Endogenous Lectins from Cultured Cells: Subcellular Localization of Carbohydrate-binding Protein 35 in 3T3 Fibroblasts

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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: immunofluorescent staining of live 3T3 cells; agglutination of suspension of 3T3 fibroblasts by specific antibodies; and 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, and the plasma membrane fraction of the postnuclear supernatant.

In previous studies, we reported the isolation, from 3T3 mouse fibroblasts, of three carbohydrate binding proteins (CBPs)[1] that 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 analogues 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 that was not structurally related to the other two lectins of lower molecular weight.

Knowledge of 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 with 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).

[1] *Abbreviations used in this paper*: CBP, carbohydrate-binding protein: LDH, lactate dehydrogenase; TK, 0.02 M Tris-HCl, 5 mM KCl buffer (pH 7.2) containing 105 mU/ml aprotinin.

Materials and Methods

Cell Growth and Radiolabeling

The growth and maintenance of 3T3 fibroblasts has been previously described (33). Metabolic labeling of 3T3 cells with [^{35}S]methionine (Amersham Corp., Arlington Heights, IL) was performed according to Roff and Wang (31).

Cell surface iodinations were performed using the iodogen method (16, 26). The 3T3 fibroblasts were grown to confluence in 100-mm tissue culture dishes in Dulbecco's modified Eagle's medium containing 10% (vol/vol) calf serum. The growth medium was discarded, and the cell monolayer was washed three times with 5 ml phosphate-buffered saline (PBS). A coverslip coated with 100 μg iodogen was suspended face-down on each culture dish containing 5 ml of PBS. Na^{125}I (0.5 mCi, Amersham Corp.) 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 PBS and extracted with 1 ml of 5 mM phosphate buffer (pH 8.0) containing 0.5% (vol/vol) Triton X-100. Phenylmethylsulfonyl fluoride was added to the extract to a final concentration of 1 mM, and the insoluble material was removed by centrifugation at 3,000 *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 (31). 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 Affigel 10 (Bio-Rad Laboratories, Richmond, CA). The column was washed extensively with coupling buffer and PBS before use. The column was then equilibrated with 2% (vol/vol) calf serum in coupling buffer to minimize nonspecific 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 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% (vol/vol) 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 PAGE in the presence of 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% [vol/vol] Tween 20 and 0.05% [wt/vol] NaN_3), and the protein bands were visualized by India ink staining (0.2% [vol/vol] 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 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 was 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 Lightning Plus intensifying screen (DuPont Instruments, Wilmington, DE).

Immunoblots stained using horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin (Bio-Rad Laboratories) 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 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 2×10^6 cells/ml. Serum samples (0.2 ml 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% (wt/vol) bovine serum albumin and buffered with 20 mM Hepes to pH 7.5 (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 Laboratories Inc., Elkhart, IN) 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 50× water immersion objective. Pictures were taken using Kodak Tri-X film.

Fixed cells were prepared and permeabilized with 0.2% (vol/vol) 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% (vol/vol) normal goat serum for 1 h at room temperature. After three washes in PBS containing 3% (vol/vol) 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% (wt/vol) of the antibleaching agent *n*-propyl galate and viewed with a Leitz epiphase fluorescence microscope with a 50× 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 4×10^6 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 NADH diaphorase (1) have been described. For 5′-nucleotidase activity, 0.2% (wt/vol) 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 min 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 (31). This antiserum 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% wt/vol) and the extracts were electrophoresed and immunoblotted with rabbit anti-CBP35, only a single polypeptide band (M_r 35,000) was observed (Fig. 1, lane *a*). Parallel analysis with preimmune rabbit serum failed to yield this band (Fig. 1, lane *b*).

