PROLIFERATION DEPENDENT EXPRESSION AND NUCLEAR LOCALIZATION OF CARBOHYDRATE-BINDING PROTEIN 35 IN CULTURED FIBROBLASTS

By

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ABSTRACT

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A highly specific polyclonal antibody against carbohydrate-binding protein 35 (CBP35), was used to analyze the subcellular distribution of the galactose specific lectin in mouse 3T3 fibroblasts. Cell surface specific labeling with anti-CBP35 and ^{125}I revealed the presence of small amounts of CBP35 externally exposed at the cell surface. However, the majority of CBP35 was localized intracellularly as revealed by immunofluorescent studies of fixed and permeabilized 3T3 cells. The staining pattern showed the presence of CBP35 on the nucleus and in the cytoplasm. Subcellular fractionation studies also indicated that CBP35 can be detected by immunoblotting procedures in the nuclear pellet, the cytoplasm, and the plasma membrane.

The levels and the localization of CBP35 in the nucleus and the cytoplasm vary depending on the proliferative state of the cells. Sparse proliferating cultures of 3T3 cells contain high levels of CBP35, prominently localized in the nucleus, relative to quiescent cultures of the same cells as

revealed by cytometric analysis of a large number of immunofluorescently labeled cells. In quiescent cells, the majority of the cells have lost their high level of nuclear staining and have undergone a general decrease in the overall fluorescence intensity. Stimulation of serum-starved quiescent cells by the addition of serum resulted in an increase in the level of CBP35. More importantly, there was an apparent translocation of CBP35 into the nucleus. Nucleolar staining reached a maximum before the onset of DNA synthesis and then decreased. This translocation into the nucleoli was blocked by hydroxyurea, a G_1/S transition blocker, and was resumed upon removal of the drug.

CBP35 exhibits similar localization, expression and molecular properties to a protein called cyclin. Certain patients with the autoimmune disease systemic lupus erythematosus produce autoantibodies to both of these antigens, and transformed cells contain higher levels of both of these proteins than their normal counterparts. However, the two proteins appear to be antigenically distinct as antibodies reactive against CBP35 and cyclin do not cross-react.

DEDICATED TO MY PARENTS

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ABBREVIATIONS

CBP	carbohydrate binding protein
LDH	lactate dehydrogenase
M6P	mannose 6-phosphate
CLL I	chicken lactose lectin I
CLL II	chicken lactose lectin II
DMEM	Dulbecco-Modified Eagle's Medium
ASF	asialofetuin
ASGP	asialoglycoprotein
PMSF	phenyl methyl sulfonylfluoride
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
Hepes	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
EDTA	(ethylenedinitrilo)-tetraacetic acid
Tris	Tris (hydroxymethyl) aminoethane
BSA	bovine serum albumin
SLE	systemic lupus erythematosus
HRP	horseradish peroxidase
PCNA	proliferating cell nuclear antigen

INTRODUCTION

Our laboratory has previously isolated three carbohydrate binding proteins (CBPs) from mouse 3T3 fibroblasts. These lectins specifically bind to galactose containing glycoconjugates, and they have been named in terms of their molecular weights as CBP35 (M_r 35,000), CBP16 (M_r 16,000) and CBP13.5 (M_r 13,500). Counterparts of CBP16 and CBP13.5 have been identified previously in a variety of other species, but CBP35 represented a newly identified lectin.

In an effort to better understand the endogenous function of CBP35, we have investigated the localization of this protein in the 3T3 cells. This thesis will describe the localization data that we have obtained using immunocytochemical and biochemical techniques. Localization data of other lectins have been used as a first approach to the study of the endogenous function. Therefore, I have chosen to review the available data on the localization of lectins and examine the way that postulated functions have been derived from them.

As it will become apparent, although detailed localization data are available for several well known lectins, the endogenous function of many of these proteins

remains uncertain. Naturally, localization data by themselves are not expected to give the answer to a complex question like the endogenous function of a polypeptide. However, they can eliminate unfavorable possibilities and in conjunction with other data (endogenous ligand, structure, biochemical characteristics, developmental regulation) they can present a more complete picture from which a function may be suggested. The localization studies of CBP35 have given us new insight into the regulation of this protein and have suggested new ideas for the elucidation of its endogenous function.

CHAPTER I

LITERATURE REVIEW

Introduction to Carbohydrate Binding Proteins

Proteins that bind carbohydrate moeities of glycoproteins, glycolipids, or regions of polysaccharides have been isolated from a variety of organisms. These carbohydrate binding proteins (CBPs) are commonly referred to as lectins if they agglutinate cells that display more than one saccharide of sufficient complementarity and do not have any known enzymatic activity (1a). Obviously, such criteria make lectins a subclass of CBPs but in the present literature review the two terms will be used interchangeably.

Although lectins were originally identified in plant extracts as agglutinins of erythrocytes (8), the past decade has unraveled a much wider distribution of these proteins in species as diverse as the slime molds and humans. Since this thesis will describe the localization and expression of a mammalian lectin, I have chosen to review data relevant to invertebrate and vertebrate lectins but not those of plant origin. Table I summarizes certain properties of the lectins that will be discussed below.

Table I

Comparison of Localization and Properties of

Non-Plant Origin Lectins

Localization	Lectin	Subunit M_r	Carbohydrate specificity	Source	References
Extracellular	Discoidin I	25 K	D-Gal	slime mold	12, 17, 18
	CLL-I	16 K	D-Gal	chicken	9, 19
	CLL-II	12 K	D-Gal	chicken	9, 20
	rat lung lectin	14.5 K	β-D-Gal	rat	14, 23, 47
	Xenopus lectin	13 K	β-D-Gal	Xenopus	25
Cell Surface	ASGP receptor	52 K	β-D-Gal	rabbit, rat	2, 27, 31
	M6P receptor	215 K	Man6P	human, bovine	34, 36, 38
	EDA	13 K	β-D-Gal	human	41
Intracellular	tumor cell lectin	34 K	β-D-Gal	B16 melanoma	44
Nuclear	bovine heart lectin	13 K	β-D-Gal	bovine, human	48,49

a) Classification

Lectins can be subdivided on the basis of whether or not they integrate into membranes. The integral membrane lectins require detergent for their solubilization but the soluble ones do not. Therefore two general categories of CBPs have been defined : a) soluble CBPs and b) membrane bound CBPs (1). It has been proposed that such a subdivision probably suggests a fundamental difference in the general functions of these classes. Integral membrane CBPs appear to have evolved to bind glycoconjugates to membranes and transport them to other cellular compartments. Well studied examples of these proteins include the asialoglycoprotein (ASGP) receptor found in mammalian liver membranes (2) and the mannose 6-phosphate (M6P) receptor (7). The function of the soluble CBPs has been more difficult to infer partly because these CBPs tend not to be as sharply localized as those in the membranes. The finding that many of these CBPs are originally localized inside cells but are ultimately externalized has led some investigators to believe that they function extracellularly to bind the complementary glycoconjugates on and around the cells that release them. Examples in this category include chicken lactose lectin I and II (CLL-I, CLL-II) (3 , 4) and the slime mold CBPs, discoidin I and II (5 , 6).

b) Endogenous Ligands

One approach for the determination of the function of a CBP is the isolation of the glycoconjugate that it binds. A biologically meaningful interaction will result only if the participants of appropriate complementarity are at the right place at the right time. A few such candidates have been identified for both the membrane bound CBPs and the soluble ones. Probably the most well understood example is that of the M6P receptor. Neufeld and coworkers (7) showed that a CBP was involved in the transport of lysosomal enzymes from the extracellular medium into the cell. This protein binds specifically to mannose 6-phosphate residues which are post-translationally incorporated into the lysosomal enzymes (33). In addition, Sly and his coworkers have since shown that the delivery of lysosomal enzymes from the rough endoplasmic reticulum, where these enzymes are synthesized, to their main residence site, the lysosome, is also mediated by intracellular M6P receptors (36, 37).

For soluble lectins, the candidacy of components as natural ligands has been supported by their abundance in tissues rich in the CBP as well as their colocalization to the same tissue or subcellular site. For example, intestinal mucin, a highly glycosylated protein that contains many terminal β-galactoside residues, appears to be an endogenous ligand for CLL-II (4). The mucin and the CBP bind well, and both are found in the secretory granules of the goblet cells and on the intestinal mucosal surface. Similar findings

suggest that a polysaccharide synthesized by D. Discoideum at a late developmental stage is an endogenous ligand for discoidin II (6). This material contains mostly galactose and N-acetylgalactosamine, binds well to the CBP, and immunohistochemical evidence suggests that both discoidin II and the polysaccharide are localized in vesicles in prespore cells and are secreted around these cells later in development.

c) Developmental regulation of expression

A recurring theme in the study of CBPs has been their temporal expression and association with certain tissues or organs. CBP amounts and localization seem to vary, depending on the developmental stage of the organism under study. For example, CLL-I levels in embryonic muscle become maximal as it differentiates and decline thereafter (9). Similarly, CLL-II levels in liver and kidney are much higher in the embryo than in the adult (9). CBP35, a lectin isolated from mouse 3T3 fibroblasts, is present in the embryonic mouse muscle, liver and skin, but absent in the corresponding tissues of the adult (10). Not in all cases, however, is the expression of a lectin maximal in embryonic tissues. CLL-I is scarce in embryonic liver but plentiful in the adult and CLL-II becomes plentiful in the intestine only late in development and is maintained at high levels thereafter (9). Since the same lectin can be differentially expressed either in embryonic or adult tissues, it appears that it does not

only participate in the process of development but also in adult processes as well.

The slime mold Dictyostelium discoideum can be induced to differentiate from a unicellular to a multicellular form by starvation. The individual amoeboid cells, which do not contain any detectable CBP activity, are then induced to produce large amounts of CBPs as they form aggregates and develop into a fruiting body (11). Among these CBPs the discoidins are the best studied . Because the synthesis of discoidin I coincides with the event of cell-cell adhesion, its possible role in this process has been examined in detail by Barondes and coworkers (11 , 12). However, recent evidence suggests that the expression of this CBP may not be necessary for aggregation and subsequent fruiting body construction since mutants that express neither discoidin I nor discoidin II can still complete morphogenesis and cytodifferentiation (16).

Recently, Jessel and coworkers have identified a series of developmentally regulated cell-surface glycoconjugates that are restricted to subsets of dorsal root ganglia (DRG) neurons. Lactoseries glycoconjugates are expressed in the cytoplasm and on the surface of small-diameter DRG neurons (13 , 14). The fact that the same DRG neurons also express two endogenous lactose binding lectins (M_rs 14,500 and 29,000) (15) suggested to the investigators that these complementary molecules contribute to the development and function of primary sensory neurons.

Localization of CBPs

A standard approach in our search for the endogenous function of CBPs has been the detailed localization of these proteins within the system that contains them. The hope of most investigators is that such a study will provide topological information that may be unique in its interpretation with respect to the function. From the description that will follow, however, it will become clear that although detailed localization studies have been performed quite successfully, the function of CBPs still remains unclear mainly due to the following reasons:

a) CBPs have been found to be localized in a variety of patterns that excludes an underlying unifying theme, and
b) CBP localization may be transient and controlled by both the state of development of the organism as well as its proliferative state.

Two main approaches have been used in the localization of CBPs. Immunohistochemical techniques and functional (usually agglutination) assays. Each approach has its limitations and it is possible that a form of the CBP detected by the one method is not detected by the other and vice versa. Immunohistochemical techniques are based on the use of polyclonal or monoclonal antibodies and the specificity of such antibodies, preservation of antigen after fixation and processing for immunocytochemistry, and masking of antigenic determinants by endogenous cellular components are all factors that have to be addressed for the

meaningful interpretation of immunocytochemical localization data. Functional assays, such as agglutination, lack the specificity of immunological methods but are important if the carbohydrate binding function of the protein is to be detected. In most cases a combination of these approaches has been used to define the localization of a CBP, thus eliminating most of the uncertainties of a single method.

I have chosen to present examples of the various CBP localizations in a pattern moving from the outside of the cell towards its center, the nucleus. However, it should be noted that a unique localization is the exception rather than the rule in the study of CBPs and therefore the following classification should be viewed with a liberal eye.

a) Extracellular CBPs

Several immunohistochemical studies show that cells frequently release their soluble CBPs which remain near the cells that make them. In this sense then, these soluble CBPs may be viewed as a class of extracellular proteins. The fact that the extracellular matrix of cells is rich in glycoconjugates with affinity for the CBPs makes it likely that such binding takes place in vivo and is biologically important.

Barondes and coworkers (17), using immunoelectron microscopy, demonstrated that discoidin I becomes localized in vesicles and multilamellar bodies as it is synthesized.

Very little labeling was found in the nucleus ,the cytoplasm, or the plasma membrane under the fixation conditions used. These multilamellar bodies appear to be the vehicle for release of the lectin around the agreggating cells where it is now postulated to play a role in cell-substratum adhesion (18). Despite the fact that secreted multilamellar bodies appear to originate from vesicles with digestive function, they contain intact discoidin I with active carbohydrate binding sites.

Many vertebrate CBPs are also externalized into the extracellular space of the cells that synthesize them. CLL-I was localized by immunohistochemical techniques in tissue samples taken at various stages of in vivo development and in primary muscle cultures (19). The lectin which was diffusely distributed in the cytoplasm of myoblasts, became localized in myotubes in a distribution similar to that of the sarcoplasmic reticulum and T tubules. Later in development, the CBP was predominantly extracellular. The investigators suggested that externalization may have occurred by migration in the T tubules which are continous with the extracellular space. Both CLL-I and CLL-II were also localized within the vesicles of the mucin-secreting intestinal goblet cells by indirect immunofluorescence and immunoperoxidase staining methods (20). The localization of CLL-II in secretory vesicles, as well as on the intestinal epithelial surface, suggested that at least a portion of the CLL-II in the vesicles was secreted and rebound on the

mucosal surface. Secretion of the CBPs may occur in conjunction with mucin because both are localized in the secretory vesicles and CLL-I and CLL-II bind strongly to purified chicken intestinal mucin.

A β-galactoside binding lectin which has been identified in several mammalian tissues including bovine (21, 22) and rat lung (23, 24) (dimer of M_r 29,000) has been immunohistochemically localized in the cytoplasm of both alveolar and smooth muscle cells of rat lung and in high concentrations in the extracellular space of elastic fibers. All lung elastic fibers including those of the lung parenchyma and blood vessels contain this CBP. Because the CBP was accumulated at an extracellular site it was assumed that it is secreted there from the cells that make it. However, no structures resembling secretory vesicles were found to be associated with the CBP (47).

Using immunoperoxidase staining and immunofluorescence, an α and β-galactoside binding CBP found in Xenopous laevis oocytes has been localized during the process of fertilization and embryo formation (25). The localization of the CBP depends on the stage of development and includes yolk platelets, cortical granules and vitelline envelope of oocytes and unfertilized eggs, and the fertilization envelope of fertilized eggs. In the embryo, however, the CBP is present in extracellular materials in the cleavage furrow region. The authors suggested that the CBP associated with the yolk platelets is a storage form, like the remainder of

the yolk materials, and that the lectin found in the cleavage furrows of the embryos is derived from yolk platelets through an externalization process that is not yet understood.

b) Cell Surface CBPs

The two major paradigms of cell surface CBPs are those of the ASGP receptor in the liver and the M6P receptor in fibroblasts and other cells. Both CBPs have been initially identified by their ability to bind and endocytose ligands carrying the appropriate carbohydrate (26, 7) and further localization studies have taken advantage of both the carbohydrate binding ability of these CBPs (27) as well as immunocytochemical methods. It should be noted that for both the ASGP and M6P receptors, only a small percentage of the total CBP is actually expressed at the cell surface. Therefore, the abundance of a CBP at a particular site does not necessarily reflect its significance in terms of a physiological function. Finally, both the ASGP and M6P receptors are not degraded in the lysosomes but each receptor seems to be separated from its ligand in some prelysosomal compartment and then recycles to the cell surface (40).

Initial subcellular fractionation studies using functional assays for the ASGP receptor from rat liver demonstrated that the protein was found on plasma membranes and in other membranous intracellular organelles like Golgi

and lysosomes (27, 28). Only a few data are available as to the quantitative distribution of ASGP receptors in rat liver cells. Measurements of ligand binding capacity of intact and solubilized cells led Steer and Ashwell (29) to suggest that about 90% of the ASGP-binding sites occur in intracellular compartments. More recently however, others estimate that about half of the receptors are at the cell surface in isolated hepatocytes (30). Geuze and coworkers have been able to visualize using double label immunoelectron microscopic technique and antibodies to the ligand, the ASGP receptor and chlathrin, the compartments in which dissociation of ligand-receptor complexes occur (31). By quantitating binding sites for their anti-ASGP receptor antibody they concluded that about 35% of the receptor is at the plasma membrane while most of the intracellular receptor was found in the Golgi complex and the smooth ER/CURL (compartment for receptor ligand uncoupling).

The M6P receptor is a membrane - associated protein required for proper targeting of lysosomal enzymes in mammalian cells and tissues (32). The receptor binds the M6P residues that occur in oligosaccharides of lysosomal enzymes (33) and was originally studied in the context of endocytosis of extracellular enzymes (34, 35). The receptor is now perceived as playing a key role in the intracellular transport of enzymes to lysosomes (36, 37). By subcellular fractionation of rat liver (38) and by ultrastuctural immunocytochemistry of Chinese hamster ovary cells (39) the

CBP has been localized primarily in the endoplasmic reticulum and Golgi regions, with a minor fraction (about 10%) on the cell surface. The properties of the receptor at the cell surface have been studied by cell- surface specific lactoperoxidase catalyzed radioiodination and immunoprecipitation. The subunit molecular weight of the cell surface receptor, its half life and its binding specificity are identical with those of the biosynthetically labeled receptor (40).

