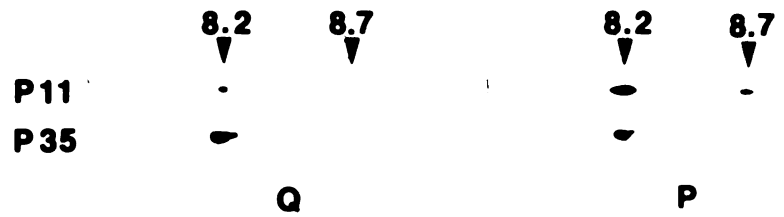

Figure 4

stimulation was most dramatically manifested by an increase in the intensity of the pI 8.2 spot, with a minor pI 8.7 spot emerging (Fig. 4). In contrast, the increase of CBP35 upon serum stimulation of mouse 3T3 cells was noticeable in terms of increases in intensity of both pI 8.2 and pI 8.7 spots (Fig. 2).

Serum-starved SL66 fibroblasts of high passage (passage 33) showed a single spot on NEPHGE gels, pI 8.2 (Fig. 4). The intensity of this spot was greater than the corresponding spot in serum-starved cells of low passage. Moreover, the intensity of this pI 8.2 does not change appreciably upon serum stimulation in the old cells. This result is consistent with our previous observations that the level of CBP35 in human SL66 fibroblasts was rather insensitive to serum/growth state modulation. Finally, there was no evidence of any pI 8.7 CBP35 species in serum-stimulated cultures of passage 33 human fibroblasts (Fig. 4). This is in direct contrast to all the results obtained with mouse 3T3 fibroblasts and passage 11 human SL66 cells, in which the extracts of cells induced into the proliferative state (sparse culture or serum stimulation) show the pI 8.7 spot.

Discussion

The general conclusions that can be derived from the results of the present study include: (a) The pI 8.7 isoelectric variant of CBP35 is found almost exclusively in the cell nucleus. (b) This pI 8.7 species is found readily in whole cell extracts and nuclei fraction of proliferating cultures; its presence in quiescent cells is detectable only in the nuclei fraction, where the specific activity of CBP35 per unit weight of total protein is highly enriched (8). (c) The cytoplasm contains mostly the pI 8.2 isoelectric variant of CBP35, although this species is also found in the nuclear pellet.

When quiescent 3T3 cells are stimulated by the addition of serum growth factors, there is an increase in the expression of CBP35. This conclusion has now been documented by analysis of the transcription of the CBP35 gene in nuclear run-off assays (18), analysis of mRNA accumulation by Northern blotting (18), analysis at the protein level by Western blotting (2,3), and analysis at the single cell level by immunofluorescence (2). These latter immunofluorescence experiments also revealed that the increased amount of CBP35 is most distinctly seen as an accumulation in the nuclei of 3T3 cells. The present results provide further information on this phenomenon: in proliferating 3T3 cell cultures, the increased CBP35 expression results in elevated amounts of the nuclear pI 8.7 species of CBP35 and some increases in the amount of pI 8.2 species of CBP35 in both the nucleus and the cytoplasm.

Human fibroblasts have a finite life span when cultured *in vitro*, suggesting that these cells, like whole organisms, are susceptible to the aging process (19). The "age" of the cells increases with passage in culture and the senescent fibroblasts lose their capacity to proliferate. We had previously compared the expression and localization of CBP35 in human fibroblasts of different replicative capacities: young (passage 11) through old (passage 33) SL66 cells (3). The results indicated that in

cells with age-acquired replicative deficiencies, the level of CBP35 was unresponsive to serum/growth state; there was no down regulation upon serum starvation nor upregulation upon serum stimulation. In contrast, the level of CBP35 in young SL66 cells was modulated by serum/growth state, as was observed with mouse 3T3 fibroblasts. Consistent with these results, our present data indicate that in SL66 fibroblasts of low passage number, serum starvation lowered the overall amount of CBP35, seen only as the pI 8.2 species, while serum stimulation elevated both pI 8.2 and pI 8.7 species of CBP35. On the other hand, in high passage SL66 cells that are replication deficient, only the pI 8.2 species is seen in both serum-starved and serum-stimulated cultures. Thus, it appears that in those senescent cells that cannot be stimulated to undergo the events of the cell cycle, there is suppression of the elevation of the pI 8.7 species of CBP35.

