Journal of Cellular Biochemistry 84:408–419 (2002)

# The Cellular Prion Protein: A New Partner of the Lectin CBP70 in the Nucleus of NB4 Human Promyelocytic Leukemia Cells

**C. Rybner,[1] S. Finel-Szermanski,[1] M. Felin,[1] T. Sahraoui,[1,2] C. Rousseau,[1] J.-G. Fournier,[3] A.-P. Sève,[1] and J. Botti[1]***

[1]INSERM U-496, Institut Universitaire d'Hématologie (IUH), Hôpital Saint-Louis, 1 Avenue Claude Vellefaux, 75475 Paris, Cedex 10, France
[2]Université Es-Sénia, Faculté des Sciences, Département de Biologie, BP 16, Oran 31100, Algérie
[3]Service de Neurovirologie, CEA-DSV-DRM, 60-68 Avenue du Général Leclerc, BP 6, 92265 Fontenay-aux-Roses, Cedex, France

**Abstract** Prion diseases are characterized by the presence of an abnormal isoform of the cellular prion protein (PrPc) whose physiological role still remains elusive. To better understand the function of PrPc, it is important to identify the different subcellular localization(s) of the protein and the different partners with which it might be associated. In this context, the PrPc–lectins interactions are investigated because PrPc is a sialoglycoprotein which can react with lectins which are carbohydrate-binding proteins. We have previously characterized a nuclear lectin CBP70 able to recognize *N*-acetyl-β-D-glucosamine residues in HL60 cells. Using confocal immunofluorescence, flow-cytofluorometry, and Western-blotting, we have found that PrPc is expressed in the nucleus of the NB4 human promyelocytic leukemia cell line. It was also found that the lectin CBP70 is localized in NB4 cell nuclei. Moreover, several approaches revealed that PrPc and CBP70 are colocalized in the nucleus. Immunoprecipitation experiments showed that these proteins are coprecipitated and interact via a sugar-dependent binding moiety. In conclusion, PrPc and CBP70 are colocalized in the nuclear compartment of NB4 cells and this interaction may be important to better understand the biological function and possibly the conversion process of PrPc into its pathological form (PrPsc). J. Cell. Biochem. 84: 408–419, 2002.


**Key words:** cellular prion protein; nucleus; hematopoietic cells; lectin

Prion protein (PrP) is an ubiquitous sialoglycoprotein synthesized in several tissues including the central nervous system (CNS). In vivo, cellular prion protein (PrPc) has been detected at synaptic sites in the CNS [Fournier et al., 1995], at the neuromuscular junction [Gohel et al., 1999], and in different extracerebral tissues such as heart, kidney [Bendheim et al.,

Abbreviations used: Ab, antibody/ies; BSA, bovine serum albumin; CBP, carbohydrate-binding protein(s); CNS, central nervous system; DAPI, 4′,6-diamino-2-phenylindole dihydrochloride; ECL, enhanced chemiluminescence; FITC, fluorescein isothiocyanate; GlcNAc, *N*-acetyl-β-D-glucosamine; Hsp, heat shock protein; Ig, immunoglobulin(s); NLS, nuclear localization signal; PBS, phosphate-buffered saline with (+) or without (–) $Ca^{2+}$ and $Mg^{2+}$; PMSF, phenylmethylsulfonylfluoride; PrP, prion protein; PrPc, cellular prion protein; PrPsc: scrapie prion protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin.

C. Rybner and S. Finel-Szermanski contributed equally to this work and are both considered as first authors.

Our colleague and friend Dr. Annie-Pierre Sève passed away (January 7, 2000). We wish here to bear testimony to her invaluable scientific contribution in initiating this project.

Grant sponsor: INSERM; Grant sponsor: The Association pour la Recherche contre le Cancer; Grant number: ARC 9884; Grant sponsor: The Ministère de l'Enseignement Supérieur, de la Recherche Scientifique et Technique (joint project with INRA and ExonHit Therapeutics); Grant sponsor: The INTAS; Grant sponsor: The Fondation pour la Recherche Médicale; Grant sponsor: The Coopération Franco-Algérienne; Grant number: ALG4B207B.

*Correspondence to: Dr. J. Botti, INSERM U-496, Institut Universitaire d'Hématologie (IUH), Hôpital Saint-Louis, 1 Avenue Claude Vellefaux, 75475 Paris, Cedex 10, France. E-mail: botti@vjf.inserm.fr

Received 16 July 2001; Accepted 20 August 2001


DOI 10.1002/jcb.10017

1992; Fournier et al., 1998], and in blood cells [MacGregor et al., 1999]. This sialoglycoprotein is localized at the plasma membrane [Caughey et al., 1989; Borchelt et al., 1990; Stahl et al., 1990; Caughey and Raymond, 1991] and also in the cytoplasmic and nuclear compartments of the cell [Taraboulos et al., 1990; Pfeifer et al., 1993; Schröder et al., 1994; Fournier et al., 2000]. Two isoforms of this protein are found: a normal cellular form (PrPc) and a pathological scrapie form (PrPsc) which is derived from PrPc by a post-translational, conformational modification [Caughey and Raymond, 1991]. This PrPsc altered form is considered to be the infectious agent responsible for the transmission of the human spongiform encephalopathies including Kuru, Creutzfeldt-Jakob disease, Gerstmann-Straüssler-Scheinker disease, and also the animal scrapie and bovine spongiform encephalopathies.