Several other CBPs, whose functions are still largely unknown, have been localized at the cell surface. During erythroid differentiation in the adult bone marrow, erythroblasts cluster together in the vicinity of a "nurse" macrophage cell until the enucleation stage when the immmature reticulocyte is released and passes through the sinusoidal wall into the circulation. A β-galactoside specific CBP, (M_r 13,000), can be extracted from erythroblast enriched marrow. This CBP has been termed erythroid developmental agglutinin (EDA) and has been shown by immunofluorescence to exist at the surface of erythroblasts and reticulocytes. It has been suggested that EDA may be responsible for the inter-erythroblast recognition and adhesion in vivo (41).

The presence of galactoside-specific CBPs in a variety of tumor cells has suggested that such molecules can influence the metastatic potential of these cells by forming tumor cell aggregates in the circulation. (42, 43). Raz and

coworkers have generated monoclonal antibodies to endogenous galactose specific CBPs from the B16 melanoma cell line. A monoclonal antibody, designated 5D7, inhibited the homotypic aggregation of these cells and was used to localize its antigen by immunofluorescence. When non-permeabilized cells were examined 5D7 showed a microclustered distribution of fluorescence on the cell surface. Pretreatment of the cells with the hapten lactose did not abolish the staining implying that the CBP is an integral membrane protein. Permeabilization of the cells prior to staining, revealed a much larger population of intracellular CBP (44).

Finally, several of the CBPs that have been proposed to function primarily extracellularly have also been identified at the cell surface. CLL-I is detectable on the surface of cultured myoblasts (45, 46) and the β-galactoside binding CBPs from rat lung (47) have been shown recently to exist at the cell surface of small DRG neurons (15).

c) Intracellular CBPs

I have previously described data concerning the localization of CBPs found in the extracellular space of cells as well as on their cell surface. Obviously since the synthesis and processing of these CBPs has to take place in the cytoplasm, even to that limited extent we have to accept that all these CBPs occur intracellularly at one time point of their existence. As I have already described, the majority of the ASGP receptor and the M6P receptor occur on intracellular membranes including those of the endoplasmic

reticulum , the Golgi complex, and the lysosomes. Since these organelles are the major biosynthetic, processing and transport centers of the cell, such a localization per se does not provide any clear-cut clues as to their function. However, knowledge of the endogenous ligands (asialoglycoproteins and lysosomal enzymes) has provided important insights in these two cases concerning their function, and the available localization data seem to fit nicely with the proposed function of these CBPs. For example, the M6P receptor is a glycoprotein that has to be processed through the Golgi complex for the attachment and processing of the carbohydrate chains (40). In addition, it has been proposed that this CBP is responsible for the intracellular transport of lysosomal hydrolases from their site of synthesis and glycosylation to the lysosomes (36). This intracellular function depends on the dissociation of enzymes from receptors in lysosomes and on recycling of free receptors to the endoplasmic reticulum or Golgi complex.

Discoidin I, which I have described as an extracellular CBP, is localized intracellularly prior to its externalization and it has been shown by immunoelectron microscopy to reside in intracellular multilammelar vesicles but not in the cytoplasm (18). Many of the other extracellular CBPs, including CLL-I and CLL-II, have also been localized intracellularly prior to their secretion (19,20).

Feizi and Raz have idependently examined tumor

associated CBPs using monoclonal antibodies generated either against CBPs initially identified in normal cells (48) or tumor cells (44). Using immunofluorescence they both observed strong cytoplasmic staining of fixed and permeabilized cells, indicating a cytoplasmic distribution of the CBP. In some cases (48) variable nuclear staining was also observed. However, these studies lacked the detailed electron microscopic localization which has been performed with some of the exracellular CBPs and therefore the association of these CBPs with specific intracellular structures remains to be determined.

Therefore, both membrane bound CBPs as well as soluble ones have been localized in intracellular locations. With the exception of the M6P receptor, however, no intracellular ligands have been identified. Although there does not appear to be an obvious intracellular function of these CBPs, accumulating evidence for intracellular carbohydrate bearing structures (which will be discussed later) may eventually lead to the identification of these ligands and the elucidation of the intracellular importance of CBPs.

d) Nuclear CBPs

The evidence of CBPs in the nucleus has been very limited and rather circumstantial. Using rabbit antisera against bovine heart -galactoside binding lectin (M_r ~13,000), Childs and Feizi have observed immunofluorescent staining of nuclei in epithelial cell cryosections (49).

Furthermore β-galactoside binding activity was demonstrated in sections of nuclei by the addition of fluorescein-conjugated bovine serum albumin derivatized with lactose (BSA-lactose). This staining was inhibited by non-fluorescent BSA-lactose but not with BSA derivatized with mannose.

A monoclonal antibody to the bovine heart CBP has been produced and it has been used to immunohistochemically localize this CBP in human lymphocytes (48) and in a variety of bovine epithelial tissues and cultured fibroblasts (50). This monoclonal antibody (designated 36/8) stains predominantly the cytoplasm and the nucleus of a variety of tissues and cells including bovine and human fibroblasts. The nuclear staining is variable and electron microscopic evidence suggests that this CBP is not associated with membranes, such as those of the ER, the Golgi complex and mitochondria (50). The results of these studies, however, are difficult to interpret since immunoblotting analysis of lymphocyte extracts with 36/8 indicated immunoreactivity with approximately ten antigenically related proteins ranging in M_r from 130,000 to 13,000. Which of these proteins is at the nucleus and whether this protein is in fact a CBP remains to be determined.

Observations consistent with the nuclear localization of β-galactoside binding CBPs in epithelial tissues have also been reported by Beyer and Barondes (51).

Intracellular CBP ligands: Do they exist?

As more and more evidence is accumulated for the intracellular localization of CBPs, the question of the existence of intracellular carbohydrate targets to which these proteins could bind becomes even more relevant to examine. Although traditionally carbohydrates in glycoproteins, glycolipids and polysacharides have been studied in the context of the extracellular space and the cell surface, recent evidence points to the existence of carbohydrate bearing proteins in the intracellular compartment (52-55). The advent of improved fractionation techniques for subcellular organelles (56), the use of monoclonal antibodies of high specificity (55, 57), and the use of glycosyltransferases and radiolabeled sugar nucleotide donors to label oligosaccharides of glycoproteins (54, 58) have given added confidence to these findings.

Gerace and coworkers have identified a M_r 190,000 protein (gp190) from rat liver nuclei (52) that is specifically localized in the nuclear envelope. This protein stains heavily on SDS gels with the periodic acid-Schiff, a carbohydrate specific stain, and it is labeled with [^{125}I] concanavalin A (Con A, a plant lectin specific for glucosyl and mannosyl residues of glycoconjugates). However, this glycoprotein apparently does not contain sialyl or terminal N-acetylglucosamine residues, since it is not labeled on nitrocellulose blots with [^{125}I] wheat germ agglutinin

(WGA). This polypeptide becomes dispersed throughout the cytoplasm of the cell in mitotic prophase when the nuclear envelope is disassembled, and subsequently returns to the nuclear surfaces during telophase when the nuclear envelope is reconstructed. Immunoelectron microscopy revealed that gp190 occurs exclusively in the nuclear pore complex, in the regions of the cytoplasmic (and possibly nucleoplasmic) pore complex annuli. Monoclonal antibodies made against the gp190 analog in Drosophila also bound specifically to the nuclear envelope in situ (55).

Another rat liver nuclear protein (p62) has been recently immunolocalized by electron microscopy at the nuclear pore complex (57). This protein was specifically labeled on nitrocellulose blots by biotinylated WGA, but not by Con A, suggesting that it contains N-acetylglucosamine (GlcNac) but not glucose or mannose. Furthermore, Holt and Hart (54) and Schindler and Hogan (59) have independently identified terminal GlcNac moieties on intracellular proteins using a galactosyltransferase to transfer radiolabeled galactose to available terminal GlcNac. Several nuclear polypeptides are labeled by this method (54), the most prominent one of which is a 74 Kd protein. These GlcNac residues represent a single GlcNac residue attached to the protein via an O-linkage (60). Nuclei and the soluble fraction of rat liver cells are particularly enriched with proteins bearing this novel linkage although virtually all organelles tested demonstrated the presence of proper

acceptor proteins (54).

Eucaryotic ribosomes have been reported to contain Con A binding sites (53). The ribosomes used for this study were free from plasma membrane contamination. Con A binding to the ribosomes was saccharide specific, saturable, and involved the 60S ribosomal subunit. When ^{3}H-labeled chicken liver ribosomes were fractionated on a Con A Sepharose column a 31 Kd protein was eluted and it was assumed to be the glycoprotein to which Con A was bound to in the 60S ribosomal subunit.

Ricin, a toxin with β-galactoside binding ability, has also been shown to bind to the rat liver 60S ribosomal subunit in a lactose inhibitable manner (61). These data indicate that ribosomes also contain a glycosylated component.

A major endoplasmic reticulum protein (ERp99) has been shown by sensitivity to Endo H (a glycosidase specific for high mannose oligosaccharides) to be a glycoprotein primarily localized in the membranes of the rough ER (62) and two glycoproteins identified by Pastan and coworkers (63) have been localized by immunofluorescence and electron microscopy on the lysosomal membranes. Whether the carbohydrate of these proteins is exposed to the cytoplasm or it is sequestered to the luminal side of these membranous organelles is not, however, known.

From this brief discussion it should be apparent that glycosylation of intracellular proteins, although not as

extensively studied as that of cell surface and of secreted proteins, is a rather common event. Whether the placement of the carbohydrate moiety of these glycoproteins with respect to the endogenous intracellular CBPs and whether the sugar specificity of the CBPs make their interaction possible remains to be seen. It seems likely though that since the important components of carbohydrate binding systems have now been discovered intracellularly (i.e. CBPs and glycoproteins) it may not be long before their in vivo interaction will be better understood.

An additional point should be made with respect to the intracellular functions of CBPs. They do not necessarily have to be carrying out their intracellular function exclusively through their carbohydrate binding activity. The fact that we recognize their carbohydrate binding property does not mean that their in vivo ligands interact only via the carbohydrate moeity. Some data to this point have been obtained with respect to an extracellular CBP, discoidin I. The cell-substratum association of slime molds mediated by discoidin I is similar to the fibronectin-mediated cell-substratum adhesion of vertebrate cells. This similarity is suggested by the finding that discoidin I contains the amino acid sequence arg-gly-asp (64) shared by fibonectin and implicated in its binding to the cell surface of fibroblasts. Synthetic peptides containing the above sequence and adjacent amino acid residues of discoidin I block attachment of aggregating (but not vegetative) D.

discoideum to a plastic substratum and block the streaming of cells into aggregates (65). Therefore, the cell-substratum adhesion and ordered cell migration depend on the cell binding site of discoidin I which is distinct from its carbohydrate binding site. Recently the receptor for that site has also been isolated (66) and it has been shown that univalent antibodies against the receptor block cell aggregation. Therefore, it seems possible that CBPs may carry more than one functional and/or regulatory domains independent of the carbohydrate binding site.

Cell Cycle and Proliferation Regulated Nuclear Proteins

In this last part of my literature review, I would like to examine in more detail a number of nuclear (and possibly also cytoplasmic) proteins which have attracted the attention of cell biologists due to their apparent regulation by the proliferative state of the cell and/or the striking differences in their localization during the cell cycle. The reason for this apparent diversion from the theme of CBPs will become clear from the data that I will present in Chapters III and IV of this thesis.

a) Cyclin or Proliferating Cell Nuclear antigen (PCNA)

During a search for differential polypeptide synthesis throughout the cell cycle of HeLa cells, Bravo and Celis identified a M_r 36000 acidic polypeptide whose synthesis increased during the S phase of the cell (67). They subsequently showed by two-dimensional gel electrophoresis of karyoplasts and cytoplasts of labeled cells that this polypeptide is localized predominantly in the nucleus, and that its relative proportion decreases in parallel with a decline in cell proliferation (68). Parallel to these studies, Tan and coworkers discovered that the serum of a small percentage of patients with the autoimmune disease systemic lupus erythematosus (SLE) reacts with a nuclear antigen of proliferating and transformed cells which they named PCNA (69, 70). Because of the apparent association of PCNA with proliferation they sought to examine in more

detail its relationship with respect to DNA synthesis in synchronized lymphocyte polulations. They made the striking observation that PCNA appears in the nucleoli of stimulated cells preceding the onset of DNA synthesis showing a maximum accumulation early in S phase. After that, the number of cells showing nucleolar staining decreased (70). These observations raised the possibility that PCNA may play some regulatory role in DNA replication.

Closer examination of cyclin and PCNA by immunoprecipitation, peptide mapping, and two-dimensional electrophoresis revealed that the two proteins are in fact identical (71). Therefore, I will use these names interchangeably or as cyclin/PCNA.

Cyclin has become the subject of some elegant immunofluorescent studies from which it has been determined that the cyclin distribution in the nucleus follows a well defined pattern during the S phase (72, 73, 74),and it has been proposed that these patterns can be used as markers for the finer subdivision of S phase (72). Early in S phase, cyclin is localized throughout the nucleoplasm with the exception of the nucleoli. A similar, but stronger, staining pattern is observed as the cells progress through the S phase. At a later stage, before maximum DNA synthesis , cyclin redistributes to reveal a punctated pattern with foci of staining throughout the nucleus. A major redistribution follows during which the protein is detected in the nucleoli. At this point DNA synthesis is at or near

its maximum. The pattern then becomes punctated and decreases in intensity. In addition, it is now known that although changes in the nuclear distribution of cyclin depend on DNA replication its synthesis does not (73). Bravo and Macdonald Bravo have used hydroxyurea (an inhibitor of the cell cycle transition from G_1 to S) to arrest cells at the G_1/S boundary. Although the increased synthesis of cyclin, which is normally seen during the S phase, was not affected by such a treatment, the sequential redistribution of cyclin to the nucleolus was inhibited until hydroxyurea was removed from the media.

These properties of cyclin/PCNA make it an attractive candidate for studying the regulatory components involved in the initiation of DNA replication and growth regulation.

b) Statin

A M_r 57,000 protein uniquely present in non-proliferating cells and senescent human fibroblasts has been recently identified using a monoclonal antibody (75). This antibody stains the nuclei of the nonproliferating cells present predominantly in the senescent cultures of human fibroblasts, whereas no reaction was observed in the cultures of the same cells during early passage. Immunoelectron microscopy localized the protein at the nucleoplasm as well as the nuclear envelope. Furthermore, it was determined that statin could be induced in young

proliferating fibroblasts if the cells were arrested at G_0/G_1 by growing them to confluency. The expression of statin could be rapidly turned off once the restriction of cell replication at G_0/G_1 was removed by passing the confluent cultures to lower density or by wounding the monolayer, thus allowing the cells to replicate in order to heal the wound (76).

It is interesting to note that, during the cell cycle, the expression of statin is inversely related to that of cyclin. Most likely, statin represents a counterpart of cyclin in the nonproliferating cells.

c) Perichromin

Another nuclear antigen, defined originally by its reaction with an SLE patient serum, is a M_r 33,000 protein called perichromin (77). By immunofluorescence microscopy, it has been shown that perichromin is associated with the nuclear envelope at interphase, and is localized on the periphery of the chromosomes at metaphase. There are also intermediate stages in the redistribution of this antigen as it moves from association with the nuclear envelope to localization on the independent chromosomes. Perichromin is a highly conserved protein across different species, and its spatial localization during the cell cycle suggests that it may have a role in the organization of the chromosome in both metaphase and interphase.

d) Oncogene Products

There are several other gene products whose expression has been known to be cell cycle dependent. Such genes include the oncogenes c-myc (78), c-fos (79, 80), and c-ras (81, 82, 83); the gene encoding the cellular tumor antigen p53 (84); genes isolated as cDNA clones on the basis of their cell cycle dependent expression (85, 86); and genes coding for well-characterized cellular proteins, such as ornithine decarboxylase (87), ß-actin (88, 81), thymidine kinase (89), and histones (90). The detailed examination of all these proteins is out of the scope of this review and therefore I would like to conclude by briefly mentioning two of the products from the oncogene family.

1) c-fos

Growth factors can act as either 'competence factors' or progression factors' (91). If fibroblasts are exposed to a 'competence' factor (e.g. platelet-derived growth factor, PDGF) for a short time, the cells become competent for growth in that upon subsequent treatment with a progression factor (e.g. platelet-poor plasma, PPP) they proceed through G_1 and reach the S-phase. Treatment of non-competent cells with PPP alone has no detectable effect on the growth state of the cells.

Among the earliest known nuclear events following stimulation of quiescent fibroblasts by peptide growth factors is the transient induction of c-fos and c-myc

proto-oncogenes (81, 79). This observation has led to the hypothesis that the c-fos and c-myc gene products may play a role in the control of cell proliferation.

The c-fos gene product (a protein localized by immunofluorescence to the nucleus) is induced only by competence factors, but not by the progression factors (92). This observation suggests that the induction of c-fos may play a role in conferring competence to the cells but may not be necessary for the maintenance of the competent state. Additional observations suggest that cells at different stages of the cell cycle (except for mitosis) are as sensitive to c-fos induction by growth factors as quiescent cells (93). However, with the simultaneous induction of c-myc and other as yet unidentified products, c-fos may contribute to remove the cells from the G_0 to the G_1.

2) p53

Another protein that has been suggested to be involved in the regulation of the mammalian cell cycle is a protein with M_r 53,000 that has been identified in mouse cells transformed by viruses, chemicals or x-ray irradiation (94, 95). Milner and Milner (96) have reported that p53 is not synthesized in nondividing G_0 lymphocytes but is synthesized in the same lymphocytes when they are stimulated to proliferate by the addition of Con A. In addition, microinjection of monoclonal antibodies directed against p53 blocks the serum-induced stimulation of cellular DNA

synthesis (97). In SV40 transformed cells, p53 is associated with the large-T antigen (98) in the form of non-covalent complexes. It seems that this association increases the half-life of p53 thus resulting in high levels of large-T/p53 complexes which may act in a positive way to maintain the unregulated growth of the transformed cell (99).