We have identified pI 8.7 as the isoelectric point of the unmodified CBP35 polypeptide (1). We have also provided evidence to indicate that phosphorylation of the CBP35 polypeptide results in the conversion of its isoelectric point from 8.7 to 8.2. The observations that the unmodified CBP35 polypeptide is found almost exclusively in the cell nucleus, while the phosphorylated derivative is found in both the cytoplasm and the nucleus, raise interesting questions concerning the regulation of the localization of the protein.

Proteins are synthesized on cytoplasmic ribosomes. Small proteins can pass freely into the nucleus, whereas large ones (~ 60 kD) cannot penetrate the nucleopore complex unless they carry specific nuclear targeting sequences (20). But the possession of such a sequence merely fulfills the probably necessary, but not sufficient condition for nuclear entry. For example, other structural motifs on the protein could negate the effect of the nuclear targeting sequence by anchoring the protein in the cytoplasm (21). The nuclear targeting sequence may be masked by binding to other proteins; alternatively, ligand binding could expose or mask the

recognition sequence. Finally, protein phosphorylation could modify the effectiveness of the nuclear targeting sequence or the interaction of the nuclear protein with its cytoplasmic anchor. One example worth noting is the transcription factor NF-κB, which is involved in the activation of the immunoglobulin κ light chain gene transcription in B lymphocytes (22). This protein is found in nuclear extracts of activated lymphocytes, but appears to reside in the cytoplasm of unstimulated cells as a complex with a cytoplasmic anchor, I-κB. Phosphorylation of the anchor I-κB by protein kinase C appears to release the transcription factor for entry into the nucleus.

Taken in this context, the identification of the dephosphorylated form of CBP35 in nuclei of stimulated cells is particularly interesting. One hypothesis is that the cytoplasmically synthesized protein is quickly phosphorylated. This pI 8.2 species distributes between the nucleus and the cytoplasm. But because the cytoplasm is, in general, much larger than the nucleus, the majority of this protein will be in the cytoplasm (23). Upon serum growth factor stimulation, a phosphatase is activated to dephosphorylate CBP35, which in turn allows the latter to concentrate within the nucleus.

Whatever the detailed mechanism, it is clear from our study, as well as other examples (24-26), that the distribution of proteins between the nucleus and cytoplasm can be controlled. It is also clear that there is still much to be learned about the molecular details of how this control is exerted and the mechanism by which this, in turn, can control the physiological state of the cell.

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Chapter IV

Expression of Carbohydrate Binding Protein 35 in Human Fibroblasts: Comparisons Between Cells with Different Proliferative Capacities

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Key Words: Nuclear proteins; lectins; *in vitro* senescence; Werner's syndrome

Abstract

Carbohydrate Binding Protein 35 (CBP35) is a galactose-specific lectin found in the nucleus and cytoplasm of mouse 3T3 fibroblasts. In these cultures, the level of expression and nuclear localization of CBP35 was correlated with the proliferative state of the cells. CBP35 is also found in human fibroblasts. We have compared the expression and localization of CBP35 in human fibroblasts of different replicative capacities: young (passage 11), intermediate (passage 19), and old (passage 33) SL66 cells, and fibroblasts derived from a patient with Werner's syndrome. The results indicate that the expression of CBP35 in cells with either age-aquired or congenital replicative deficiences was unresponsive to serum stimulation, in contrast with that found in young normal human fibroblasts and in 3T3 cells.