PrPc interacts with several proteins according to its subcellular localization(s). For example, the 37 kDa laminin-receptor precursor was found to interact with PrPc at the plasma membrane [Rieger et al., 1997]. It was hypothesized that this precursor protein could act as a receptor, or co-receptor, for PrPc in mammalian cells. PrPc also interacts with molecular chaperones of the Hsp60 family [Edenhofer et al., 1996]. The physiological relevance of this interaction is not yet known but it was postulated that Hsp60 might be involved in the propagation of PrPsc, for example in blocking the conversion of PrPc to PrPsc or by preventing advanced glycation of PrPc. In the yeast two-hybrid system, PrPc also seems to bind to the anti-apoptotic protein Bcl-2 [Kurschner and Morgan, 1995]. Furthermore, the β-galactoside-binding protein galectin-3, a plurilocalized lectin, is among the proteins that bind to PrPc protein for the regulation of PrP mRNA [Schröder et al., 1994].

Lectins are carbohydrate-binding proteins (CBP) which interact reversibly with specific sugar residue(s) of glycoproteins. In addition, the potential information encoded by different carbohydrate structures is higher than possibilities offered by the genetic code. These proteins play an important part as partners in the regulation of various physiological activities [Hubert et al., 1989]. Most of these proteins are plurifunctional, with the different functions being determined by their different subcellular localization(s) and the interacting partner(s). For example, the β-galactoside-binding protein galectin-3 is implicated: i) in the adhesion of the cells to the extracellular matrix by interacting with laminin [Woo et al., 1990; Kuwabara and Liu, 1996], ii) in apoptosis through its interaction with Bcl-2 [Yang et al., 1996; Akahani et al., 1997], and iii) in splicing [Dagher et al., 1995]. Several years ago, our group co-isolated, with galectin-3, another lectin called CBP70 from HL60 cell nuclei [Sève et al., 1993]. CBP70 is an *N*-acetyl-β-D-glucosamine (GlcNAc)-binding protein [Felin et al., 1994]. These two lectins are complexed in the cell nucleus via protein–protein interactions which are disrupted when each lectin interacts with its specific sugar [Sève et al., 1993, 1994].

In order to elucidate the possible role(s) of PrPc in cells, it is important to determine its different subcellular localization(s) and its putative partners. In this context, the PrPc-lectins interactions have been explored in the human promyelocytic leukemia cell line NB4 [Lanotte et al., 1991], which expresses the lectin CBP70 [S. Finel-Szermanski, personal communication].

In this report, we demonstrate for the first time that PrPc is expressed in the nucleus of NB4 cells. We also identified the lectin CBP70 as a new putative nuclear partner for PrPc.

## MATERIALS AND METHODS

### Cell Culture

The human promyelocytic leukemia cell line NB4 [Lanotte et al., 1991] was cultured as follows. Briefly, cultures were initiated by seeding $2 \times 10^5$ cells/ml in fresh RPMI 1640 medium (Gibco, Life Technology, Cergy-Pontoise, France) supplemented with 10% fetal calf serum (Bayer, Puteaux, France), 2 mmol/L L-glutamine, 50 IU/ml of penicillin, and 50 μg/ml of streptomycin (Gibco, Life Technology). Cells were incubated at 37°C in a humidified 5% $CO_2$ atmosphere and maintained at a density of $3.5 \times 10^5$ cells/ml by resuspending the cells in fresh culture medium every day.

### Antibodies

Anti-CBP70 polyclonal antibody (Ab) was obtained as previously described [Sève et al., 1994]. Anti-prion monoclonal Ab 3F4, which recognizes both PrPc and PrPsc [Kascsak et al., 1987], was purchased from Clinisciences (Montrouge, France). Anti-P53 monoclonal Ab was

purchased from Pharmingen (Becton-Dickinson, Le Pont-de-Claix, France). Anti-calnexin-C rabbit polyclonal Ab against carboxyl terminus of canine calnexin was purchased from StressGen (Tebu, Le Perray-en-Yvelines, France). Anti-lamin B was applied from Tebu. Anti-LAP2 (thymopoïetin β) was a generous gift from Dr. Courvalin, Institut Jacques Monod, Paris, France. Cy5-conjugated goat anti-mouse and peroxidase-conjugated goat anti-mouse immunoglobulins (Ig) Ab were furnished by Jackson ImmunoResearch Laboratories (West Grove, PA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig Ab, peroxidase-conjugated goat anti-rabbit Ig Ab and streptavidin-labeled peroxidase were supplied by Sigma (St. Louis, MO). FITC-conjugated swine anti-rabbit Ig Ab were purchased from Dako A/S (Glostrup, Denmark).