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Chapter II

ENDOGENOUS LECTINS FROM CULTURED CELLS

Subcellular Localization of Carbohydrate-Binding Protein 35 in 3T3 Fibroblasts

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FOOTNOTES

1. The abbreviations used are: CBP, carbohydrate-binding protein; PBS, phosphate buffered saline (8 g NaCl,0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 per liter, pH 7.4); SDS, sodium dodecyl sulfate; TK, 0.02 M Tris-HCl, 5 mM KCl buffer (pH 7.2) containing 105 m units/ml aprotinin; NADH, reduced nicotinamide-adenine dinucleotide; LDH, lactate dehydrogenase.

ABSTRACT

In previous studies, a lectin designated as carbohydrate-binding protein 35 (CBP35) has been isolated from cultured 3T3 fibroblasts. In the present study, rabbit antibodies directed against CBP35 were used to analyze the subcellular distribution of CBP35 in 3T3 cells. Several lines of evidence indicate that CBP35 is found externally exposed at the cell surface: (a) immunofluorescent staining of live 3T3 cells; (b) agglutination of suspension of 3T3 fibroblasts by specific antibodies; and (c) isolation, by immunoaffinity chromatography, of a M_r = 35,000 component from cells surface-labeled with ^{125}I. In addition to the plasma membrane, CBP35 could also be found intracellularly, as revealed by immunofluorescence studies of fixed and permeabilized 3T3 cells. The staining pattern showed the presence of CBP35 on the nucleus and in the cytoplasm. These results are consistent with the finding that among several subcellular fractions, CBP35 can be found by immunoblotting procedures in the nuclear pellet, the soluble fraction, as well as the plasma membrane fraction of the postnuclear supernatant.

INTRODUCTION

In previous studies, we reported the isolation, from 3T3 mouse fibroblasts, of three carbohydrate binding proteins (CBPs) which are specific for galactose-containing glycoconjugates (31,32). The molecular weights of these CBPs are 35,000 (CBP35), 16,000 (CBP16) and 13,500 (CBP13.5). On the basis of several biochemical and immunological criteria, it was proposed that CBP16 and CBP13.5 may be the murine analogs of lectins previously isolated from embryonic chicken (7,27), electric eel (25), bovine (3,11,14) and human tissues (10,11,28). CBP35, however, represented a newly-identified lectin which was not structurally related to the other two lectins of lower molecular weight.

A knowledge concerning the subcellular localization and tissue distribution of these lectins may provide useful clues to the analysis of their endogenous function. CBP35 has been identified in several tissues of the adult and embryonic mouse; the expression of this protein in tissues such as liver, muscle, and skin was developmentally regulated (13). We now report data relating to the subcellular localization of CBP35 in Swiss 3T3 fibroblasts from which the lectin was initially isolated. Our results showed that CBP35 could be quantitatively and reproducibly found in the nuclear, cytoplasmic and plasma membrane fractions. These results are particularly striking when compared to similar analyses, carried out on the counterparts of CBP13.5 in bovine and human tissues, which showed nuclear and cytoplasmic localizations of the lectin (9,11).

MATERIALS AND METHODS

Cell Growth and Radiolabelling

The growth and maintenance of 3T3 fibroblasts has been previously described (33). Metabolic labeling of 3T3 cells with [^{35}S]methionine (Amersham) was performed according to Roff and Wang (32).

Cell surface iodinations were performed using the iodogen method (16,26). The 3T3 fibroblasts were grown to confluent density in 100 mm tissue culture dishes in Dulbecco's modified Eagle's Medium containing 10% (v/v) calf serum. The growth medium was discarded and the cell monolayer was washed three times with 5 ml of phosphate-buffered saline (PBS). A coverslip coated with 100 µg of iodogen was suspended face-down on each culture dish containing 5 ml of PBS. Na^{125}I (0.5 mCi, Amersham) was added and the iodination was carried out at room temperature for 15 min with gentle rotation. The coverslip was removed and the iodinating medium was discarded. The cell monolayer was then washed five times with 5 ml of PBS and extracted with 1 ml of 5 mM phosphate buffer (pH 8.0) containing 0.5% (v/v) Triton X-100. Phenyl methyl sulfonylfluoride (PMSF) was added to the extract to a final concentration of 1 mM and the insoluble material was removed by centrifugation at 3000 g for 10 min. The supernatant was analyzed for the presence of CBP35 by affinity chromatography as described below.

Affinity Chromatography Procedures

Antiserum against CBP35 isolated from 3T3 cells was raised in New Zealand white female rabbits as previously described (32). This antiserum will be designated rabbit anti-CBP35. The immunoglobulin fraction of rabbit anti-CBP35 (15 mg), isolated by ammonium sulfate precipitation and DEAE-cellulose ion exchange chromatography, was dissolved in 4 ml of 0.1 M sodium bicarbonate buffer pH 8.0 (coupling buffer) and coupled to 1 ml of Affigel 10 (Bio-Rad). The column was washed extensively with coupling buffer and PBS before use. The column was then equilibrated with 2% (v/v) calf serum in coupling buffer to minimize non-specific binding. Identical procedures were used to prepare affinity columns using the immunoglobulin fractions from rabbit anti-succinyl concanavalin A (19) and normal rabbit sera. Triton X-100 extracts of radioactively labeled cells were passed over the column; the nonbound material was reapplied to the column. After four rounds of reapplication, the column was then washed extensively with coupling buffer until the radioactivity level reached background. The column was washed further with 0.1 M sodium phosphate buffer (pH 7.0) containing 0.5% (v/v) Tween-20. The detergent was washed away with 20-30 bed volumes of coupling buffer and the bound proteins were eluted with 0.1 M citrate buffer (pH 3.0). The eluted material was dialyzed overnight against water, lyophilized, and subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) (24).

Immunoblotting Procedures

Proteins, separated on 10% or 12.5% polyacrylamide gels in the presence of SDS, were electrophoretically transferred to

nitrocellulose paper (400 mA for 2 h at room temperature) (13,34). The nitrocellulose paper was then incubated twice (30 min each) in saturating buffer (PBS containing 0.5% (v/v) Tween 20 and 0.05% (w/v) NaN_3) and the protein bands were visualized by India ink staining (0.2% (v/v) in saturating buffer) (20).

The nitrocellulose paper was then destained with several washes of saturating buffer and incubated in the same buffer overnight at room temperature. The paper was then incubated for 3 h in 15 ml of saturating buffer containing rabbit anti-CBP35 serum (1:250 dilution). The paper was washed five times with saturating buffer and 15 ml of ^{125}I-labeled goat anti-rabbit immunoglobulin (10^6 cpm) in the same buffer were added and incubated at room temperature for 2 h. The nitrocellulose paper was then washed five times with buffer and dried under vacuum. Autoradiography was performed using Kodak XRP-5 film and a Lighting Plus intensifying screen (DuPont).

Immunoblots stained using horseradish peroxidase conjugated goat anti-rabbit immunoglobulin (Bio Rad) were processed and stained according to the protocol supplied by the manufacturer. Pictures of the stained nitrocellulose filter were taken using Tri X Pan Kodak film.

Agglutination Assay

3T3 fibroblasts were removed from 100 mm tissue culture dishes by treatment with 5 mM ethylene glycol bis (β-aminoethyl ether)-N,N,N',N',-tetraacetic acid (EGTA) in PBS on a rotary shaker for 1 h at 37°C. The cells were collected by centrifugation at 500 g for 3 min and resuspended in PBS to a final concentration of 2 x 10^6

cells/ml. Serum samples (0.2 ml of rabbit anti-CBP35 or preimmune) or concanavalin A (0.2 ml of a 50 μg/ml solution) was added to an equal volume of the cell suspension. After 10 min at room temperature, a drop of the solution was examined under the microscope for cell agglutination.

Immunofluorescence

3T3 cells seeded on a microscope slide coverslip were rinsed with ice-cold Dulbecco's modified Eagle's medium containing 0.5% (w/v) bovine serum albumin and buffered with 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) to pH 7.4 (Buffer A). In some experiments 0.1 M lactose was included in Buffer A to inhibit possible lectin binding to carbohydrate structures. The coverslip was then inverted on 200 μl of a 1:10 dilution of antiserum in Buffer A and incubated at 4° for 2 h. Unbound primary antibody was removed by three 5-min incubations with Buffer A at 4°C and the cells were then incubated with 1:30 dilution of rhodamine conjugated goat anti-rabbit immunoglobulin (Miles) in Buffer A for 30 min at 4°C. Unbound fluorescent antibody was removed with three 10-min washes in Buffer A and the cells were immediately observed on a Leitz epiphase fluorescence microscope using a 50X water immersion objective. Pictures were taken using Kodak Tri-X film.

Fixed cells were prepared and permeabilized with 0.2% (v/v) Triton X-100 as previously described (18). Each coverslip was incubated with 100 μl of a 1:10 dilution of rabbit anti-CBP35 in PBS containing 3% (v/v) normal goat serum for 1 h at room temperature. After three washes in PBS containing 3% (v/v) normal goat serum, the

coverslip was incubated with rhodamine conjugated goat anti-rabbit immunoglobulin (1:30 dilution) for 1 h at room temperature. Stained preparations were mounted in 70% glycerol-PBS containing 5% (w/v) of the antibleaching agent n-propyl galate and viewed with a Leitz epiphase fluorescence microscope with a 50X objective lens. A parallel analysis of intracellular staining was carried out with a rabbit antiserum directed against pig muscle lactate dehydrogenase (LDH). This antiserum was a gift from Dr. John Wilson of Michigan State University.

Subcellular Fractionation and Marker Enzyme Assays.

Homogenates were prepared from 15 confluent 100 mm plates, each containing 4 X 10^6 3T3 cells. The homogenization and fractionation protocols have been described in detail previously (12,29). Assays for certain marker enzymes were carried out on all subcellular fractions.

Protein was measured by the Bradford assay (8) using crystalline bovine serum albumin as a standard. The procedures for assaying for LDH (21) and reduced nicotinamide-adenine dinucleotide (NADH) diaphorase (1) have been described. For 5'-nucleotidase activity, 0.2% (w/v) deoxycholate was added to the samples to solubilize the enzyme (2,23). Samples of each fraction, containing 6-15 μg of total protein, were incubated for 30 minutes at 37°C in an assay mixture containing 100 mM glycine-NaOH, pH 9.0, 10 mM $MgCl_2$, 0.1 mM AMP and ^{3}H-AMP (2 μCi/ml) as a tracer. Unhydrolyzed AMP was precipitated with $Ba(OH)_2$ and $ZnSO_4$ (22) and the amount of free ^{3}H-adenosine in the supernatant was determined by liquid scintillation counting.

RESULTS

Characterization of Antibodies Against CBP35

The initial characterization of the rabbit anti-CBP35 involved the specific precipitation of CBP35 from a partially purified preparation of lectins (CBP35, CBP16, and CBP13.5) from 3T3 cells (32). This anti-serum was also shown to recognize CBP35 in Triton X-100 extracts of 3T3 cells and mouse lung tissue by immunoblotting techniques (13). When 3T3 cells were extracted with SDS (4% w/v) and the extracts were electrophoresed and immunoblotted with rabbit anti-CBP35, only a single polypeptide band (M_r 35,000) was observed (Fig. 1a). Parallel analysis with preimmune rabbit serum failed to yield this band (Fig. 1b).

To complete the characterization of this antibody, it remained to be ascertained whether the polyclonal antiserum would specifically recognize only CBP35 from a more complex, non-denatured, protein mixture. The immunoglobulin fraction of rabbit anti-CBP35 was coupled to Affigel 10 beads. A Triton X-100 extract of 3T3 cells labeled with [^{35}S]methionine was prepared in a hypotonic buffer (5 mM phosphate buffer, 0.5% (v/v) Triton X-100, pH 8.0). This extract was then fractionated on the Affigel column containing rabbit anti-CBP35. In parallel, the extract was also fractionated on Affigel columns containing rabbit anti-succinyl concanavalin A or normal rabbit immunoglobulin. The polypeptides bound to the respective columns were examined by polyacrylamide gel electrophoresis. The material bound and eluted from the rabbit anti-CBP35 column yielded three major components (M_rs 35,000, 48,000 and 57,000) (Fig. 2b). Two of

Figure 1: Immunoblots of SDS (4% w/v) extracts of 3T3 cells with rabbit anti-CBP35 and rabbit anti-LDH. Approximately 100 µg of protein was electrophoresed on a 12.5% polyacrylamide gel and transferred to nitrocellulose paper as described in the Materials and Methods. Following incubation with 1:250 dilution of the appropriate antiserum, the nitrocellulose papers were incubated with horseradish peroxidase conjugated goat anti-rabbit immunoglobulin and developed using 4-chloronaphthol as substrate. (a) rabbit anti-CBP35; (b) preimmune serum control for (a); (c) rabbit anti-LDH; (d) preimmune serum control for (c). The arrows indicate positions of migration corresponding to the molecular weights of authentic CBP35 and LDH.

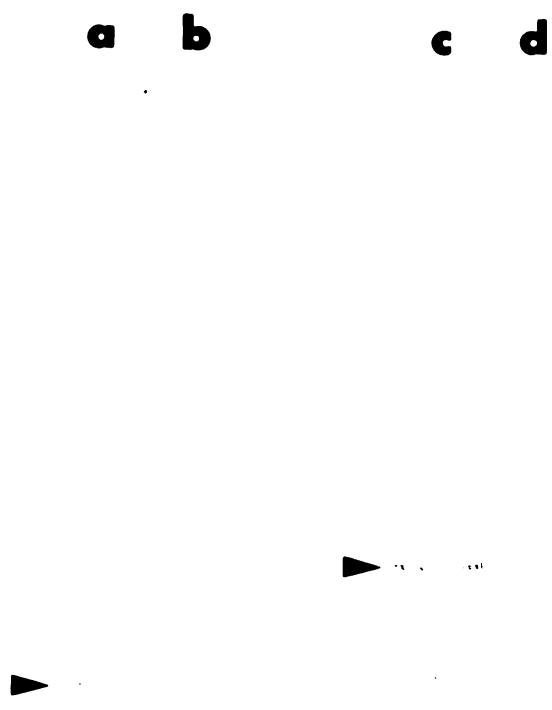

Figure 1

Figure 2: Polyacrylamide gel electrophoresis in SDS of ^{35}S-labeled polypeptides bound on a column (1.5 x 2 cm) of Affigel 10 covalently derivatized with the immunoglobulin fraction of rabbit anti-CBP35. Triton X-100 extracts of 3T3 cells, labeled with [^{35}S]methionine (1.2 x 10^7 total cpm per column) were fractionated as detailed in Materials and Methods. The radioactive components bound to the column were eluted and subjected to polyacrylamide gel electrophoresis. (a) authentic ^{35}S-labeled CBP35 from 3T3 cells; (b) polypeptides bound on rabbit anti-CBP35 column; (c) polypeptides bound on rabbit anti-succinyl concanavalin A column; (d) polypeptides bound on normal rabbit immunoglobulin column. Approximately 15,000 cpm were electrophoresed in lanes b-d and the fluorogram was exposed for two days. The arrow on the left highlights the position of migration of CBP35. Molecular weight markers are indicated on the right.

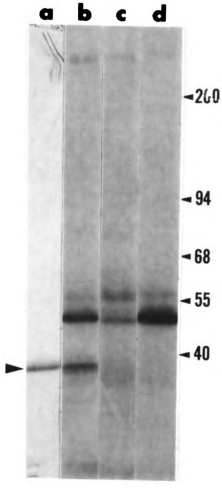

Figure 2

these components (M_rs 48,000 and 57,000) were also found in the material from the control columns containing anti-succinyl concanavalin A (Fig. 2c) or normal rabbit immunoglobulin (Fig. 2d). In contrast, the M_r = 35,000 band, which co-migrated with an authentic sample of CBP35 (Fig. 2a), was found only in the material bound by the column containing rabbit anti-CBP35. Therefore, under these conditions of extraction and fractionation, the anti-CBP35 column recognizes and binds specifically CBP35 out of a complex mixture of proteins present in the cell extract.

Evidence for CBP35 at the Cell Surface

Live 3T3 fibroblasts were stained with rabbit anti-CBP35 using indirect immunofluorescence. The staining and washings were carried out at 4°C to minimize internalization of the antibody by endocytosis. Weak fluorescent staining of the cell surface was observed. At different focal planes, the periphery of the cell was outlined by numerous fluorescent patches (Fig. 3A and 3C), a rather characteristic pattern of cell surface staining in live cells. Staining the cells with normal rabbit serum did not result in any significant amount of labeling (Fig. 3B). Similar results were obtained when these experiments were carried out in the presence of lactose (0.1 M) (Fig. 3C and 3D). Therefore, it was unlikely that the antigenic target detected by immunofluorescence staining with rabbit anti-CBP35 was due to lectin, released by dead cells, that bound to cell surface glycoconjugates. These observations suggest the possibility that CBP35 can be detected on the external surface of 3T3 fibroblasts.

Figure 3: Indirect immunofluorescence detection of the binding of rabbit anti-CBP35 to the surface of 3T3 fibroblasts. The binding of the rabbit antibody (4°C, 2 h) was detected by rhodamine-labeled goat anti-rabbit immunoglobulin (4°C, 30 min). Pattern of fluorescence labeling when the cells are stained with rabbit anti-CBP35 in the absence of (A) or presence of 0.1M lactose (C). Staining with normal rabbit serum in the absence (B) or presence of 0.1M lactose (D). Bar = 50 μm.