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, nondenatured, 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% [vol/vol] 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 PAGE. The material bound and eluted from the rabbit anti-CBP35 col-

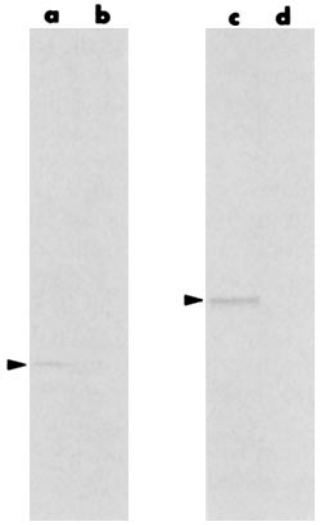

Figure 1. Immunoblots of SDS (4% wt/vol) extracts of 3T3 cells with rabbit anti-CBP35 and rabbit anti-LDH. Approximately 100 μg protein was electrophoresed on a 12.5% polyacrylamide gel and transferred to nitrocellulose paper as described in Materials and Methods. After incubation with 1:250 dilution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

umn yielded three major components (M_r's 35,000, 48,000 and 57,000) (Fig. 2, lane *b*). Two of these components (M_r's 48,000 and 57,000) were also found in the material from the control columns containing anti-succinyl concanavalin A (Fig. 2, lane *c*) or normal rabbit immunoglobulin (Fig. 2, lane *d*). In contrast, the M_r 35,000 band, which co-migrated with an authentic sample of CBP35 (Fig. 2, lane *a*), 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.

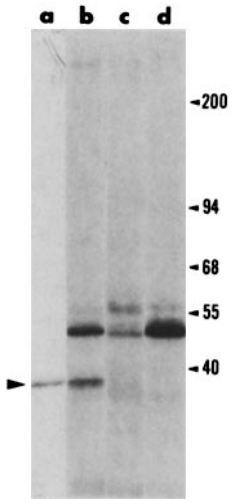

Figure 2. SDS PAGE of ^{35}S-labeled polypeptides bound on a column (1.5 × 2 cm) of Affigel 10 covalently derivatized with the immunoglobulin fraction of rabbit anti-CBP35. Triton X-100 extracts of 3T3 cells labeled with [^{35}S]methionine (1.2×10^7 total cpm per column) were fractionated as detailed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

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. 3, *A* and *C*), 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. 3*B*). Similar results were obtained when these experiments were carried out in the presence of lactose (0.1 M) (Fig. 3, *C* and *D*). Therefore, the antigenic target detected by immunofluorescence staining with rabbit anti-CBP35 was probably not 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.

This conclusion was corroborated by the observation that 3T3 cells can be agglutinated by rabbit anti-CBP35. The 3T3