Introduction

Carbohydrate Binding Protein 35 (CBP35), a lectin specific for galactose-containing glycoconjugates, was originally identified in and purified from cultured mouse 3T3 fibroblasts (Roff and Wang, 1983). By the use of a specific antiserum directed against CBP35, the protein was subsequently shown to occur in a variety of tissue types and cell cultures, including SL66 human fibroblasts (Roff *et al.*, 1983; Crittenden *et al.*, 1984). Immunoblotting and immuno- fluorescence studies revealed that the level and subcellular location of CBP35 in mouse 3T3 fibroblasts depended on the proliferative state of the cells: in quiescent cultures, the protein was located primarily in the cytoplasm, whereas in proliferating cultures, it increased in amount and was found predominantly in the nucleus (Moutsatsos *et al.*, 1987). In synchronized cultures, the percentage of cells containing nuclear CBP35 was highest at the onset of the S phase of the cell cycle. CBP35 has now been cloned and sequenced (Jia *et al.*, 1987; Jia and Wang, 1988). The structural results, along with immunochemical analysis of subnuclear fractions (Laing and Wang, 1988), indicate that CBP35 is a component of the heterogeneous nuclear ribonucleoprotein complex (hnRNP).

The identification of CBP35 as a nuclear component and the correlation of its expression with the proliferative state of 3T3 cultures prompted us to compare the level and localization of the protein in normal proliferating human fibroblasts (SL66, passage number 11) with that in two cultures with diminished proliferative capacities: (i) late-passage SL66 human fibroblasts and (ii) human fibroblasts from a patient with Werner's syndrome (WS), a heritable disease characterized by segmental premature aging (Salk, 1982). Our results, documented in the present paper, indicate that the expression of CBP35 in fibroblasts with either age-acquired

or congenital replicative deficiencies was unresponsive to serum stimulation, in contrast with that found in young normal fibroblast cultures.

Materials and Methods

Cell Culture Conditions

The growth and maintenance of 3T3 fibroblasts have been previously described (Steck *et al.*, 1979). SL66 human skin fibroblasts were a gift from Drs. J.J. McCormick and V.M. Maher (Drinkwater *et al.*, 1982) and were used at passages 11, 19 and 33. Werner's syndrome fibroblasts AG6300 were obtained from the NIA Aging Cell Repository, Camden, N.J. The culture conditions for the human fibroblasts have been previously described (Cowles *et al.*, 1987). Cells were seeded at a density of 1.5 x 10^4 cells/cm^2 in growth media; the cells were allowed to attach overnight. The 3T3 cells were synchronized by serum starvation and readdition of serum (Laing and Wang, 1988). The human cells were synchronized by serum starvation in Eagle's minimal essential medium containing 0.2% fetal bovine serum for 48 hours followed by readdition of serum (20%, v/v) for 16 hours.

Immunoblotting and Immunofluorescence Procedures

Quiescent and stimulated cells were washed with phosphate buffered saline (PBS; 0.14 M NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). The cells were scraped off the culture flasks into PBS with a rubber policeman. The cells were isolated by centrifugation for 3 minutes at 1330 x g in a tabletop centrifuge, and resuspended in a 10 mM Tris-HCl buffer, pH 7.5, containing 2 mM EGTA, 5 mM benzamidine, 10 mM N-ethylmaleimide, 1 mM phenylmethylsulfonylfluoride, 1 μg/ml soybean trypsin inhibitor and 30 KIU/ml aprotinin (Boehringer-Mannheim Biochemicals). The cells were incubated on ice for 20 minutes and sonicated for 15 seconds four times. Protein was estimated by the method of Bradford (1976). Aliquots of the cellular extracts (50 μg total protein) were mixed with sample buffer (Laemmli, 1970) and separated on a 12.5%

polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE). Quantitative immunoblotting procedures have been described in detail (Moutsatsos *et al.*, 1987).

Immunofluorescence studies were carried out on 3T3 and human fibroblasts seeded onto coverslips. The binding of the primary antibody, rabbit anti-CBP35, was detected by either fluorescein-conjugated or rhodamine-conjugated goat anti-rabbit immunoglobulin. The details of these procedures have been reported (Moutsatsos *et al.*, 1986; Moutsatsos *et al.*, 1987).