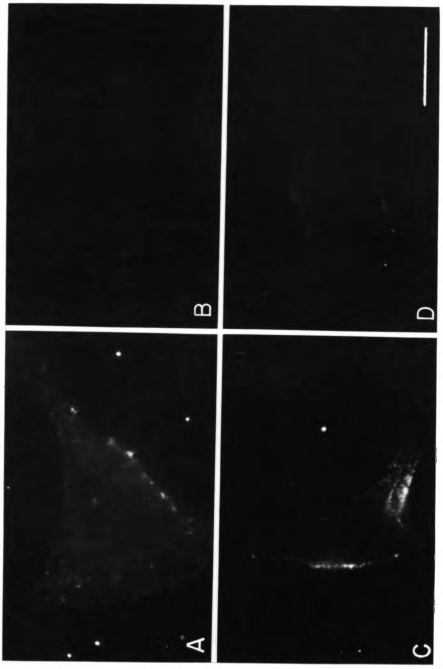

Figure 3

Figure 4: Agglutination of 3T3 fibroblasts in suspension by rabbit anti-CBP35. The final concentration of the cells in the assay was 1 x 10^6 cells/ml. (A) Rabbit anti-CBP35 serum; (B) Normal rabbit serum; (C) Concanavalin A (25 µg/ml); (D) PBS.

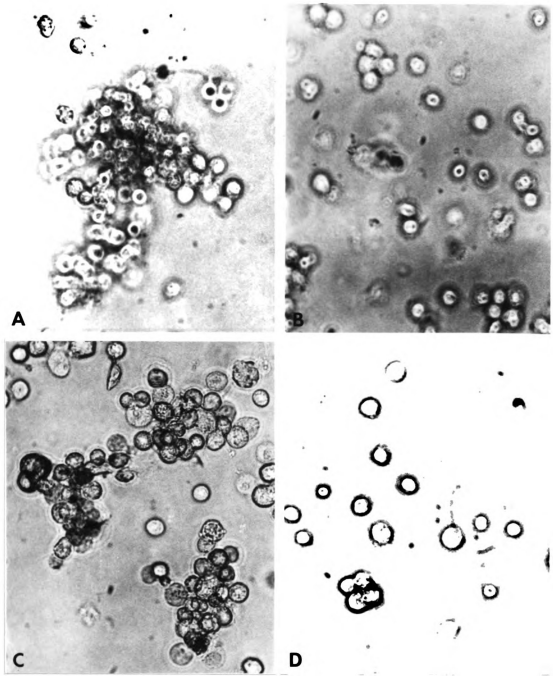

Figure 4

This conclusion was corroborated by the observation that 3T3 cells can be agglutinated by rabbit anti-CBP35. The 3T3 fibroblasts were removed from their substratum with EGTA in order to preserve the integrity of the cell surface components. Rabbit anti-CBP35 serum, normal rabbit serum and PBS were tested for their ability to agglutinate the cells. Concanavalin A, a lectin known to agglutinate 3T3 cells by binding to cell surface carbohydrates, was also used as a positive indicator of agglutination. The results indicated that the rabbit anti-CBP35 serum agglutinated the cells strongly (Fig. 4A), as did concanavalin A (Fig. 4C). Normal rabbit serum (Fig. 4B) and PBS (Fig. 4D), however, failed to agglutinate the cells. These results also indicate that an immunoreactive component (presumably CBP35) exists at the cell surface of 3T3 fibroblasts.

Molecular Identification of the Cell Surface Component Reactive with Rabbit Anti-CBP35.

Proteins externally exposed on the surface of 3T3 fibroblasts were labeled with ^{125}I using the insoluble chloramide, iodogen (16,26). A Triton X-100 extract of the labeled surface components was then chromatographed on an Affigel column conjugated with rabbit anti-CBP35 and the bound proteins were examined by polyacrylamide gel electrophoresis. The autoradiogram of the SDS gel showed that a protein of M_r = 35,000 was bound on the anti-CBP35 column (Fig. 5a, arrow) but not on the control column (Fig. 5b), which contained covalently coupled rabbit anti-succinyl concanavalin A. Both columns bound identically two other polypeptides of higher molecular weight; these were assumed to represent non-specific binding. These results

Figure 5: Polyacrylamide gel electrophoresis in SDS of ^{125}I-labeled polypeptides bound on a column (1 ml bed volume) of Affigel 10 covalently derivatized with the immunoglobulin fraction of rabbit anti-CBP35. Cells were surface-labeled with ^{125}I and iodogen; Triton X-100 extracts were fractionated as detailed in Materials and Methods. The radioactive components bound and eluted were subjected to polyacrylamide gel electrophoresis: (a) polypeptides bound on rabbit anti-CBP35 column; (b) polypeptides bound on rabbit anti-succinyl concanavalin A column. Approximately 2000 cpm and 1000 cpm were electrophoresed in lanes a and b, respectively; the fluorogram was exposed for 26 days. The arrow on the left indicates the position of migration of CBP35. Molecular weight markers are indicated on the right.

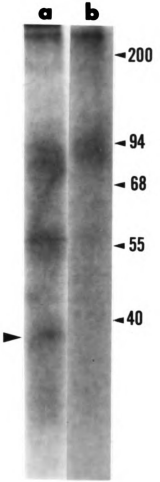

Figure 5

parallel that obtained when ^{35}S-labeled extracts of whole 3T3 cells were subjected to affinity chromatography on columns containing rabbit anti-CBP35 or rabbit anti-succinyl concanavalin A (Fig. 2b and 2c). Therefore, the present data indicate that the immunoreactive component on the cell surface of 3T3 fibroblasts that was initially implicated by immunofluorescence and cell agglutination is in fact CBP35.

Several control experiments were carried out to ascertain that the CBP35 detected on the cell surface was not actually derived from internal proteins (released by a low percentage of lysed cells) which become adsorbed to the cell surface. First, medium conditioned by exposure to 3T3 fibroblasts, analyzed by polyacrylamide gel electrophoresis and immunoblotting, failed to yield a M_r =35,000 component cross-reactive with rabbit anti-CBP35. In addition, immunoblotting analysis of the medium after incubation with lactose (0.1 M) for 30 min did not show any CBP35. Finally, 3T3 cells were labeled with either [^{35}S]methionine or with ^{125}I and then chased in unlabeled medium. There was no evidence of release of the lectin from the labeled cells. Together with the immunofluorescence staining obtained in the presence and absence of lactose and with the agglutination results, the present data strongly suggest that CBP35 is externally exposed at the cell surface.

Immunofluorescence Staining of CPB35 in Permeabilized Cells

The intracellular localization of CBP35 was examined by indirect immunofluorescence staining of formaldehyde fixed and Triton X-100 permeabilized 3T3 fibroblasts using rabbit anti-CBP35. For

Figure 6: Immunofluorescence staining of 3T3 fibroblasts after fixation with formaldehyde (3.7%) and permeabilization with Triton X-100 (0.2%). (A) rabbit anti-CBP35 (1:10 dilution of antiserum); (B) normal rabbit serum (1:10 dilution). (C) rabbit anti-LDH (1:10 dilution); (D) preimmune serum control for anti-LDH staining. The binding of the primary immunoglobulin was detected by rhodamine-labeled goat anti-rabbit immunoglobulin. Incubations with both the primary antibody and the secondary antibody were carried out at room temperature for 1 h. Bar = 50 μm.

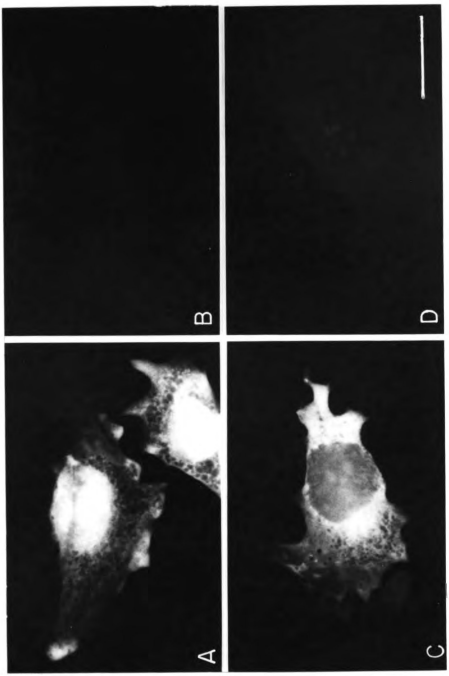

Figure 6

comparison, we carried out the identical staining protocol with a rabbit antiserum raised against pig muscle LDH. Immunoblotting analysis of SDS extracts of 3T3 cells showed that this antiserum reacted with a single polypeptide (Fig. 1c), migrating at a position corresponding to the molecular weight of mouse LDH (M_r ~37,000). Since LDH is a generally accepted marker enzyme for the cytosol fraction, this staining provided a reference pattern expected for a cytoplasmic protein in cells with a prominent cytoskeleton.

Fixed and permeabilized 3T3 fibroblasts stained with anti-rabbit CBP35 showed intracellular labeling in all cells (Fig. 6A). There was prominent labeling of the nucleus and variable staining of the cytoplasm. Cytoplasmic areas devoid of phase-dense intracellular vesicles stained diffusely in a uniform manner, while areas rich in vesicular bodies stained in a highly reticular manner. We suspect this is due to the exclusion of the fluorescent stain from the vesicles themselves (Fig. 6A). In contrast, staining with normal rabbit serum resulted in insignificant labeling. (Fig. 6B).

In parallel experiments, 3T3 fibroblasts stained with rabbit anti-LDH showed a diffuse distribution of fluorescence within the cytoplasm of the cells (Fig. 6C). Cytoplasmic staining was again variable and gave a reticular pattern in areas rich in vesicular bodies as previously observed for anti-CBP35 staining. In general, the cytoplasmic staining of the two antibodies was very similar but more important for our present study, there was only weak diffuse staining of nuclei, most likely resulting from overlying or underlying cytoplasm, when the anti-LDH antibody was used for staining. This is in direct contrast to the results obtained with

rabbit anti-CBP35, which showed prominent labeling of the nucleus (compare Fig. 6A and 6C).

Quantitation of CBP35 in Subcellular Fractions

Homogenates were prepared from 3T3 cells swollen in hypotonic TK buffer. After low speed centrifugation to remove nuclei and remaining intact cells, the postnuclear supernatant was separated into a high speed P150 pellet and a soluble S150 fraction (Table I). The subcellular fractions were then subjected to polyacrylamide gel electrophoresis and immunoblotting with rabbit anti-CBP35 and ^{125}I-labeled goat anti-rabbit immunoglobulin (Table I inset). After autoradiography, the intensity of the band corresponding to CBP35 was quantitated by densitometric scanning.

One important point should be noted in the interpretation of the immunoblot shown in Table I. Equal amounts (100 µg) of total protein from each subcellular fraction were electrophoresed in the individual lanes. Therefore, the intensity of the band corresponding to CBP35 reflects the proportion of the lectin relative to the total protein content of the subcellular fractions and comparisons of the relative amount of the lectin from one subcellular fraction to another must take into account of the total protein contents of the fractions.

CBP35 was found predominantly in the S150 fraction, along with the majority of the total cellular proteins as well as with 98% of the total LDH activity, a marker for soluble proteins of the cell (Table I). There were, however, reproducible and significant amounts of CBP35 detectable in the nuclear pellet and fractions derived from the P150 pellet. The nuclear pellet, which was free of gross

contamination as indicated by the low levels of marker enzymes, contained approximately 5% of the CBP35. These results are consistent with the intracellular immunofluorescence staining patterns (Fig. 6A), localizing CBP35 within the cytoplasm and on the nucleus.

The particulate fraction P150, containing large fragments of plasma membrane, intracellular membranes such as endoplasmic reticulum and Golgi, and organelles such as lysosomes and mitochondria, were further fractionated by discontinuous sucrose gradient centrifugation. Nearly all of the CBP35 of the particulate fraction (accounting for ~4% of the total CBP35 recovered) was found at the 20%/35% sucrose interface, along with the plasma membrane marker 5'-nucleotidase (Table I). There was little or no CBP35 in the other two heavier membrane fractions. For example, the 40%/50% sucrose interface, which contained 77% of the marker enzyme NADH diaphorase, showed no CBP35. These results are consistent with our finding that some CBP35 is associated with the plasma membrane, exposed at the cell surface.

TABLE 1

Distribution of CBP35 in Subcellular Fractions of Homogenates Prepared in Hypotonic TK Buffer

		Percentage of total recovered			
	lane*	CBP35	Protein	Lactate Dehydrogenase	5'-Nucleotidase	NADH Diaphorase
S150	a	90.2	58.4	98.0	38.0	2.6
P150						
50%/40% sucrose interface	b	-	19.3	0.7	16.5	77.0
40%/35% sucrose interface	c	-	12.6	0.5	13.2	16.0
35%/20% sucrose interface	d	4.4	8.4	0.4	31.0	4.0
Nuclear Pellet	e	5.4	1.3	0.4	1.3	0.4

*Lanes a through e correspond to the immunoblot autoradiogram shown in the inset. Various subcellular fractions (100 µg) were subjected to polyacrylamide gel electrophoresis, transferred to nitrocellulose paper, and blotted with rabbit anti-CBP35 (1:250 dilution of antiserum) followed by ^{125}I-labeled goat anti-rabbit immunoglobulin (10^6 cpm). The arrow indicates the position of migration of CBP35.

DISCUSSION

The results of both fractionation and immunofluorescence analysis indicate that CBP35 is found on the nucleus, in the cytoplasm, and on the cell surface of 3T3 cells. The distribution of marker enzymes in the various subcellular fractions that we obtained is very similar to that reported by Radke et al.(29), who studied the localization of a 36,000-dalton substrate for tyrosine phosphorylation in chicken embryo fibroblasts transformed by avian sarcoma viruses. Thus, approximately 90% of the total LDH activity was associated with the S150 soluble fraction while 60% and 97% of the 5'-nucleotidase and NADH diaphorase activities, respectively, were found in the P150 pellet of the postnuclear supernatant.

The presence of CBP35 in the various subcellular fractions is qualitatively consistent with the patterns obtained from immunofluorescence. However, an apparently much higher level of CBP35 is detected in the nucleus by indirect immunofluorescence than by analysis of nuclei obtained after subcellular fractionation. The nuclear staining with rabbit anti-CBP35 was striking when compared with parallel staining with rabbit anti-LDH, an enzyme marker of cytosolic proteins. It is possible that the conditions employed for the subcellular fractionation, low ionic strength buffers and absence of metal ions, do not favor the association of CBP35 with the nucleus, thus releasing it into the cytoplasm in a soluble form. Nevertheless, even under the buffer conditions used for subcellular fractionation, the presence of CBP35 in the nuclear pellet was significant, particularly in view of the fact that 5% of the total

lectin recovered was found in a fraction accounting for only 1% of the total protein. It is possible that the CBP35 found here is due to the presence of intact cells in the nuclear pellet but the amount of LDH activity in the pellet argues against this notion.

The detection of CBP35 in the nuclear fraction should be compared with the findings of Feizi and co-workers, who have localized a lectin corresponding to CBP13.5 at the nucleus using a polyclonal (11) and a monoclonal (9) antibody. Moreover, observations consistent with the nuclear localization of β-galactoside-binding lectins in epithelial tissues have also been reported by Beyer and Barondes (6). Glycosylated proteins have been localized in the nucleus both at the ultrastructural (17) and light microscope (15) levels and monoclonal antibodies prepared against the major nuclear matrix-pore complex-lamina glycoprotein bind specifically to the nuclear envelope in situ.

CBP is also localized in the cytoplasm, as detected by immunofluorescence. The staining pattern of the cytoplasm obtained with rabbit anti-CBP35 is similar to that observed with rabbit anti-LDH, representative of cytosolic proteins. Consistent with these results, 90% of the total CPB35 behaves as a soluble, cytoplasmic component under our subcellular fractionation conditions. Several other lectins of bovine and avian origin have also been predominantly localized in the cytoplasm (3,27).

Finally, a small amount of CBP35 is also found at the cell surface. The finding that a lectin is simultaneously surface exposed and localized within the cytoplasm of the cell is strikingly similar to the recent report of Raz et al. (30). Using monoclonal antibodies

directed against tumor cell lectins (M_r 34,000 and 68,000), they have shown that the lectin(s) is exposed at the cell surface. Staining of viable B16-F1 melanoma cells was in the form of microclusters distributed randomly at the cell circumference, a result mimicked by the staining of rabbit anti-CBP35 on 3T3 cells. Moreover, Raz et al. (30) also found that the majority of the lectin(s) is inside the cell, as revealed by immunofluorescence after fixation and permeabilization. The relationship between CBP35 from 3T3 cells and the tumor cell lectin(s) remains to be elucidated.

The mode of anchorage of CBP35 on the plasma membrane is not known. The observations that the presence of lactose did not affect surface staining by rabbit anti-CBP35 and that lactose can not wash the lectin off the membrane in quantities detectable by immunoblotting procedures (1-5 ng (13)) argues against the notion that it is bound to cell surface glycoconjugates. It is possible that the lectin is anchored through interactions other than carbohydrate-binding. This in turn implies that the surface-exposed CBP35 has carbohydrate-binding sites free to interact with extracellular matrix components or other cells. Barondes and co-workers have reported surface localization and externalization of the chicken lactose lectin I (5) and have postulated that the function of this lectin may be to organize glycoconjugate networks (4).

ACKNOWLEDGEMENTS

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Chapter III

ENDOGENOUS LECTINS FROM CULTURED CELLS

Proliferation-dependent Expression and Nuclear Localization of Carbohydrate-Binding Protein 35

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FOOTNOTES

1. The abbreviations used are: CBP, carbohydrate-binding protein; SDS, sodium dodecyl sulfate; HRP, horseradish peroxidase; PBS, phosphate-buffered saline (10 mM sodium phosphate, 0.14 M NaCl, 4 mM KCl, pH 7.4); PCNA, proliferating cell nuclear antigen.