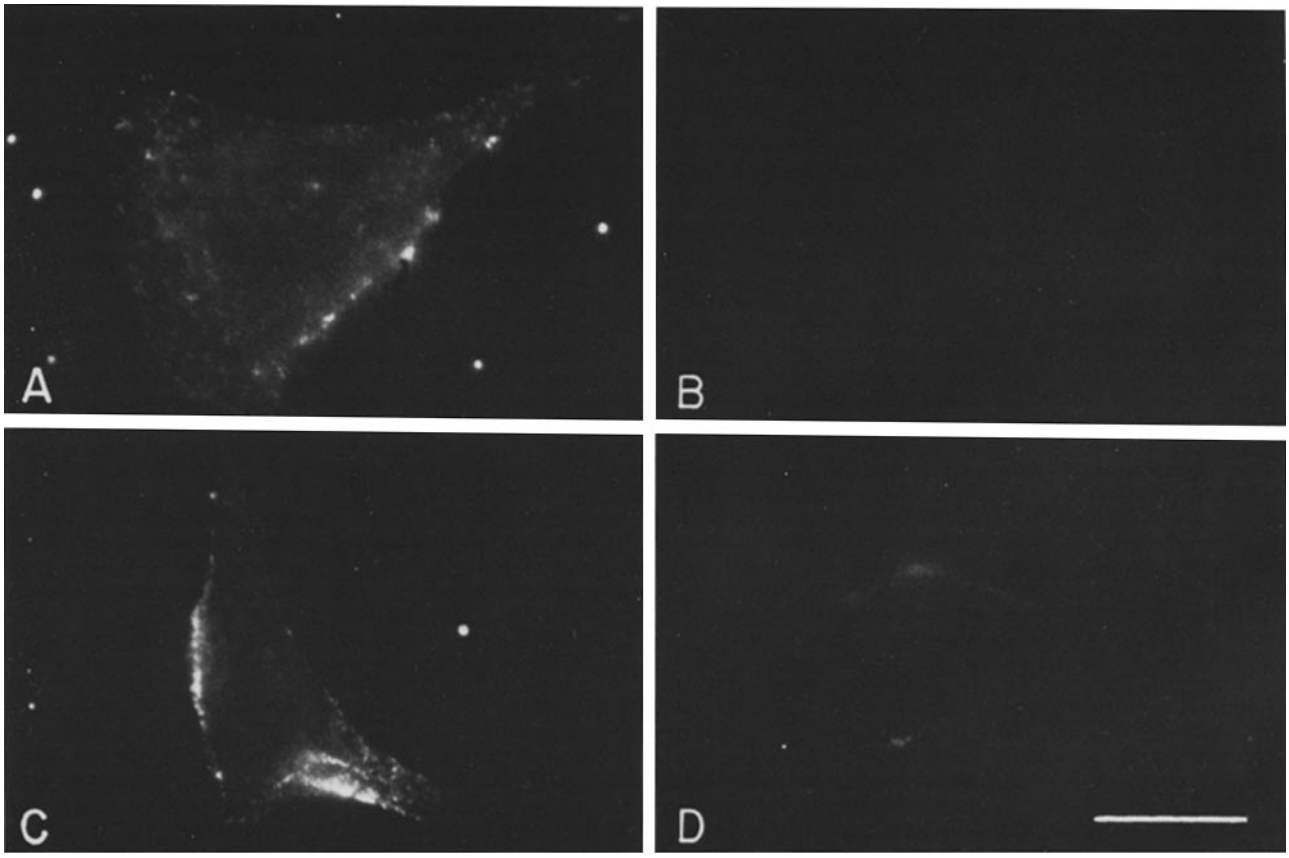

Figure 3. Indirect immunofluorescence detection of the binding of rabbit anti-CBP35 to the surface of live 3T3 fibroblasts. The binding of the rabbit antibody (4°C, 2 h) was detected by rhodamine-labeled goat anti-rabbit immunoglobulin (4°C, 30 min). The figure shows the pattern of fluorescence labeling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

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. 4*A*), as did concanavalin A (Fig. 4*C*). Normal rabbit serum (Fig. 4*B*) and PBS (Fig. 4*D*), 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 PAGE. The autoradiogram of the SDS gel showed that a protein of M_r 35,000 was bound on the anti-CBP35 column (Fig. 5, lane *a*) but not on the control column (Fig. 5, lane *b*), 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 nonspecific binding. These results 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. 2, lanes *b* and *c*). 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.

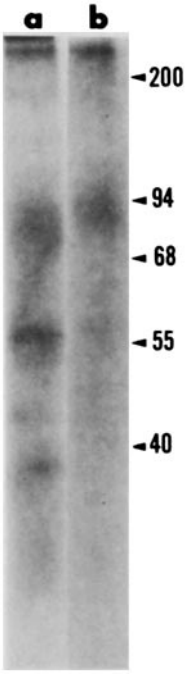

Figure 5. SDS PAGE of ^{125}I-labeled polypeptides bound on a column (1-ml bed volume) of Affigel 10 covalently derivatized with the immunoglobulin fraction of rabbit anti-CBP35. Cells were surface-labeled with ^{125}I and iodogen; Triton X-100 extracts were fractionated as detailed in Materials and Methods. The radioactive components bound and eluted were subjected to PAGE. Lane *a*, polypeptides bound on rabbit anti-CBP35 column; lane *b*, polypeptides bound on rabbit anti-succinyl concanavalin A column. Approximately 2,000 and 1,000 cpm were electrophoresed in lanes *a* and *b*, respectively; the fluorogram was exposed for 26 d. Molecular weight markers are indicated on the right.