Results

Analysis of the Immunofluorescence Patterns for CBP35 at Sparse and Confluent Culture Densities

In previous immunofluorescence studies, we had observed intracellular staining of mouse 3T3 fibroblasts by rabbit anti-CBP35 (Moutsatsos *et al.*, 1986). In certain instances, the staining was localized mainly in the nucleus of the cell; in other cases, the staining was diffusely distributed in the cytoplasm. We observed that the labeling patterns were apparently dependent on the density of the culture on which the immunofluorescence was performed. Cultures of 3T3 cells were seeded at different densities; after an overnight period of attachment, the cultures were subjected to immunofluorescence analysis with rabbit anti-CBP35. Representative epi- fluorescence photographs for two different cell densities are shown in Figure 1. In confluent monolayers ("high" cell density 5×10^4 cells/cm^2), there was weak labeling of the cytoplasm (Fig. 1A). In contrast, cells of sparse cultures ("low" cell density, 3×10^3 cells/cm^2) showed intense staining, predominantly in the nucleus (Fig. 1B). Consistent with previous experiments (Moutsatsos *et al.*, 1986), control samples stained with pre-immune rabbit serum showed no fluorescence labeling.

These results indicate that the overall expression of CBP35, as well as its subcellular distribution, are highly dependent on the condition of the cell culture under analysis. Sparse cultures of 3T3 cells proliferate with a doubling time of approximately 20 hours, while the same cells are subject to density-dependent inhibition of growth in confluent cultures (Todaro and Green, 1963). Our results suggest the possibility, therefore, that the level of CBP35 polypeptide and its nuclear localization may be dependent on the proliferation state of the cell.

Figure 1. **Representative immunofluorescence staining patterns of 3T3 fibroblasts after fixation with formaldehyde (3.7%) and permeabilization with Triton X-100 (0.2%).** The binding of rabbit anti-CBP35 (1:10 dilution of antiserum, 1 hour at room temperature) was detected by fluorescein-labeled goat anti-rabbit immunoglobulin (1:30 dilution, 1 hour at room temperature). (A) 5×10^4 cells/cm^2 (confluent). (B) 3×10^3 cells/cm^2 (sparse). Bar = 50 μm.

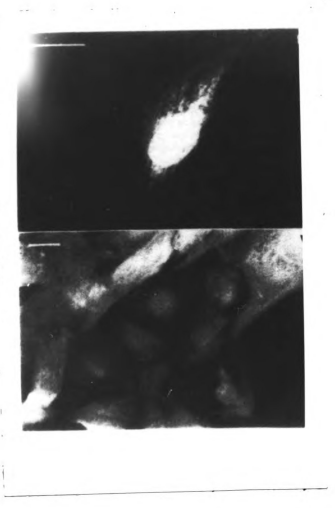

Figure 1

Kinetics of the Expression of CBP35 Upon Serum Stimulation of Quiescent 3T3 Cells

When serum-starved, quiescent cultures of 3T3 cells were stimulated by the addition of serum, the first wave of DNA synthesis in the synchronized population was observed between 16 and 28 hours after serum addition (Fig. 2a). Samples from parallel cultures were subjected to SDS-PAGE and quantitative immunoblotting as follows: (a) Extracts of 3T3 cells were subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted with rabbit anti-CBP35; (b) The immunoreactive material was identified with horseradish peroxidase-conjugated goat anti- rabbit immunoglobulin; (c) The nitrocellulose strip containing the peroxidase-positive polypeptide band was excised, incubated with ^{125}I-labeled Protein A, and its level of radioactivity was quantitated in a γ-spectrometer. The assay was linear over the range of protein concentrations used in our experiment.

Using this assay, we found that there was a two-fold increase in the level of CBP35, beginning at about 8 hours after serum addition. At times thereafter, the level of CBP35 remained in a plateau (Fig. 2b). In parallel experiments, we have also prepared immuno- fluorescence slides, stained with rabbit anti-CBP35, from cultures stimulated with serum. We found that the percentage of labeled cells increased monotonically with time after serum addition (Fig. 2c).