2. I.K. Moutsatsos, R.P. Cleveland, and J.L. Wang unpublished observations.

ABSTRACT

Sparse, proliferating cultures of 3T3 mouse fibroblasts contain high levels of the lectin, Carbohydrate-binding Protein 35 (CBP35), relative to quiescent cultures of the same cells. An ACAS 470 Fluorescence Workstation was employed in conjunction with a rabbit antiserum directed against CBP35 to map the cellular fluorescence distribution in a large population of cells under different growth conditions. This cytometric analysis showed that the lectin is prominently localized in the nucleus of the proliferating cells. In quiescent 3T3 cultures, the majority of the cells have lost their high level of nuclear staining and have undergone a general decrease in the overall fluorescence intensity. Stimulation of serum-starved quiescent 3T3 cells by the addition of serum resulted in an increase in the level of CBP35. The percent of cells showing nucleolar staining reached a maximum about five hours before the onset of the first S-phase of the cell cycle. Finally, a comparison of the levels of CBP35 in normal 3T3 cells and 3T3 cells transformed with Kirsten murine sarcoma virus showed that the transformed cell expressed more CBP35 than its normal counterpart. All of these results suggest that CBP35 may be a protein whose presence in the nucleus is coordinated with the proliferation state of the cell.

INTRODUCTION

In previous studies, we reported the isolation, from 3T3 mouse fibroblasts, of three carbohydrate-binding proteins (CBPs) which are specific for galactose-containing glycoconjugates (16,17). These were designated as CBP35 (M_r 35,000), CBP16 (M_r 16,000) and CBP13.5 (M_r 13,500). A polyclonal rabbit antiserum, highly specific against CBP35, was used to analyze the subcellular localization (14) and tissue distribution (8) of the lectin. These studies revealed that the majority of CBP35 was associated with the cytoplasmic fraction and the nucleus of the 3T3 cell. In addition, CBP35 was identified in adult and embryonic mouse tissues that contained proliferating cell populations (e.g. embryonic liver and skin). In contrast, the lectin was not detected in several adult tissues whose predominant cell population was quiescent (e.g. adult liver and brain).

The association of CBP35 with the nucleus and the apparent correlation with proliferating cell populations prompted us to analyze the expression of this protein in 3T3 mouse fibroblasts under proliferating and quiescent conditions. In the present communication, we document proliferation-dependent expression and nuclear localization of CBP35. This expression and nuclear translocation are apparently regulated during the G_1 phase of the cell cycle.

MATERIALS AND METHODS

Cell Culture and Synchronization

Swiss 3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium (K.C. Biological Inc., Lenexa, KS) containing 10% calf serum (Microbiological Associates, Walkersville, MD). Cells cultured at a density below 5 x 10^4 cells/cm^2 were proliferative and incorporated [^{3}H]thymidine; above this density, the cells remained in a quiescent monolayer state (21). Cells at low density were arrested by removal of serum and maintenance in medium containing 0.2% calf serum for 48 hours. Upon re-addition of serum (10%), the cells were stimulated to proceed into the cell cycle in a synchronous fashion.

Assays of DNA Synthesis

DNA synthesis was assayed by the incorporation of [^{3}H]thymidine (1.9 Ci/mmol, Schwarz-Mann, Spring Valley, NJ). Cultures were pulse-labeled with radioactive thymidine (2 uCi/culture) for 1 h, washed with phosphate-buffered saline (PBS) and detached from the growth surface with trypsin (19). After centrifugation and resuspension in PBS, the number of cells was determined in a corpuscle counting chamber (Hausser Scientific, Blue Bell, PA). Aliquots of the cell suspension corresponding to 5 x 10^5 cells were centrifuged in a Beckman microfuge for 8 sec and the pelleted cells were solubilized in 0.1 N NaOH - 1% sodium dodecyl sulfate (SDS) and subjected to scintillation counting (19).

Immunoblotting and Quantitation of CBP35

Cells were removed from the culture dish with trypsin, washed and centrifuged. After resuspension in PBS, the number of cells was determined in a corpuscle counting chamber and aliquots corresponding to 5 x 10^5 cells were centrifuged in a Beckman microfuge for 8 sec. The pelleted cells were solubilized in 40 ul of SDS-PAGE sample buffer and heated to 90 C° for 15 min. The samples were then analyzed in SDS-PAGE (10,14).

After electrophoresis, the separated proteins were transferred to nitrocellulose filters and immunoblotted with a rabbit antiserum directed against CBP35. The generation and characterization of the specificity of this antiserum have been reported previously (14,17). The binding of rabbit anti-CBP35 to the M_r 35,000 polypeptide on the nitrocellulose paper was revealed with the colored product of the horseradish peroxidase (HRP) reaction in HRP-conjugated goat antibodies directed against rabbit immunoglobulin (Bio-Rad Laboratories, Richmond, CA). The stained bands corresponding to CBP35 were excised and incubated in 75 mM Tris-HCl, 0.5 M NaCl, pH 7.5 containing bovine serum albumin (1%, w/v) for 10 min. The nitrocellulose strips were then incubated in the same buffer containing 2.5 x 10^5 cpm/ml of ^{125}I-labeled (9) Protein A (Miles Laboratories, Elkhart, IN) for 1 h at room temperature. The nitrocellulose strips were washed three times (10 min each) and then counted for gamma radioactivity. Only bands from the same immunoblot were compared directly with each other.

Immunofluorescence and Digital Image Analysis

The details of culture and labeling of 3T3 cells with rabbit anti-CBP35 and rhodamine or fluorescein conjugated goat anti-rabbit immunoglobulin (Miles) have been described (14). To obtain a quantitative assessment of antibody labeling in a large number of cells, we employed the ACAS 470 Fluorescence Workstation (Meridian Instruments, Inc. Okemos, MI), an automated, laser-based digital imaging system designed to perform quantitative and distributional analysis of fluorescence intensity in single cells.

The standard instrument was equipped with a 2W argon ion laser (for rhodamine, dichroic and barrier filters at 580 nm, for fluorescein, dichroic 510 and barrier filter at 515 nm), inverted phase contrast microscope, microstepping stage and 16 bit microcomputer for data acquisition. As the stage moves in an x-y raster pattern, the attenuated laser beam, focused to approximately 1 micron, excites the fluorescence in cells at 1.5 micron step intervals. The emission is recorded by the photomultiplier tube and digitized by the computer where the information is displayed on the video screen as a 16 color image of the fluorescence distribution. Several fields of view were analyzed using the same instrument settings and the 40X phase objective (total magnification = 400X). The data were plotted in histogram form.

Analysis of the Immunofluorescence Patterns for CBP35 at Different Cell Densities

In previous immunofluorescence studies, we had observed intracellular staining of mouse 3T3 fibroblasts by rabbit anti-CBP35 (14). This antiserum had been characterized in terms of specificity in the following experiments: (a) immunoblotting of a single polypeptide (M_r 35,000) in SDS extracts (14) and in Triton X-100 extracts (8) of 3T3 cells; (b) specific isolation of CBP35 from Triton X-100 extracts of 3T3 cells by immunoaffinity chromatography (14); and (c) specific immunoprecipitation of CBP35 out of a partially purified preparation of endogenous lectins derived from 3T3 cells (17). Therefore, rabbit anti-CBP35 was monospecific in its recognition of CBP35 in both denatured and nondenatured protein mixtures. Using this antiserum, we observed that 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. Therefore, a more detailed quantitative and distributional analysis of the intracellular staining pattern was carried out at the level of individual cells.

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 three different cell densities are

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 (1:5 dilution of antiserum, 2 h at room temperature) was detected by rhodamine-labeled goat anti-rabbit immunoglobulin (1:30 dilution, 0.5 h at room temperature). Cells at three densities were used: (A) 3×10^3 cells/cm^2 (sparse); (B) 2×10^4 cells/cm^2 (subconfluent); and (C) 5×10^4 cells/cm^2 (confluent). Bar = 50 um.

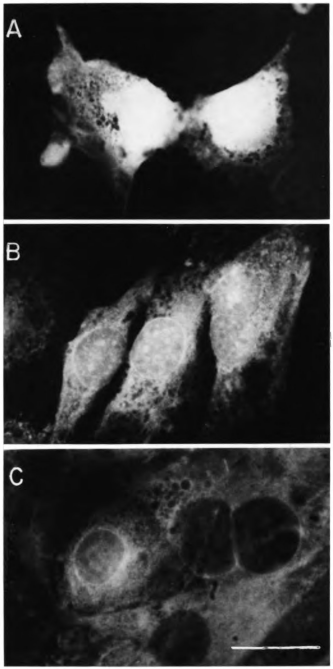

Figure 1

shown in Figure 1. Qualitatively, these photographs indicate the following main points: (A) in sparse cell cultures ("low" cell density 3 x 10^3 cells/cm^2); most cells showed strong staining and the fluorescence is localized predominantly in the nucleus; (B) in subconfluent cultures ("medium" cell density 2 x 10^4 cells/cm^2), the overall staining intensity has decreased and the nuclear localization is less distinct; and (C) in confluent monolayers ("high" cell density 5 x 10^4 cells/cm^2), there was only weak labeling of the cytoplasm. Consistent with previous experiments (14), control samples stained with pre-immune rabbit serum showed no fluorescence labeling under all three culture conditions.

Using the ACAS 470 Fluorescence Workstation, we attempted to examine the distribution of CBP35 in labeled cells by fluorescence digital imaging, taking advantage of the instrument's analytical capabilities to display a population analysis of CBP35 concentration per cell in a large number of cells (~60 cells per analysis). Figure 2 shows a series of pseudo-color fluorescence intensity maps of the distribution of CBP35 in cells from sparse (column A), subconfluent (column B), and confluent (column C) cultures. The scale on the left side provides the intensity color code. A number of such image fields were recorded and a histogram was generated representing the total number of cells recording a particular integrated fluorescence intensity (Fig. 2). All histograms were scaled to a maximum intensity value of 50,000 arbitrary fluorescence units.

This quantitative analysis on single cells yielded conclusions which are consistent with the qualitative observations made on the epi-fluorescence photographs (Fig. 1). First, more than 50% of the

Figure 2. Analysis of the rabbit anti-CBP35 immunofluorescence patterns on 3T3 cells using the ACAS 470 Fluorescence Workstation. The cells were labeled as described in legend to Figure 1. (A) 3 x 10^3 cells/cm^2 (sparse); (B) 2 x 10^4 cells/cm^2 (subconfluent); and (C) 5 x 10^4 cells/cm^2 (confluent). Top row: pseudo-color fluorescence intensity maps (scale on left provide the fluorescence intensity color code). Bottom row: histograms representing the number of cells recording a particular integrated fluorescence intensity. All histograms were scaled to a maximum intensity value of 50,000 arbitrary fluorescence units.

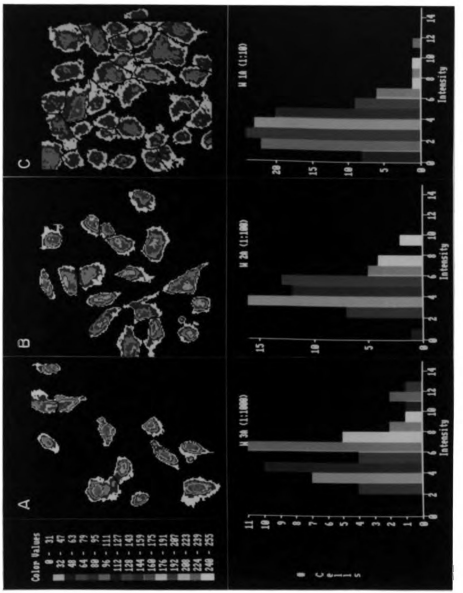

Figure 2

cells in sparse cultures were intensely labeled (arbitrarily defined as >6 on the intensity scale); only a few cells in the sparse culture sample had little or no CBP35 labeling. In labeled cells, the intensity was highest at the cell nucleus, with limited intensity in the cytoplasm. Second, as the cell density is increased to sub-confluence, a large population of cells have lost their high level of nuclear staining and have undergone a general decrease in overall fluorescence intensity. Only 18% of the cells were intensely labeled. Finally, 3T3 cells in a confluent monolayer no longer showed nuclear localization and demonstrated an overall fluorescence intensity that is slightly above background. Less than 8% of the cells were intensely labeled.

All of these results indicate that the overall expression of CBP35, as well as its subcellular localization, are highly dependent on the condition of the cell culture under analysis. These results did not permit us to distinguish, however, whether this expression is strictly dependent on the cell density or on the proliferation state of the cell since 3T3 cells in sparse cultures proliferate with a doubling time of approximately 20 hours while the same cells are subject to density-dependent inhibition of growth in confluent cultures (21).

Quantitation of CBP35 in Proliferating and Quiescent 3T3 Cells

To determine the amount of CBP35 in proliferating and quiescent 3T3 cells, the immunoblotting assay that we used previously (14) was modified 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 HRP-conjugated goat anti-rabbit immunoglobulin; (c) The nitrocellulose strip containing the HRP-positive polypeptide band was excised, incubated with ^{125}I-labeled Protein A, and its level of radioactivity, due to bound ^{125}I-labeled protein A, was quantitated in a γ-spectrometer. This assay was linear over the range of protein concentrations used in our experiments.

Using this assay, the expression of CBP35 in proliferating and quiescent 3T3 cells was compared. Two different protocols were used: (a) density-arrested confluent monolayers of 3T3 cells were stimulated to undergo one round of DNA synthesis and cell division by the addition of serum; and (b) sparse cultures of 3T3 cells were deprived of serum to induce quiescence and restimulated by the addition of serum. In both sets of experiments, the change in the level of CBP35 was compared with the change in the level of DNA synthesis, as measured by the incorporation of [^{3}H]thymidine into cellular DNA.

Confluent monolayers of 3T3 cells exhibit density-dependent inhibition of growth (21); a low level of DNA synthesis was observed (Fig. 3a). Upon addition of serum, this monolayer of cells was stimulated to traverse the cell cycle. The level of [^{3}H]thymidine increased approximately six fold 20 h after the addition of serum. Over the same period of time, serum addition also increased the amount of CBP35 by about 3.5-fold (Fig. 3b). These results indicate that the level of expression of CBP35 may be correlated with the proliferative state of the cell.

Figure 3. Comparison of the increase in (a) level of DNA synthesis and (b) level of CBP35 when density inhibited 3T3 monolayers (5×10^4 cells/cm^2) were stimulated by the addition of calf serum (10%). DNA synthesis was assayed by the incorporation of [^{3}H]thymidine (2 uCi/culture, 1 h, 37°, left ordinate). CBP35 was quantitated by SDS-PAGE of extracts of cultures followed by immunoblotting analysis as described in Materials and Methods (right ordinate). The data for CBP35 quantitation represent the average of triplicate determinations (+ standard error of the mean). The assays were carried out 20 h after the addition of calf serum (+ serum; ▨). Control cultures that received no calf serum (- serum; ☐) were analyzed in parallel.

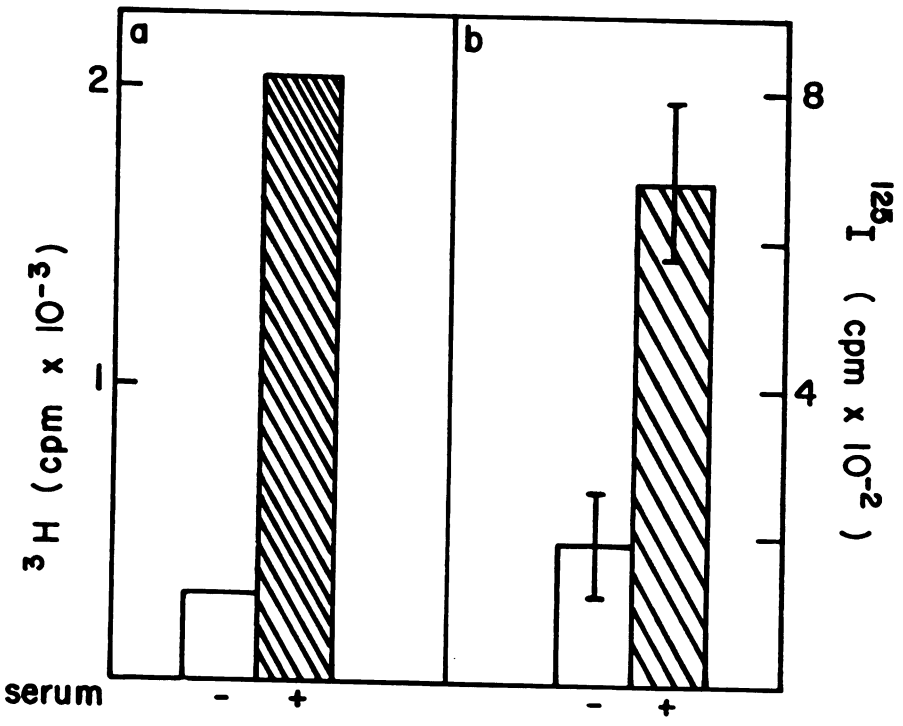

Figure 3

Similar results were also obtained in sparse cultures of 3T3 cells. At low cell densities, the quiescent state was induced by serum starvation. Addition of serum to these quiescent cells increased DNA synthesis as well as the expression of CBP35 (see below). Thus, it appears that the correlation of CBP35 expression with proliferating 3T3 cells is independent of the cell density of the cultures and of the method by which quiescence was achieved.

Kinetics of the Increase of CBP35 in Synchronized 3T3 Cells

When serum-starved, sparse cultures of 3T3 cells were stimulated by the addition of serum, the first wave of DNA synthesis in the synchronized cells was observed between 16 and 28 h after serum addition (Fig. 4a). Samples from parallel cultures were subjected to SDS-PAGE and immunoblotting analysis with rabbit anti-CBP35. There was a two-fold increase in the level of CBP35, beginning at about 8 h after serum addition. At times thereafter the level of CBP35 remained in a plateau (Fig. 4b).