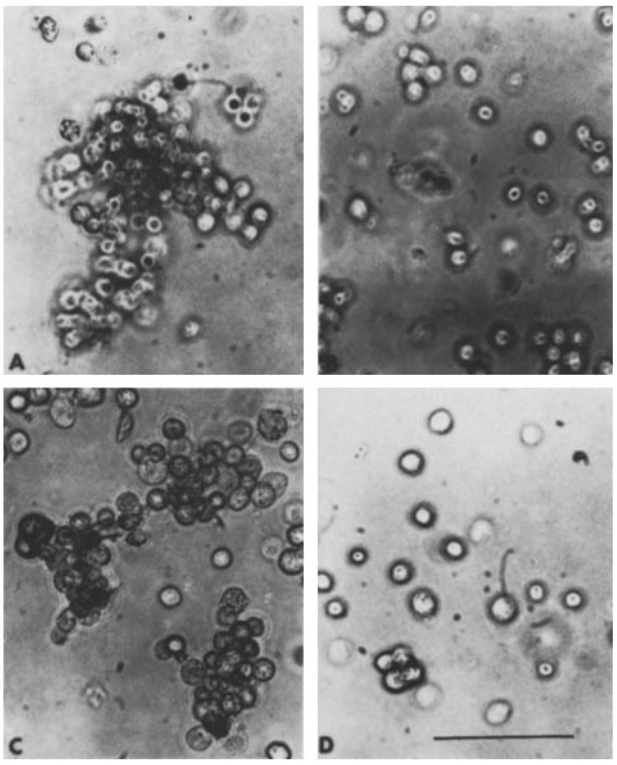

Figure 4. Agglutination of 3T3 fibroblasts in suspension by rabbit anti-CBP35. The final concentration of the cells in the assay was 1 × 10^6 cells/ml. (*A*) Rabbit anti-CBP35 serum; (*B*) normal rabbit serum; (*C*) concanavalin A (25 μg/ml); (*D*) PBS. Bar, 10 μm.

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) that become adsorbed to the cell surface. First, medium conditioned by exposure to 3T3 fibroblasts, analyzed by PAGE and immunoblotting, failed to yield an 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 CBP35 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 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. 1, lane *c*), migrating at a position that corresponded 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 rabbit anti-CBP35 showed intracellular labeling in all cells (Fig. 6*A*). 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, whereas 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. 6*A*). In contrast, staining with normal rabbit serum resulted in insignificant labeling (Fig. 6*B*).

In parallel experiments, 3T3 fibroblasts stained with rabbit anti-LDH showed a diffuse distribution of fluorescence within the cytoplasm of the cells (Fig. 6*C*). 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, probably 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. 6, *A* with *C*).

Quantitation of CBP35 in Subcellular Fractions

Homogenates were prepared from 3T3 cells swollen in hypotonic 0.02 M Tris-HCl, 5 mM KCl buffer (pH 7.2) containing 105 mU/ml aprotinin. 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 PAGE and immunoblotting with rabbit anti-CBP35 and ^{125}I-labeled goat anti-rabbit immunoglobulin (Table I and Fig. 7). After autoradiography, the intensity of the band corresponding to CBP35 was quantitated by densitometric scanning.

One important point should be noted in interpreting the immunoblot shown in Fig. 7. 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 most of the total cellular proteins and with 98% of the total LDH activity, a marker for soluble proteins of the