A comparison of the bulk quantitation of the CBP35 polypeptide (Fig. 2b) with the percentage of fluorescent cells at the level of individual cells (Fig. 2c) revealed the following two contrasting points: (a) Although the percent of labeled cells (based on an arbitrary cut-off point) was very low in serum-starved cells, there was an appreciable amount of CBP35 detectable by immunoblotting; (b) The percent of labeled cells continued to increase 8 hours after serum addition, whereas the bulk levels of CBP35 appeared to remain in a plateau. The reasons for these differences between the two methods are not known, but the results do raise the

Figure 2. **Comparison of the changes in (a) level of DNA synthesis, (b) level of CBP35 quantitated by immunoblotting, and (c) percent of cells stained with rabbit anti-CBP35 in synchronized 3T3 cell populations during the cell cycle.** 3T3 cells (10^4 cells/cm^2) were synchronized by serum starvation (48 hours at 0.2% calf serum). Cells were restimulated by the addition of calf serum (10%) and, at various times thereafter, samples containing 5 x 10^5 cells were analyzed for DNA synthesis by the incorporation of [^{3}H]thymidine (2 μCi/culture, 1 hour, 37°C) and for CBP35 by SDS-PAGE and immunoblotting analysis. At the same time points, cells were fixed and processed for immunofluorescence staining by rabbit anti-CBP35 and fluorescein-labeled goat anti-rabbit immunoglobulin.

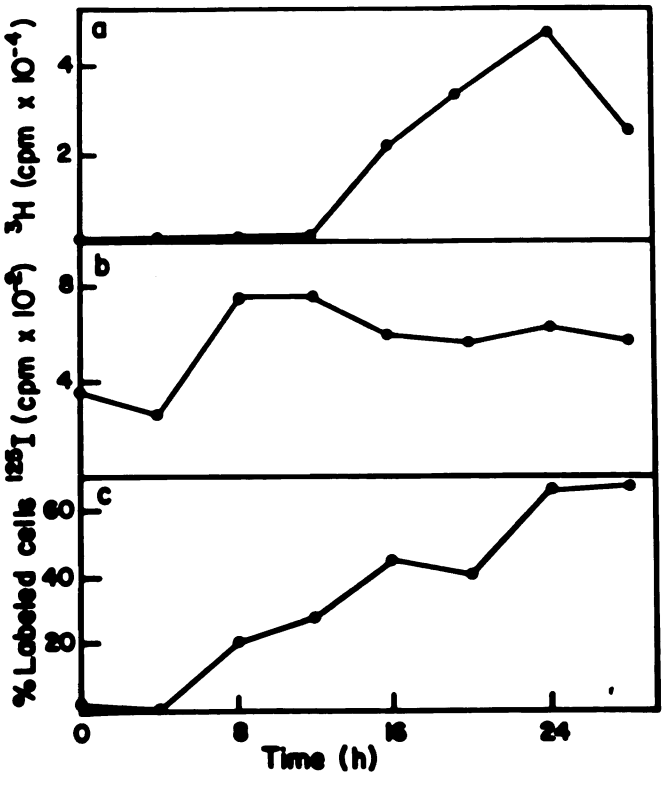
a
b
c
4
2
8
4
60
40
20
0
8
16
24
Time (h)
% Labeled cells
^{125}I (cpm x 10^{-3})
^{3}H (cpm x 10^{-4})

Figure 2

possibility that there may be CBP35 molecules sequestered from detection by immunofluorescence but are detectable after denaturation in the SDS-PAGE analysis. It is the uncovering of these cryptic CBP35 molecules that may account for the monotonic increase in percent of fluorescently-labeled cells after serum stimulation. In any case, it is important to note that both quantitation procedures show that the level of CBP35 rises well before the onset of S phase in the synchronized cell population.

CBP35 Expression in Response to Serum Stimulation of Human Fibroblasts

CBP35 is also found in human fibroblasts (Roff *et al.*, 1983; Crittenden *et al.*, 1984). Using the paradigm developed in the 3T3 cell system, we wanted to compare the expression and localization of CBP35 in human fibroblasts of different replicative capacities. CBP35 in quiescent and serum-stimulated human SL66 and WS fibroblasts was visualized by indirect immunofluorescence, and compared to mouse 3T3 cells. In quiescent 3T3 cells, the staining was light and diffusely distributed, both in the cytoplasm and the nucleus (Fig. 3, A), whereas, consistent with previous studies (Moutsatsos *et al.*, 1987), the nuclei exhibited intense staining in a significant proportion of the serum-stimulated 3T3 cells (Fig. 3, B). In contrast to the 3T3 cells, WS fibroblasts exhibited no increase in immunofluorescence upon serum stimulation (Fig. 3, compare panels A and B).