In parallel experiments, we have also prepared immunofluorescence slides, stained with rabbit anti-CBP35, from cultures stimulated with serum. These slides were analyzed by the ACAS 470 Fluorescence Workstation and histograms representing the number of cells recording a particular integrated fluorescence intensity were generated in the same manner as the experiments shown in Figure 2. All cells with an integrated fluorescence intensity of >4 were defined as labeled cells. Using this arbitrary cut off point, we found that the percentage of labeled cells increased monotonically with time after serum addition (Fig. 4c).

A comparison of the bulk quantitation of the CBP35 level (Fig. 4b) with the quantitation of labeled cells at the level of individual cells (Fig. 4c) 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 appreciable amount of CBP35 detectable by immunoblotting; (b) The percent of labeled cells continued to increase 8 h after serum addition whereas the bulk level 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 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 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.

Nuclear Localization of CBP35 in Synchronized 3T3 Cells

The general pattern of intracellular staining in serum-starved, quiescent 3T3 cells and in serum-stimulated, proliferating cells was comparable to those obtained for cells in confluent and sparse cultures (Fig. 1). When we examined the fluorescence in the nucleus of cells at various times after serum addition, however, an interesting kinetic profile was observed. Serum-starved, quiescent cells showed little fluorescence staining with rabbit anti-CBP35 (Fig. 4c). Of the stained cells, approximately 50% showed staining of the

Figure 4. 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 h at 0.2% calf serum). Cells were restimulated by the addition of calf serum (10%) and at various time thereafter, samples containing 5 x 10^5 cells were analyzed for DNA synthesis by the incorporation of [^{3}H]thymidine (2 uCi/culture, 1 h, 37°) and for CBP35 by SDS-PAGE and immunoblotting analysis as described in Materials and Methods. At the same time points, cells were fixed and processed for immunofluorescence staining by rabbit anti-CBP35 and fluorescein labeled goat anti-rabbit immunoglobulin. The cultures were then analyzed using the ACAS 470 Fluorescence Workstation. Scans of cultures and their corresponding fluorescence intensity histograms were constructed. The percent of cells registering a fluorescence intensity of 4 or above (on an arbitrary scale scale of 1 to 12) were considered as staining positively for CBP35.

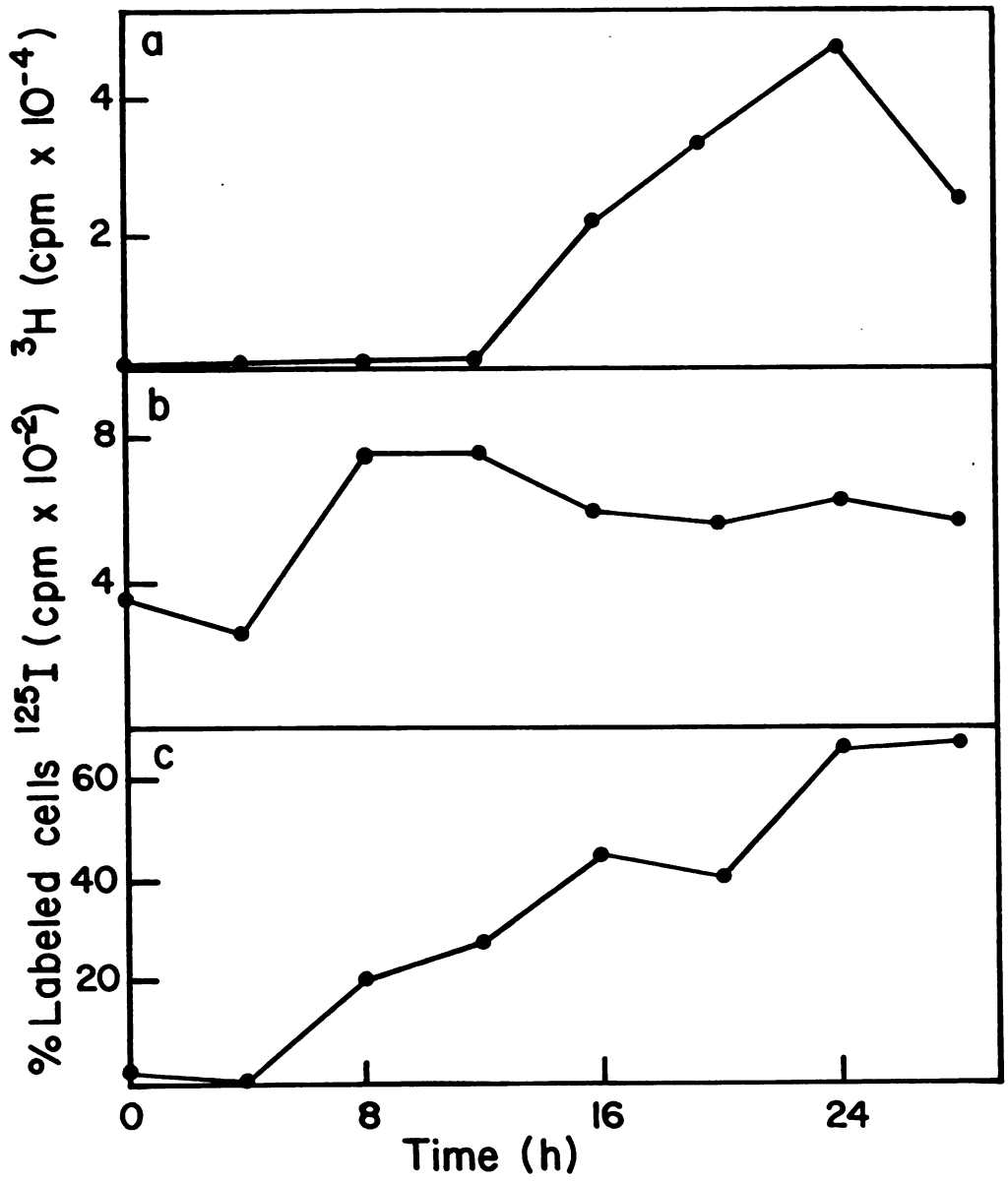
a
b
c
4
2
8
4
60
40
20
0
8
16
24
Time (h)
% Labeled cells
^{125}I (cpm x 10^{-2})
^{3}H (cpm x 10^{-4})

Figure 4

nucleus. In Figure 5A, a nucleus with a granular staining pattern is shown along side a diffusely and weakly labeled nucleus (indicated by arrow). Sixteen hours after serum stimulation, 85% of the fluorescent cells showed nuclear staining. Nucleolar staining was prominent in about half of the labeled nuclei (Fig. 5B). Twenty hours after serum addition, 68% of the fluorescent cells had stained nuclei. Characteristic patterns include diffuse nuclear staining (62% of nuclear positive cells) and nucleolar staining (38% of nuclear positive cells) (Fig. 5C).

When the percent of nucleolar staining cells was plotted as a function of time after serum stimulation, there was a distinct maximum at about 16 h (Fig. 6). A similar profile was observed when the percent of nuclear positive cells was plotted as a function of time after the addition of serum. Thus, it appears that although the overall level of CBP35 increased with serum stimulation, the nuclear localization (or nucleolar localization) showed a rise and a fall, possibly reflecting a cycling phenomenon.

Comparison of the Expression of CBP35 in Normal and Transformed 3T3 Cells

The amount of CBP35 in normal 3T3 fibroblasts was compared to that in 3T3 cells transformed by Kirsten Murine Sarcoma Virus under both sparse and dense culture conditions. In confluent monolayers, normal 3T3 cells are subject to density-dependent inhibition of growth; these quiescent cells contain little CBP35. By contrast, the virally-transformed cells continue to proliferate at high density and these cells have approximately 18 times more CBP35 than their normal

Figure 5. Fluorescence staining patterns of rabbit anti-CBP35 in serum-starved 3T3 cells at various times after serum stimulation. Cells were synchronized and stained for immunofluorescence as described in legend to Figure 4. (A) Serum-starved quiescent 3T3 cells showing a nucleus with a granular staining pattern and a weak, diffusely stained nucleus (white arrow). Most of the cells were not fluorescent; of the fluorescent cells, approximately 50% showed nuclear staining. (B) Serum-starved 3T3 cells treated with serum for 16 h showed prominent nucleolar staining. Approximately 85% of fluorescent cells showed nuclear staining; of this, 53% showed nucleolar labeling. (C) Serum-starved 3T3 cells treated with serum for 20 h showed diffuse nuclear staining as well as nucleolar staining. Approximately 68% of the fluorescent cells showed diffuse nuclear staining while 38% had distinct nucleolar labeling. Bar = 50 um.

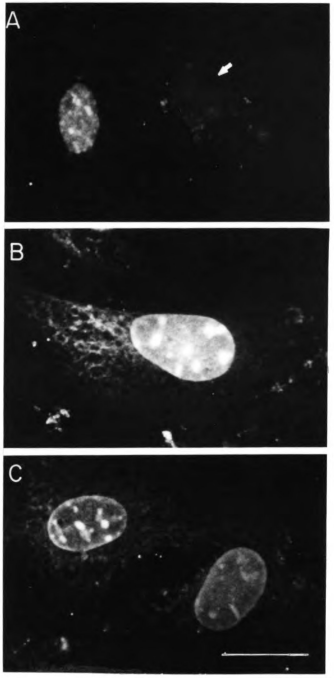

Figure 5

Figure 6. Percent of nucleolar staining by rabbit anti-CBP35 in synchronized 3T3 cells during the cell cycle. Cells were synchronized and stained for immunofluorescence as described in legend to Figure 4. Nuclear and nucleolar staining were scored and the percent of labeled nuclei exhibiting a nucleolar staining pattern is shown.

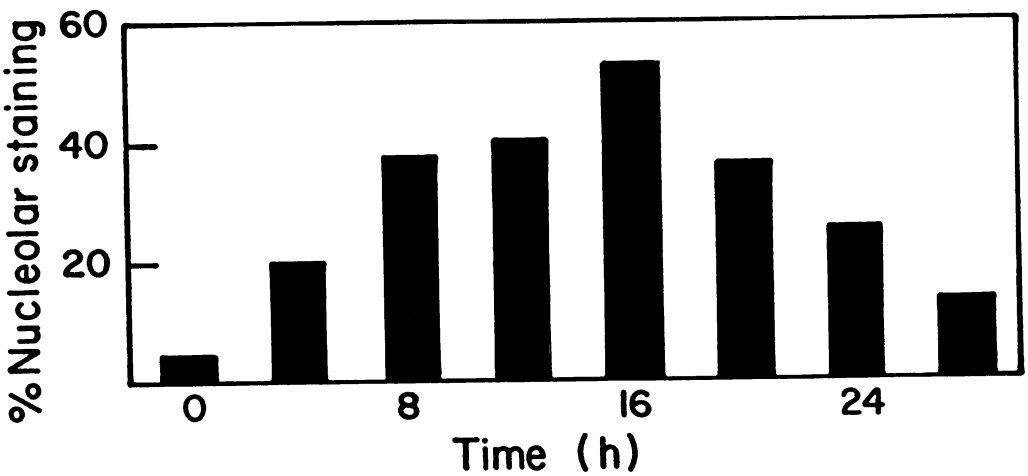

Figure 6

Figure 7. Comparison of the levels of CBP35 in normal 3T3 fibroblasts (▨) and 3T3 cells transformed by Kirsten murine sarcoma virus (□). Samples for the two cell types were obtained from cultures at densities of 5×10^4 cells/cm^2. CBP35 was quantitated by SDS-PAGE and immunoblotting analysis as described in Materials and Methods.

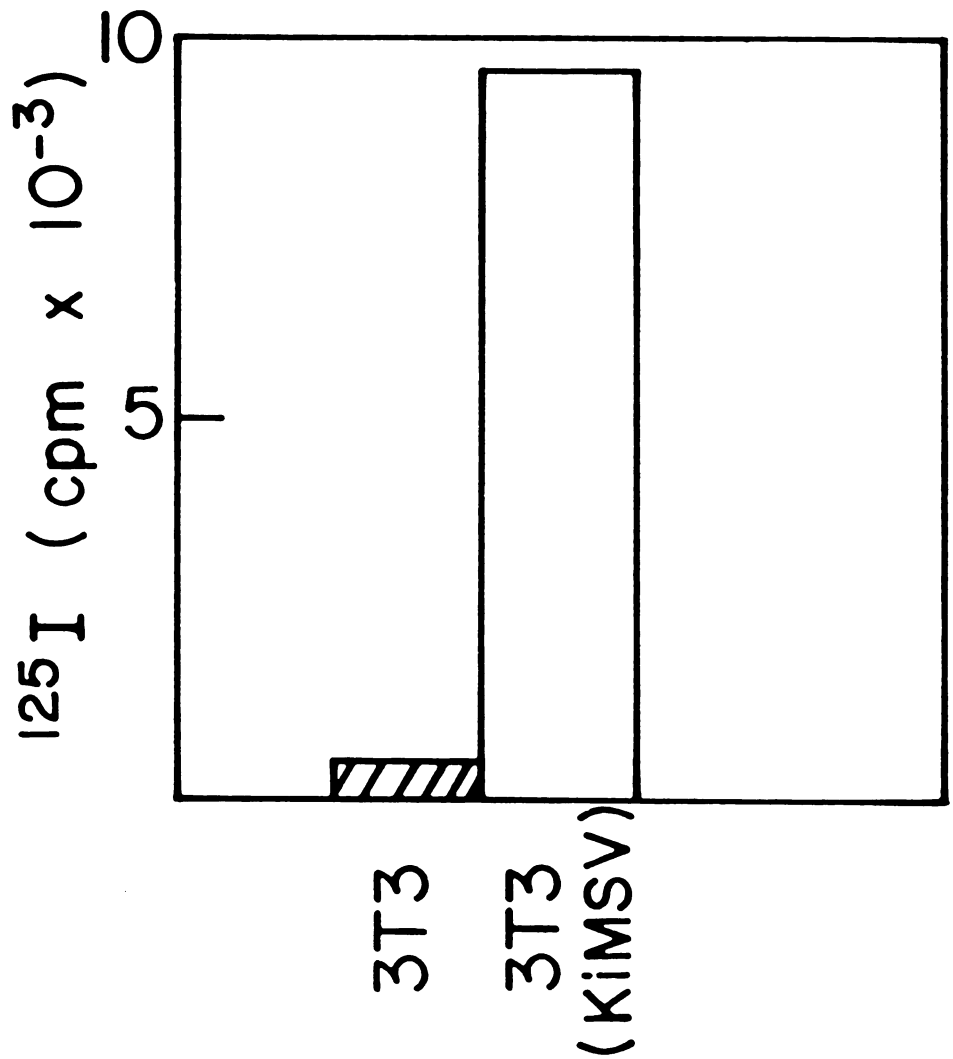

Figure 7

counterparts (Fig. 7). In sparse cultures, when both 3T3 cells and its virally-transformed counterpart are proliferating, there was also a 20 fold higher level of CBP35 in the transformed cells.

DISCUSSION

In the present communication we document that the expression and localization of the mammalian lectin CBP35 are closely correlated with the proliferative state of the cell. A higher level of the protein is expressed in proliferating cells and in this case the protein is primarily localized in the nucleus. When the cells cease to proliferate, due to either serum starvation or to density dependent inhibition of growth, the level of CBP35 becomes minimal and the protein is primarily cytoplasmic.

These results provide a basis for understanding several observations made in previous studies. First, we had noted that an apparently higher level of CBP35 was detected in the nucleus of 3T3 fibroblasts by indirect immunofluorescence than by analysis obtained after subcellular fractionation (14). We now understand that this is due, at least in part, to the fact that for subcellular fractionation studies, we had used high density (but not confluent) cultures in order to maximize the number of cells as starting material for the sucrose gradients. Second, we had also observed that the yields of CBP35 in our original isolation procedure from confluent monolayers of 3T3 cultures were rather low (16,17), compared to the level expected from an immunoblot analysis of extracts of 3T3 cells (8). Moreover, the level of radiolabeling with [^{35}S]methionine was considerably lower for CBP35 (16,17) than the labeling obtained for a growth regulatory factor (18) isolated from the same culture. These

observations can now be rationalized in terms of the fact that confluent monolayers of 3T3 cells contain a low level of CBP35.

Finally, the present results are also consistent with the results of immunoblotting analysis of CBP35 in various cell lines and several tissues of the mouse (8). On the basis of an equal amount of protein (100 ug) per cell type analyzed, there was more CBP35 in Kirsten murine sarcoma virus transformed 3T3 cells than in 3T3 fibroblasts. Similarly, there was more CBP35 detectable in Rous sarcoma virus transformed chicken embryo fibroblasts than the normal counterparts. Raz et al. (15) have identified two lectins (M_r 34,000 and 68,000) that are present in high amounts in tumor cells and have termed them tumor cell lectins. This may reflect elevated levels of expression of the normal cell gene product in the transformed cells. They have also found that most of the lectin(s) is inside the B16-F1 melanoma cell, as is the case for CBP35 in 3T3 fibroblasts (14).

More strikingly, there were drastic differences between embryonic and adult tissues when the presence of CBP35 was compared in the liver, muscle, and skin (8). Whereas the protein was abundant in the embryonic tissues, it was not found or hardly detectable in the adult. The present studies provide a more refined correlation between the expression of CBP35 and the proliferation state in a single cell line.

An additional aspect of this communication is that it presents the first use of the ACAS 470 Fluorescence Workstation to automatically examine large populations of fluorescently labeled cells to

derive cell histograms for antigen quantitation and distribution. When we examined the expression of CBP35 during the cell cycle in a synchronized population of 3T3 fibroblasts, we found that the expression of the lectin increased during the early part of the G_1 period. The level of CBP35, quantitated bulk wise by immunoblotting or quantitated on a single cell basis by immunofluorescence, was appreciable well before the onset of S phase. There was no apparent decrease in CBP35 expression upon entry into S phase. In contrast to the monotonic overall increase in CBP35 expression upon serum stimulation, there was a distinct rise and fall in the localization of CBP35 in the nucleus (and nucleolus) during the same time course. This may indicate that the nuclear localization of CBP35 is cell cycle dependent.