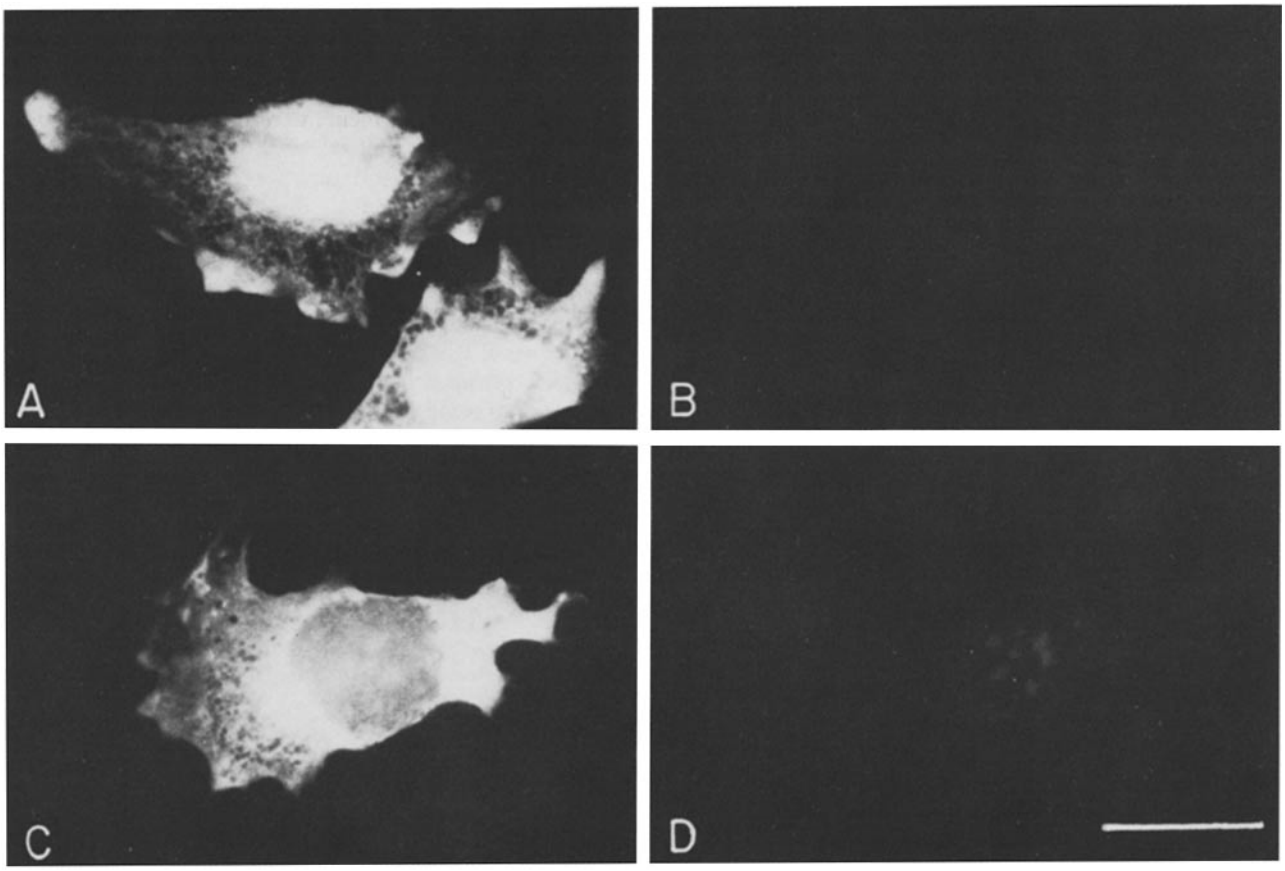

Figure 6. Immunofluorescence staining of 3T3 fibroblasts after fixation with formaldehyde (3.7%) and permeabilization with Triton X-100 (0.2%). (*A*) Rabbit anti-CBP35 (1:10 dilution of antiserum); (*B*) normal rabbit serum (1:10 dilution). (*C*) Rabbit anti-LDH (1:10 dilution); (*D*) preimmune serum 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.

Table I. 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

TK, 0.02 M Tris-HCl, 5 mM KCl buffer (pH 7.2) containing 105 mU/ml aprotinin.
* Lanes *a* through *e* correspond to the immunoblot autoradiogram shown in Fig. 7.

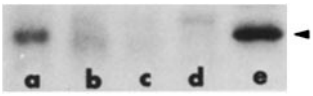

Figure 7. Immunoblot autoradiogram. Various subcellular fractions (100 μg) were subjected to PAGE, transferred to nitrocellulose paper, and blotted with rabbit anti-CBP35 (1:250 dilution of antiserum) followed by ^{125}I-labeled goat anti-rabbit immunoglobulin (10^6 cpm). The arrowhead indicates the position of migration of CBP35.

cell (Table I). There was, 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 ~5% of the CBP35. These results are consistent with the intracellular immunofluorescence staining patterns (Fig. 6*A*), localizing CBP35 within the cytoplasm and on the nucleus.

The particulate fraction P150, containing large fragments of plasma membrane, intracellular membranes such as the endoplasmic reticulum and the Golgi complex, 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.

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-D substrate for tyrosine phosphorylation in chicken embryo fibroblasts transformed by avian sarcoma viruses. Thus, ~90% of the total LDH activity was associated with the S150-soluble fraction, whereas 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 as 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 a 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 microscopic (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 CBP35 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 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 most 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 cannot wash the lectin off the membrane in quantities detectable by immunoblotting procedures (1–5 ng [13]) argue 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).

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