The staining for CBP35 was weak for serum-starved SL66 cells at low, intermediate and high passage numbers (passage 11, 19, and 33, respectively). There was a slight increase in intensity in late-passage cells (Fig. 4, A). Upon serum stimulation, the overall staining intensity of young (passage 11) SL66 cells greatly increased, and in some cells markedly so (Fig. 4, compare panel B with panel A). However, in contrast with the staining pattern of 3T3 cells, the increase in staining

Figure 3. **Comparison of the immunofluorescence staining patterns of CBP35 in mouse 3T3 fibroblasts (3T3) and human fibroblasts of a Werner's syndrome (WS) patient.** (A) Serum-starved, quiescent cultures; (B) cultures 16 hours following serum addition. ph, phase contrast; fl, fluorescence. The immunofluorescence was carried out as described in the legend to Figure 1, except that rhodamine-labeled goat anti-rabbit immunoglobulin was used as the second antibody. Bar = 20 μm.

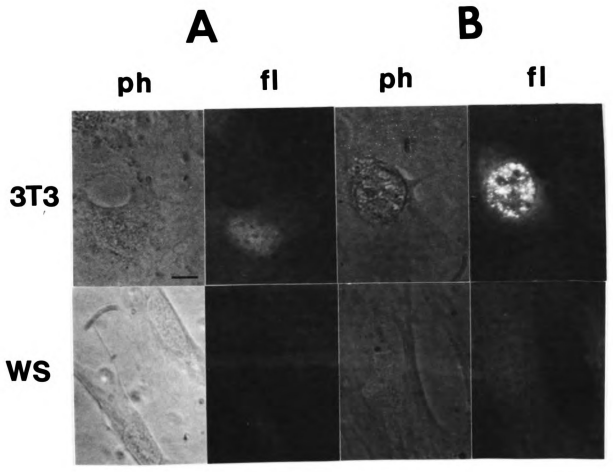

Figure 3

Figure 4. **Comparison of the immunofluorescence staining patterns of CBP35 in human SL66 fibroblasts at passage number 11, 19 and 33. (A) Serum-starved, quiescent cultures; (B) cultures 16 hours following serum addition. ph, phase contrast; fl, fluorescence. The immunofluorescence staining was carried out as described in the legend to Figure 3. Bar = 20 μm.**

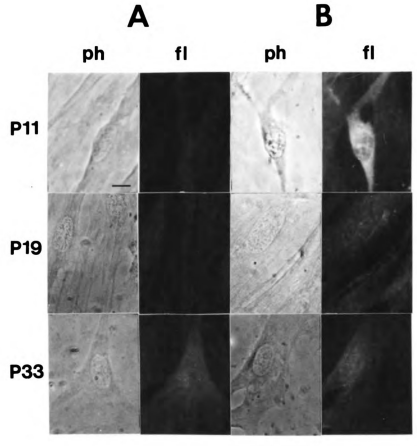

Figure 4

intensity was not restricted to the nucleus. In older SL66 cells (passages 19 and 33), serum addition led to no dramatic change in staining intensity.

The relative abundance of CBP35 in SL66 cells was also examined by immunoblotting. In quiescent cultures of SL66, the amount of CBP35 per milligram of total cellular protein was apparently greater as the passage number increased (Fig. 5, compare lanes 3, 5 and 7). This is consistent with the immunofluorescence results of serum-starved cultures, which showed slightly stronger staining with increasing passage number of the SL66 fibroblasts (Fig. 4, A). The addition of serum to the cultures markedly increased the amount of CBP35 in young (passage 11) SL66 cells (Fig. 5, compare lane 4 with lane 3). In late passage cells, however, the amount of CBP35 appeared to remain unchanged (passage 19, Fig. 5, lanes 5 and 6) or even decreased (passage 33, Fig. 5, lanes 7 and 8) after serum addition.