In this connection, it should be noted that the biochemical properties (M_r 35,000 and pI ~ 4.7), tissue distribution, and subcellular localization of CBP35 parallel those of another well-studied protein named cyclin or "proliferating cell nuclear antigen" (PCNA). Cyclin is a nuclear protein (M_r 35,000 and pI ~ 4.9) identified by its position in two-dimensional gel separation of cell proteins (1,2). It is transformation sensitive and is expressed at elevated levels in tumor cells (5). In synchronized cultures, the level and distribution of cyclin fluctuate through the cell cycle, with a striking accumulation in the nucleolus in late G_1 and early S phase (3,6). Many of these properties of cyclin are shared by PCNA. This antigen is defined by reaction with an antibody found in approximately 3% of patients with the autoimmune disease systemic lupus erythematosus

(13,20). Recently, Mathews et al. have established that cyclin and PCNA are indeed identical molecules (11). Still another protein with similar biochemical (M_r 33,000) and localization properties has been studied on the basis of recognition by a human autoantiserum. This protein, designated "perichromin," is present both on the nuclear envelope of interphase cells and the periphery of metaphase chromosomes (12).

Recently, we have also found that certain autoimmune sera will bind to CBP35.[2] On the basis of these properties and the similar molecular and distributional properties, CBP35, cyclin/PCNA, and perichromin may be related, if not identical molecules. If this turns out to be the case, the availability of an isolation procedure and the establishment of a biological activity, both based on the carbohydrate-binding property of CBP35, should facilitate future studies on the protein(s). Conversely, the detailed information accumulated on the nuclear localization of cyclin/PCNA and perichromin should also provide insights concerning the function of this and other endogenous lectins. In this connection, it is noteworthy that Feizi and co-workers have localized a bovine heart lectin (M_r 13,000, corresponding to CBP13.5) at the nucleus of cells using a polyclonal antibody on cryostat sections of tissues (7) and a monoclonal antibody on lymphoid cells (4). The level and pattern of expression of their lectin in lymphoid cells changed in association with transformation, or after stimulation with mitogens.

ACKNOWLEDGMENT

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Chapter IV

The Relationship of Carbohydrate Binding Protein 35 to the Proliferating Cell Nuclear Antigen/Cyclin

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ABSTRACT

Carbohydrate Binding Protein 35 (CBP35), a lectin purified on the basis of its binding to galactose containing glycoconjugates, and the "proliferating cell nuclear antigen" (PCNA, also known as cyclin), a protein defined by reaction with antibodies found in a small percentage of patients with the autoimmune disease systemic lupus erythematosus, share a number of similar if not identical properties: a) molecular weight (M_r ~35,000); b) isoelectric point (pI ~4.9); c) subcellular localization, including the nucleus; d) increased expression in proliferating cells; and e) tissue distribution. Despite these similarities, and the fact that patients with autoimmune diseases also produce antibodies against CBP35, rabbit antibodies prepared against CBP35 do not react with PCNA and conversely, human autoantibodies to PCNA do not react with CBP35. A side by side comparison of the two proteins indicated that they also differ in terms of their molecular weight, PCNA exhibiting a M_r=37,000 compared to a M_r=35,000 for CBP35. These data suggest that, although these proteins may belong to the same family of growth related proteins, they are distinct polypeptides.

INTRODUCTION

In previous studies, we had described the purification of three galactose specific carbohydrate binding proteins (CBPs) from cultured 3T3 fibroblasts (1) and mouse lungs (2). The molecular weight of these lectins were 35,000, 16,000, and 13,500. In two dimensional gel electrophoresis, CBP35 yielded two spots that had the same molecular weight (M_r 35,000) but different isoelectric points (pI 4.5 and 4.7). Using a highly specific rabbit antiserum directed against CBP35, we had shown by immunoblotting procedure that CBP35 was found in a number of mouse tissues and that the protein was developmentally regulated in the liver, muscle and skin. CBP35 was detected in these embryonic tissues but was undetectable in the corresponding tissues of the adult (2). Analysis of the subcellular distribution in 3T3 fibroblasts localized the protein primarily in the cytoplasm and the nucleus (3). More recently we have found that a high level and a predominantly nuclear localization are associated with proliferating cells while the expression of CBP35 decreases and the protein is predominantly cytoplasmic in quiescent cells (4). The level of CBP35 is modulated during the cell cycle in synchronized cell populations.

These biochemical and distributional properties of CBP35 parallel those of another well studied protein named cyclin or "proliferating cell nuclear antigen" (PCNA). Cyclin is a nuclear protein identified by its position in

two-dimensional gel separation of cell proteins (5-7). It is transformation sensitive, being expressed at elevated levels in tumor cells. In synchronized cultures, the level and distribution of cyclin fluctuate through the cell cycle, with a striking accumulation in the nucleolus in late G_1 and early S phase (6,9,13). Many of these properties of cyclin are shared by PCNA which is defined by its reaction with an antibody found in approximately 3% of patients with the autoimmune disease systemic lupus erythematosus (SLE) (8,9). Recenty, Mathews et. al. have established that cyclin and PCNA are indeed identical molecules (10).

Table I summarizes a comparison of CBP35 and cyclin/PCNA in terms of their molecular and distributional properties. This information has prompted us to ask whether CBP35 and cyclin/PCNA might be related or similar proteins. In this report we present further evidence substantiating the similarity of CBP35 and cyclin/PCNA but we also present evidence indicating that the two proteins are distinct polypeptides.

TABLE I

COMPARISON OF CBP35 WITH CYCLIN (PCNA)

PROPERTIES	CARBOHYDRATE BINDING PROTEIN 35	CYCLIN/PCNA
M_r (SDS-PAGE)	35,000 (1)	35,000 (10)
Isoelectric point(s)	4.5,4.7 (1)	4.9 (10)
Biological Properties	Galactose specific lectin (1)	None known
Subcellular localization	Nucleus, cytoplasm plasma membrane (3)	Mainly nuclear, some cytoplasmic (5,6,9)
Expression regulation	Increased expression in proliferating and transformed cells; nucleolar accumulation during G_1 phase of cell cycle (2,4)	Increased expression in proliferating and transformed cells; nucleolar accumulation during G_1 phase of cell cycle (6-9)
Immunological probes	Rabbit antiserum (1)	3% of SLE patient sera (9,10)
Species specificity	None (2)	None (8)
Tissue distribution	Expressed in mouse lungs, intestine, thymus and spleen; absent in adult liver and kidney (2)	Present in mouse intestine, thymus and spleen; absent from liver and kidney (8)

Numbers in parentheses indicate references cited at the end of the manuscript.

MATERIALS AND METHODS

SLE Sera Screening

Sera from patients exhibiting a variety of autoimmune diseases were obtained from the Laboratory of Clinical Immunology at Michigan State University. Thirty sera were initially chosen on the basis of their exhibiting a positive antinuclear antibody (ANA) reaction using an indirect immunofluorescent technique on mouse kidney substrate (Behring Diagnostics, San Diego, CA). Binding of patient sera to affinity purified mouse lung CBPs (2) was assayed in 96 well PVC Cooke microtiter plates following procedures previously described (11), with minor modifications. The plates were treated for 5 minutes at room temperature with 0.25% (v/v) glutaraldehyde in phosphate buffered saline (PBS) . The wells were then drained and blotted dry. 25 µl of protein solution containing 50 ng CBPs were added to each well and the plates were incubated at 37^{o} overnight. Remaining binding sites were blocked for 1 hour with 3% (w/v) bovine serum albumin (BSA) in PBS. The wells were incubated with 25 µl of 1:10 dilution of patient serum in PBS containing 5 mg/ml BSA for 2 hours at room temperature. After three washes with PBS, the binding of antibodies was detected by incubation with ^{125}I-Protein A (10^{5} cpm/well in PBS containing 5 mg/ml BSA and 0.1 M lactose) for 30 minutes at room temperature. The wells were washed five times with PBS containing 0.1% (v/v) Tween-20, blotted

dry, cut, and counted for radioactivity in a Riagamma LKB counter.

Immunoprecipitations and Immunoblotting

Immunoprecipitations were performed as previously described (12) except that protein A coupled to agarose beads (EY Laboratories, San Mateo, CA) was used as immunoabsorbent. The following sera were used in the immunoprecipitations: a) human sera from patients with autoimmune diseases, designated here as SLE patient sera; b) a specific human serum reactive with cyclin/PCNA (10), designated here as antiserum to cyclin/PCNA (this was a gift of Drs. M. Kostura and M. Mathews of Cold Spring Harbor Laboratory, Cold Spring Harbor, New York); and c) a rabbit antiserum against CBP35 (rabbit anti-CBP35). The production and characterization of rabbit anti-CBP35 have been previously described (1,2,3).

For immunoprecipitations using SLE patient sera, a mixture of affinity purified mouse lung CBPs (2) was used. The immunoprecipitate was solubilized and subjected to SDS-PAGE; the presence of CBP35 among the immunoprecipitated proteins was revealed by immunoblotting with rabbit anti-CBP35. The procedure for immunoblotting has been described previously (2,3). For a comparison of the proteins precipitated by anti-cyclin and rabbit anti-CBP35, extracts from 3T3 cells metabolically labeled for 24 hours with [^{35}S]methionine (20 μCi/ml, Amersham, Arlington Heights,

IL) were used. Extracts containing 8 X 10^6 cpm were subjected to immunoprecipitation, SDS-PAGE (12.5% polyacrylamide gels), and fluorography to reveal the radioactive polypeptides precipitated by the respective sera.

Cell Culture and Synchronization

The conditions for cell culture and synchronization have been described (4). Inhibition of DNA synthesis by hydroxyurea was performed essentially as described by Bravo and Macdonald-Bravo (13). Briefly, cells were seeded on glass coverslips (22 X 22 mm), or in 96-well plates in Dulbecco's modified Eagle's medium (DME) (K.C. Biological Inc. Lenexa, KS) containing 10% calf serum (Microbiological Associates, Walkersville, MD) at a density of 3 X 10^4 cells/cm^2 for 48 hours. The medium was changed to DME containing 0.2% calf serum and the cells were used two days later. Cells were stimulated by the addition of DME containing 10% calf serum and hydroxyurea (Sigma, St. Louis, MO) was added 8 hours after stimulation to a final concentration of 1 mM. Parallel cultures were assayed for CBP35 localization by immunofluorescence and DNA synthesis by the incorporation of [^{3}H]thymidine (3,11) every four hours. To remove hydroxyurea, cells were washed three times with DME and the medium was replaced with DME containing 10% calf serum.

Immunofluorescence

The details of labeling of fixed and permeabilized 3T3 cells for immunofluorescence have been described in detail (3,4). Immunofluorescent slides were observed using a Leitz microscope equipped with epifluorescence optics and a 50X water immersion objective. Photographs were taken using Kodak TriX-Pan film at 3200 ASA.

RESULTS

Inhibition of DNA Synthesis in 3T3 Cells by Hydroxyurea

We have previously noted that rabbit anti-CBP35 stains the nucleus and the cytoplasm of 3T3 cells (3). When synchronized populations of 3T3 cells were stained with anti-CBP35 we observed that CBP35 accumulated at the nucleus, and more specifically the nucleoli of the cells, approximately 4-5 hours prior to the S phase (4). This observation prompted us to examine whether the nucleolar localization of CBP35 depends on the events that lead to the initiation of DNA synthesis. Subconfluent populations of 3T3 cells were arrested at G_0 by serum starvation for 48 hours. The quiescent cells were then stimulated with 10% calf serum, and hydroxyurea (1 mM) was added 8 hours after stimulation. Two parallel assays were carried out: a) localization of CBP35 was examined by indirect immunofluorescence every 4 hours; and b) DNA synthesis was monitored every four hours by pulse labeling with [^{3}H]thymidine for 1 hour.

In the absence of hydroxyurea, DNA synthesis in the synchronized cultures began 20 hours after serum stimulation. The addition of hydroxyurea completely inhibited the incorporation of [^{3}H]thymidine (Figure 1a). However, the cells tranversed through the G_1 in the presence of hydroxyurea since upon removal of the drug the cells rapidly moved into the S phase, as shown in Figure 1b.

FIGURE 1 DNA synthesis of quiescent 3T3 cells (a) after serum stimulation in the absence and presence of 1 mM hydroxyurea and (b) after removal of hydroxyurea inhibition. (a):Quiescent cells were stimulated with 10% serum and DNA synthesis was monitored by [^{3}H]thymidine incorporation every four hours (●—●). 8 hours after stimulation the medium was replaced with DME containing 10% serum and 1 mM hydroxyurea. DNA synthesis was monitored as described above at intervals of four hours (□—□). The arrow indicates the time of addition of hydroxyurea. (b): 14 hours after hydroxyurea addition the drug was removed by washing the cells with DME, and the cells were incubated at 37^{o} with fresh medium not containing hydroxyurea. At two hour intervals thereafter, DNA synthesis was monitored by [^{3}H]thymidine incorporation.

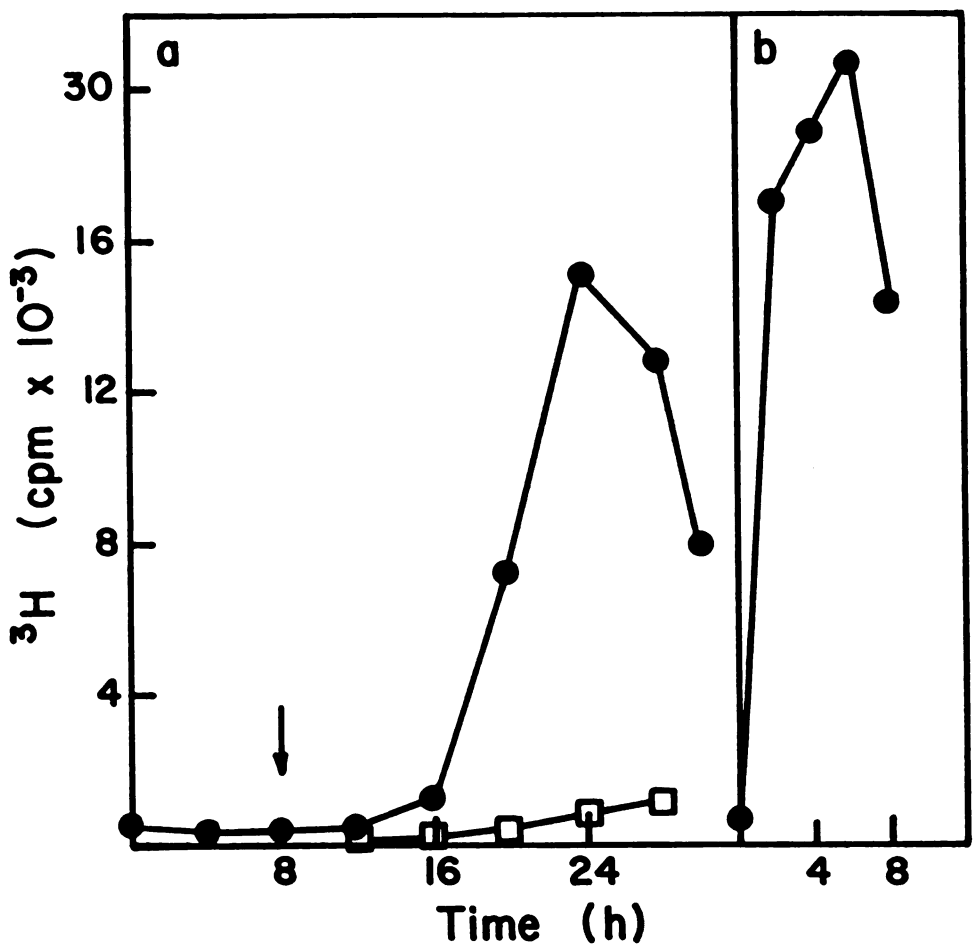

Figure 1

These results are consistent with the known effects of hydroxyurea on arresting cells at the G_1/S boundary (14). Moreover, at the concentration of the drug used (1 mM) the effects of hydroxyurea are reversible.

Inhibition by Hydroxyurea of Nucleolar Localization of CBP35

When the immunofluorescent patterns of the nuclear distribution of CBP35 in the absence of hydroxyurea were examined, we observed a sequence of patterns as depicted in Figure 2. In the early stages of the stimulation of arrested cells (<8 hours), the majority of the labeled cells show a diffuse or granular pattern of nuclear staining (Figure 2A). As we have previously documented, an increase in the nucleolar staining is observed next (4). This nucleolar staining reaches a maximum approximately 16 hours after stimulation and before the onset of maximum DNA synthesis. In the present experiment we observed two overlapping patterns of nucleolar staining. The one involves nucleolar staining with simultaneous ring-like staining of the nuclear envelope (Figure 2B) and the other nucleolar staining within a diffusely stained nucleus. (Figure 2C). Invariably, both types of staining are detected in the same population suggesting that the one succeeds the other. However, we have been unable to determine the exact sequence of these two patterns. The percent of cells exhibiting CBP35 nucleolar staining decreases as the cells progress through the S phase

(4) and the staining pattern returns to a diffuse or granular pattern (Figure 2D).

After 8 hours of hydroxyurea treatment (16 hours after stimulation) the cells still exhibit the characteristic pattern of the early stimulation period. Diffuse granular staining of the nucleus is observed (Figure 2E) while the majority of the untreated cells are now exhibiting nucleolar staining (Compare Figure 2B,C with 2E). In the presence of hydroxyurea this diffuse staining pattern remains unchanged even after 20 hours of serum stimulation (Figure 2F). Thus, it appears that hydroxyurea, besides inhibiting DNA synthesis, has also inhibited the accumulation of CBP35 in the nucleoli. It should be noted that in the presence of hydroxyurea, nucleoli are not generally present, and therefore the absence of CBP35 from such structures is not the result of the exclusion of CBP35 from the nucleolar structure but rather the result of the absence of nucleoli.