The results obtained with WS cells were comparable to SL66 at high passage number. There was no apparent change in the amount of CBP35 upon addition of serum. This conclusion is derived from both the immunofluorescence staining (Fig. 3) and the immunoblotting analysis (Fig. 5, lanes 9 and 10). Although WS and high passage SL66 cells have detectable amounts of CBP35 upon immunoblotting, the immunofluorescence staining was weak. There may be antigenic determinants in CBP35 that are cryptic in immunofluorescence, but become exposed upon denaturation in SDS-PAGE analysis, as cited previously for 3T3 cells. In any case, it appears that WS cells, like the *in vitro* aged SL66 cultures, are unresponsive to serum stimulation of CBP35 expression.

Figure 5. **Immunoblotting analysis for CBP35 in extracts of various cells.** Lanes 1 and 2: mouse 3T3 fibroblasts. Lanes 3 and 4: human SL66 fibroblasts (passage 11). Lanes 5 and 6: human SL66 fibroblasts (passage 19). Lanes 7 and 8: human SL66 fibroblasts (passage 33). Lanes 9 and 10: fibroblasts derived from a patient with Werner's syndrome. The odd-numbered lanes contained extracts derived from serum-starved, quiescent cultures. The even-numbered lanes contained extracts derived from cultures 16 hours after serum addition. Each lane contained 50 μg of protein. Immunoblotting was carried out with rabbit anti-CBP35 (1:250 dilution) and revealed with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin.

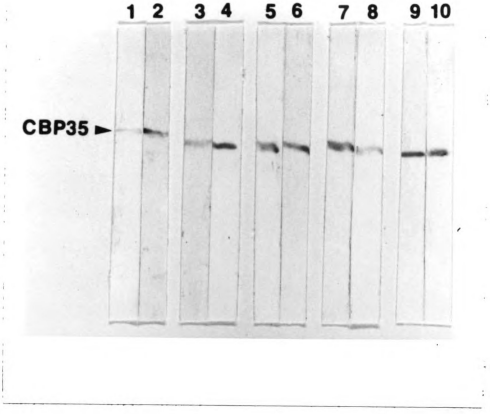

Figure 5

Discussion

We previously showed that CBP35 in quiescent mouse 3T3 fibroblasts was found primarily in the cytoplasm, whereas, after stimulation of replication by the addition of serum, CBP35 was predominantly localized in the nucleus of a high proportion of the cells (Moutsatsos *et al.*, 1987). The data presented here indicate that CBP35 in young (passage 11) human SL66 fibroblasts also occurs at a low level in quiescent cells and increases markedly 16 hours after serum stimulation (Fig. 4 and Fig. 5). However, it appears that the increase in CBP35, in response to serum addition, was not restricted to the cell nucleus, as was observed in 3T3 cells (Fig. 3). Since CBP35 has been identified as a component of hnRNP in 3T3 cells (Laing and Wang, 1988; Jia and Wang, 1988), the apparent lack of a specific nuclear location in SL66 cells is a matter of interest.

In contrast to the findings with young SL66 cells, there was no appreciable increase in the amount of CBP35, as determined both by immunofluorescence staining (Fig. 4) and immunoblotting after SDS-PAGE (Fig. 5), in old (late-passage) SL66 cells. Similar results were also obtained with WS cells (Fig. 3), which have a reduced replicative life span (Salk, 1982). This finding is of interest because of the demonstrated correlation of CBP35 level with the proliferative state in 3T3 cultures (Moutsatsos *et al.*, 1987).

The basis for this apparent unresponsiveness to serum stimulation is not known. It should be noted that, on a per milligram total cell protein basis, there is a considerable amount of CBP35 in the quiescent cultures of the unresponsive cells as detected by immunoblotting (Fig. 5, lanes 5, 7 and 9). This was true for both old SL66 fibroblasts as well as WS cells. These results raise the possibility that the level of CBP35 is not decreased in quiescent cultures of these unresponsive cells. Alternatively, however, it is also possible that the absolute level of CBP35 is still low

in the quiescent cultures, but its abundance relative to other proteins has increased (so that on a per milligram total protein basis, it is more readily detected). The absence of any intense immunofluorescence staining in these cultures is consistent with the latter possibility. In any case, the unresposiveness to serum stimulation of CBP35 expression is interesting because it is correlated with a decreased replicative capacity of the cells.

Other proteins have been identified that are associated with either the proliferative or nonproliferative state of cells. Cyclin (proliferating cell nuclear antigen, PCNA; Mathews *et al.*, 1984) is a M_r 36,000 protein expressed in the S phase of the cell cycle and found specifically in the nuclei of replicating cells (Celis and Celis, 1985); it is now known to be an auxiliary protein of DNA polymerase-δ (Bravo *et al.*, 1987; Prelich *et al.*, 1987). Statin is a M_r 57,000 protein found in the nuclear lamina of nonproliferating cells that are in the G_0 phase (Wang, 1985; Wang and Lin, 1986). Recently, proteins associated specifically with replicative incompetence (senescent cells) (Sottile *et al.*, 1987; Ching and Wang, 1988) and three other proteins associated specifically with replicative competence (young cells) (Ching and Wang, 1988) have been found in the conditioned medium of human fibroblast cultures. It would be of interest to further characterize the functional role of CBP35 in 3T3 and human cells, and to ascertain if that role includes involvement in the determination of replicative competence.

Acknowledgments

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CLOSING STATEMENT

When my thesis project was initiated, there were two observations made regarding the isolelectric points of CBP35. First, the pI of the CBP35 polypeptide was predicted to be 8.7 on the basis of the sequence of the cDNA clone (1). Second, purified CBP35 yielded two pIs of 4.5 and 4.7 upon IEF two-dimensional gel electrophoresis (2).

These observations led to the following questions: why hadn't the pI 8.7 species been observed on the two-dimensional gels? and what modifications could account for the difference between the calculated and the observed pIs?

The first question was answered by comparing two different two-dimensional analyses, IEF and NEPHGE. IEF gels are able to resolve proteins with acidic pIs, but proteins with basic pIs fail to enter the basic end of the IEF gradient and may be missed. rCBP35, a recombinant CBP35 polypeptide expressed in E. *coli*, yielded a single spot of pI 8.7 on NEPHGE gels but was not detected on IEF gels. The observed pI value for rCBP35 agrees with the pI value calculated from the deduced amino acid sequence. Extracts of 3T3 fibroblasts upon NEPHGE analysis, also yielded the pI 8.7 species as well as a pI 8.2 varaint. However, CBP35 was not found on the IEF gels, in contradiction to the previous observation that the pIs of CBP35 were 4.5 and 4.7. These two species of pIs 8.2 and 8.7 were not seen previously because NEPHGE gels, which resolve basic proteins, had not been used (2). The pI 8.2 variant represents a post-translationally modified form of the pI 8.7 CBP35 polypeptide by the addition of a single phosphate group.

The second question, on the nature of the modification of the CBP35 polypepetide to yield pI values of 4.5 and 4.7, was also answered; the results indicate that the previously observed pI values were due to an artifact of carbamylation.

I then wanted to determine if the expression of certain isoelectric variants was stimulated by serum growth factors and/ or was associated with the specific subcellular localization of CBP35. Three types of quiescent and proliferation states were studied, 1) confluent versus sparse densities, 2) serum-starved and serum-stimulated cultures, and 3) senescent versus young human fibroblasts. Several observations were made. First, the CBP35 pI 8.7 isoelectric species was found exclusively in the nucleus. Second, the pI 8.2 phosphorylated variant was localized in the nucleus and cytoplasm. Third, proliferating cells exhibit more overall CBP35 than quiescent cells and have both pI species, while quiescent cells have mainly the pI 8.2 variant. Finally, old, senescent cells cannot be serum-stimulated and do not have the pI 8.7 species.

These results imply that phosphorylation of CBP35 may play a role in its cellular localization. CBP35 may be anchored in the cytoplasm when phosphorylated, and released upon dephosphorylation. Alternatively, phosphorylation might mask a nuclear targetting sequence, thus preventing CBP35's movement into the nucleus.

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