Removal of hydroxyurea resulted in the quick redistribution of CBP35 into distinct nucleolar structures. This redistribution is evident as early as 2 hours after hydroxyurea removal (Figure 2G). Coupled with the rapid onset of DNA synthesis after hydroxyurea removal (Figure 1b) these data suggest that the events in preparation of DNA synthesis are necessary for the formation of nucleolar structures containing CBP35. A comparison of DNA synthesis in the absence of hydroxyurea and after removal of the chemical agent (Figures 1a and 1b) suggests that the cells

FIGURE 2 Patterns of the nuclear distribution of CBP35 in synchronized 3T3 cells in the absence (A,B,C,D) and presence (E, F) of 1 mM hydroxyurea, and after release from the hydroxyurea inhibition (G,H). Quiescent 3T3 cells grown on glass coverslips were stimulated with 10% serum and 8 hours later 1 mM hydroxyurea was added to the medium. At 4 hour intervals coverslips from hydroxyurea treated and control cultures were removed and processed for immunofluorescent staining with rabbit anti-CBP35 as described in the Materials and Methods. Characteristic nuclear CBP35 staining at different times after serum stimulation is shown at: t=8 (A); t=12 (B); t=16 (C); and t=20 (D). Characteristic nuclear CBP35 staining in the presence of hydroxyurea is shown at t=12 (E) and t=16 (F). After hydroxyurea release the cells rapidly demonstrated nucleolar staining (G ; 2 hours after hydroxyurea release). 8 hours after hydroxyurea release the cells demonstrate diffuse/granular nuclear staining (H).

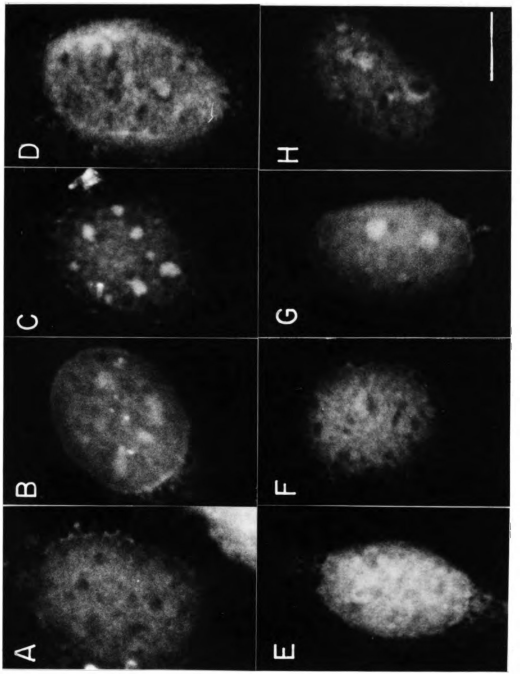

Figure 2

are at approximately the same point of the S phase at 20 hours after serum stimulation in the absence of hydroxyurea and during the first 2 hours after hydroxyurea removal. This is also reflected in the nucleolar accumulation of CBP35. Twenty hours after serum stimulation, less than 40% of the cells show nucleolar staining, a level similar to that observed in cells 2 hours after hydroxyurea removal. The nucleolar staining declines quite rapidly as the cells traverse the S phase after hydroxyurea removal suggesting that it is only a brief state associated with late G_1 and early S phase.

Reaction of CBP35 with Certain Autoimmune Sera

The detailed sequence of the translocation of CBP35 to the nucleus, the formation of the nucleolar structures and DNA synthesis, as well as the effects of hydroxyurea, indicated that CBP35 behaves similarly to cyclin/PCNA (6,13). Because cyclin/PCNA reacts with antibodies found in 3% of patients with the autoimmune disease SLE, we wanted to further examine the relationship of these proteins by examining whether sera from patients with SLE and a variety of other autoimmune diseases react with CBP35.

Thirty sera were initially chosen on the basis of their exhibiting a positive antinuclear antibody reaction using an indirect immunofluorescence technique on mouse kidney substrate. A radioimmune binding assay (11) was used to test for reaction of these sera with affinity purified mouse lung

CBPs (a mixture of CBP35, CBP16, and CBP13.5). Representative results from this screening assay are shown in Fig. 3A. In this assay, rabbit anti-CBP35 showed a strong positive reaction, relative to normal rabbit serum, with the antigen mixture containing CBP35 (Fig. 3A, lanes 8, and 9). Approximately 25% of the patient sera tested also yielded positive reactions (Fig. 3A, lanes 4-6), relative to a normal human control serum (Fig. 3A, lane 1). These positive sera showed 1.6-2.5 fold higher binding. The remaining sera showed no reaction (Fig. 3A, lanes 2, and 3). These results prompted us to identify the CBP reactive with the sera yielding positive reactions in the screening assay.

Because all of the sera available to us failed to immunoblot the CBPs after SDS gel electrophoresis and transfer to nitrocellulose filters (due, most probably, to their inability to recognize denatured antigens), an alternative approach was attempted. A native mixture of partially purified CBPs (2) was subjected to immunoprecipitation using patient or control sera and rabbit anti-CBP35 in parallel experiments. The immunoprecipitates were analyzed for the presence of CBP35 by SDS-PAGE and immunoblotting with rabbit anti-CBP35. Patient sera that reacted positively in the radioimmune binding assay (Fig. 3A, lane 4-6) also precipitated CBP35 (Fig. 3B, lanes 4-6). Authentic CBP35, purified from mouse lungs (2), was immunoblotted in lane 7 (Fig. 3B) to indicate the position of migration of the lectin. In this assay, rabbit anti-CBP35

FIGURE 3 Binding of antibodies from patients with autoimmune diseases to immobilized CBPs (A), and immunoprecipitation of CBP35 using human autoimmune sera (B). Mouse lung CBPs (50 ng/well) were immobilized in 96-well microtiter plates and reacted with a 1:10 dilution of different SLE patient sera. Binding of human antibodies was detected using ^{125}I labeled protein A. The results shown represent the average and standard deviation of triplicate samples. Immunoprecipitations were performed as described in the Materials and Methods. The immunoprecipitates were analyzed by SDS-PAGE on a 12.5% mini-gel, and immunoblotted with rabbit anti-CBP35 serum. Lane (1) Normal human serum; lanes (2, and 3) SLE sera not reacting with CBPs; lanes (4,5,6) SLE sera reactive with CBPs; lane (7) affinity purified CBP35; lane (8) rabbit anti-CBP35 serum; lane (9) normal rabbit serum.

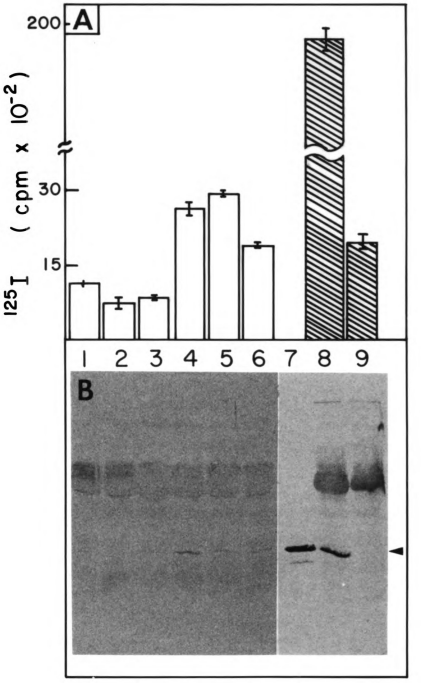

Figure 3

precipitated the same protein from the partially purified mixture of CBPs (Fig. 3B, lane 8). Normal human serum (Fig. 3B, lane 1), patient sera which were negative in the radioimmune binding assay (Fig. 3B, lanes 2 and 3), and normal rabbit serum (Fig. 3B, lane 9) all failed to precipitate CBP35.

These data provide strong evidence that a certain percentage of patients who exhibit positive ANA reaction produce autoantibodies directed against CBP35.

CBP35 and Cyclin/PCNA are Antigenically Distinct

The above data, in conjuction with the data presented in Table I, indicated a strong similarity between CBP35 and cyclin/PCNA. However, we felt it was important to be able to compare the two proteins in a side by side test if a conclusive statement concerning their identity were to be made. We obtained a human antiserum from an SLE patient that had been previously used to confirm the identity of PCNA and cyclin (10). This antiserum immunoprecipitates cyclin/PCNA from extracts of HeLa cells.

^{35}S-labeled extracts of 3T3 cells were immunoprecipitated in parallel experiments with rabbit anti-CBP35 and antiserum to cyclin/PCNA. The immunoprecipitated material was subjected to SDS-PAGE and fluorographic analysis. The results are shown in Figure 4. Antiserum to CBP35 immunoprecipitated a M_r 35,000 protein (Fig. 4, lane a), comigrating with an authentic sample of

FIGURE 4 Immunoprecipitations of ^{35}S labeled 3T3 extracts with rabbit anti-CBP35 and anti-cyclin antibodies. 3T3 extracts were prepared in pH 7.5 buffer containing 150 mM Nacl, 5 mM EDTA, 50 mM Tris, and 0.5% NP-40. 150 l of this extract containing $8X10^6$ cpm were immunoprecipitated as described in the Materials and Methods using 10μl of the corresponding serum. The immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography. The region of interest is shown. Lane (a): rabbit anti-CBP35 immunoprecipitated proteins; lane (b) anti-cyclin immunoprecipitated proteins. The arrowhead indicates the position of migration of an authentic CBP35 sample isolated from mouse lungs. Following the initial immunoprecipitation proteins remaining in the supernatant were immunoprecipitated with the complementary antiserum. Lane (c): rabbit anti-CBP35 immunoprecipitated proteins from anti-cyclin preabsorbed 3T3 extract; Lane (d) anti-cyclin immunoprecipitated proteins from rabbit anti-CBP35 preabsorbed 3T3 extract.

Figure 4

CBP35 from mouse lungs (position indicated by an arrowhead). The antiserum to cyclin/PCNA immunoprecipitated a ^{35}S labeled polypeptide migrating with a M_r 37,000 in our gel system (Figure 4, lane b).

The supernatants of the immunoprecipitates shown in lanes a and b were then subjected to a second immunoprecipitation using the complementary antiserum. The supernatant from the immunoprecipitation with the anti-cyclin/PCNA immunoprecipitation was immunoprecipitated with anti-CBP35, and the supernatant from the anti-CBP35 immunoprecipitation was immunoprecipitated with antiserum to cyclin/PCNA.

The results, shown in Figure 4 lanes c and d, again indicated that the anti-CBP35 antiserum only precipitates the M_r 35,000 polypeptide (Fig. 4, lane c), while antiserum to cyclin/PCNA only precipitates the M_r 37,000 polypeptide (presumably cyclin/PCNA, Fig. 4, lane d). The antisera did not show any sign of cross-reaction indicating that the two proteins, CBP35 and cyclin/PCNA, do not share any antigenic determinants recognized by the available antisera.

DISCUSSION

CBP35 and cyclin/PCNA have been shown to share a variety of properties (Table I) . Both proteins are acidic polypeptides of similar molecular weight (approximately 35,000) (1,10); they have similar subcellular distribution, as detected by immunofluorescence (3,5,6,9), and similar tissue expression (2,8). We have recently observed that the subcellular distribution and levels of expression of CBP35 are correlated with the proliferative state of the cell (4). Proliferating cells contain high levels of CBP35 and show a predominant nuclear localization, while quiescent cells contain lower levels of the lectin which is then predominantly localized in the cytoplasm. Transformed cells also have been shown to contain higher levels of CBP35 than their normal counterparts (4). An apparent association with growth and transformation has also been demonstrated for the nuclear protein cyclin/PCNA (7-9). In addition, the localization of this protein varies during the cell cycle, with a striking accumulation in the nucleolus in late G_1 and early S phase (6,9,13).

We have also noted a nucleolar accumulation of CBP35 before the onset of DNA synthesis (4), and we have now demonstrated that this nucleolar accumulation depends on the events immediately preceding DNA synthesis. Cells whose DNA replication is blocked by hydroxyurea do not show nucleolar accumulation of CBP35. This can be reversed by the removal

of the chemical agent. In this case, nucleolar accumulation is evident again before DNA synthesis resumes . This result is identical to that obtained by Bravo and Mcdonald-Bravo who have demonstrated that changes in the nuclear distribution of cyclin, but not its synthesis, depend on DNA replication (13).

Our data provide evidence that a certain percentage of patients who exhibit positive ANA reaction produce autoantibodies to CBP35. Of the five patients that demonstrated reactivity with CBP35, two are SLE patients but the remaining three are patients, with autoimmune diseases of unknown origin, who are not classified as lupus patients. The clinical significance of this finding is presently unknown. PCNA has been previously identified solely on the basis of reactivity with serum from patients with SLE using a radial immunodiffusion technique (8). The greater sensitivity of the radioimmunoassay described here may account for the broader spectrum of reactivity in the sera of these patients.

On a side by side comparison of CBP35 and cyclin/PCNA, using antisera against each protein, we have demonstrated that the two proteins are antigenically distinct. Antiserum to CBP35 does not immunoprecipitate PCNA and conversely, a human autoantiserum reactive with cyclin/PCNA does not react with CBP35. In the course of these studies, we have also demonstrated that cyclin/PCNA exhibits a higher molecular weight than CBP35 (37,000 as opposed to 35,000 for CBP35) on

our gel system. Identical results have also been obtained by Dr. Kostura in immunoprecipitation assays using extracts from HeLa cells and the same set of specific antisera as described here (personal communication).

There are several other observations arguing against the identity of CBP35 and cyclin/PCNA. These include: a) the anti-CBP35 serum as it has been shown previously (2,3) recognizes only a M_r 35,000 protein in a variety of tissue and cell extracts. Cyclin exhibits a M_r of 37,000 in our gel system and this difference is easily detectable as it shown in Fig. 4. If CBP35 and cyclin were structurally related a M_r 37,000 would also be detected, but clearly this is not the case; b) extracts from HeLa cells that have been enriched for cyclin/PCNA do not contain any CBP35 as determined in an immunoblotting assay; c) CBPs purified by affinity chromatography on an asialofetuin column do not contain any cyclin/PCNA as determined by two dimensional gel analysis (Moutsatsos and Wang unpublished observations).

Despite their molecular non-identity, CBP35 and cyclin/PCNA share many properties that suggest they may belong to a common family of polypeptides, possibly involved in the regulation of DNA replication. The knowledge of a biochemical property for CBP35, the availability of an isolation procedure, and the availability of highly specific polyclonal antibodies for it make CBP35 an attractive molecule for studies concerning the endogenous function of this and other lectins.

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CLOSING STATEMENT

When this thesis project was initiated, CBP35 was a protein that had been purified to homogeneity, and whose specific binding to carbohydrates had been defined. There were two main approaches to explore the endogenous function of this lectin: (a) determination of the tissue and subcellular distribution of the protein; and (b) analysis of its chemical structure.

The results of my studies have defined in sufficient detail the expression and localization of CBP35 in 3T3 cells to suggest that this lectin may be involved in the mechanism of regulation of DNA synthesis. This hypothesis forms the basis for several specific ideas amenable to experimental analysis: (a) Microinjection of purified CBP35 and/or antibodies against CBP35 into cells at different proliferative states can be performed to examine the effects of the lectin on DNA synthesis and cell proliferation; (b) The effects of CBP35 and/or antibodies against CBP35 can also be studied <u>in vitro</u> if an appropriate cell free DNA replication system is available; and (c) The identification and isolation of an endogenous ligand to which CBP35 binds can now be focused to a nuclear component that may affect DNA replication.

Some initial progress has also been made on the second approach to the question of function. We have screened a λgt11 expression library of cDNAs derived from 3T3 cells. Several positive clones have been isolated (Fig. 1). That any of these clones represents a cDNA clone for CBP35 remains to be ascertained: (a) by hybrid selection of mRNA for cell free translation studies; (b) by filter hybridization with oligonucleotide probes synthesized on the basis of amino acid sequence information. The successful molecular cloning of CBP35 will allow us to study its regulation of expression at the transcriptional level and to analyze its primary structure. Such structural information, coupled with data derived from the localization approach, may provide important clues to the endogenous function of the protein.

Figure 1 (a) Specific binding (revealed by the horseradish peroxidase catalyzed reaction) of affinity purified anti-CBP35 antibodies to a λgt11 expression clone. A λgt11 expression library of mouse 3T3 fibroblast cDNA was screened using affinity purified antibodies to CBP35. A clone (clone 1), positive upon initial screening, was purified and amplified twice. (b) DNA from clone 1 was digested with the restriction endonuclease EcoR1 to excise the cDNA insert. The digest was analyzed on 1% agarose gels, and visualized by ethidium bromide staining. Lane 1, DNA digest from clone 1; Lane 2, Lambda DNA (Hind III digest) and PhiX174 DNA (Hae III digest) DNA size markers. The position of migration of the cDNA insert is indicated by an arrowhead, and is estimated to be approximately 900 base pairs.

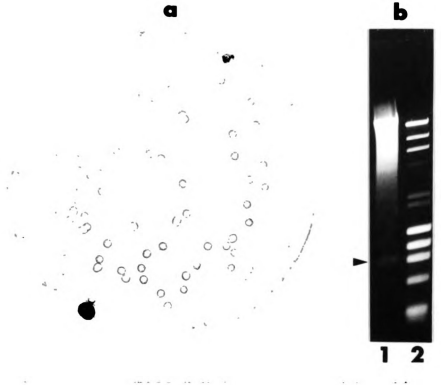

Figure 1