### Cell Fractionation and Protein Extraction

Membrane-depleted nuclei were isolated from NB4 cells according to a previously reported method for HL60 cells [Facy et al., 1990]. Briefly, exponentially growing NB4 cells ($5 \times 10^7$) were washed twice in 10 mmol/L Tris-HCl (pH 7.5) containing 20 mmol/L KCl, 2 mmol/L $CaCl_2$, 2 mmol/L $MgCl_2$, and 0.2 mmol/L spermidine (TKCM buffer), and pelleted by low-speed centrifugation (800*g* for 10 min). The cell pellets were resuspended in 20 ml of TKCM buffer containing 1 mmol/L phenylmethylsulfonylfluoride (PMSF), Protease Inhibitor Cocktail containing 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF), pepstatin A, trans-epoxysuccinyl-L-leucylamido (4-guanidino)butane (E-64), bestatin, leupeptin, and aprotinin (Sigma). Triton X-100 was added at a final concentration of 1%. Cells were homogenized and membrane-depleted nuclei were pelleted by centrifugation at 1,000*g* for 10 min. The pellets were washed twice in 20 ml of TKCM buffer and pelleted again by centrifugation. Nuclear proteins were extracted from membrane-depleted nuclei with NaCl adjusted to 2 mol/L (final concentration) in TKCM buffer for 1 h at room temperature. Excess NaCl was removed by overnight dialysis against $Ca^{2+}$ and $Mg^{2+}$ free PBS ($PBS^-$) at 4°C, and the proteins were concentrated on Centricon-10 filters (Grace and Co., Danvers, MA). All steps of the isolation procedure were carried out at 4°C. These nuclear protein extracts were tested for eventual contamination by endoplasmic reticulum or nuclear membrane proteins, by using Ab raised against a membrane resident protein from endoplasmic reticulum (calnexin) and an inner membrane nuclear protein LAP2. The integrity of the nuclei was tested by the presence of lamin B. Microsome preparation from human B lymphoblastoid cell line AHH1 was purchased from Gentest (Interchim, Montluçon, France).

### Immunofluorescence Labeling and Confocal Analyses

NB4 cells ($3 \times 10^5$) were harvested by low-speed centrifugation, washed twice in $Ca^{2+}$-$Mg^{2+}$-PBS ($PBS^+$) supplemented with 1% bovine serum albumin (BSA) ($PBS^+$-1% BSA), permeabilized in acetone kept at −20°C for 10 min, washed in $PBS^+$-1% BSA and then incubated for 1 h at 4°C in the presence of: i) anti-PrP (3F4) Ab (1/50) or ii) anti-CBP70 Ab (1/200) or iii) anti-PrP and anti-CBP70 Ab, diluted 1/50 and 1/200, respectively. After several washes in $PBS^+$-1% BSA, the cells were incubated for 30 min at 4°C with: i) Cy5-conjugated goat anti-mouse Ig Ab (1/200) or ii) FITC-conjugated goat anti-rabbit Ig Ab (1/200) or iii) both secondary Ab. The cells were then washed 10 times in $PBS^+$-1% BSA and once in $PBS^+$ before being spread between slide and coverslip in a glycerol/DAPI/$PBS^+$ solution. Samples were examined by confocal laser scanning microscopy using a Bio-Rad MRC-1024 confocal imaging system (Bio-Rad Microscience Ltd., Hertfordshire, UK) and an inverted Diaphot 300 Nikon microscope. Images were collected using an oil immersion lens (60 ×, NAI.4 plan Apochromat). For FITC and Cy5 excitation, a krypton/argon ion laser (Ion Laser Technology, Inc., Salt Lake City, UT) was used at the 488 nm wave length. For DAPI (4′,6-diamino-2-phenylindole dihydrochloride) excitation, an enterprise ion laser (Coherent Laser Group, Santa Clara, CA) was used at the 353 nm wave length. FITC, Cy5, and DAPI images were digitalized to obtain pseudocolored green for FITC, red for Cy5, and blue for DAPI, which specifically labels cell nuclei. Yellow coloration results from the overlapping of green and red. Each image represents a single section for which the confocal system was adjusted to allow a field depth of about 0.8 μm. Two controls were performed: i) incubation of NB4 nuclei with the secondary Ab alone and ii) incubation with the pre-immune serum in place of anti-CBP70 Ab.

### Quantitative Flow-Cytofluorometry

NB4 cells ($6 \times 10^7$) grown in suspension were collected by low-speed centrifugation, and membrane-depleted nuclei were isolated according to a previously reported method [Facy et al., 1990]. Isolated nuclei were washed twice in $PBS^+$ and then incubated for 1 h at 4°C in the presence of i) anti-PrP Ab (1/1,000), ii) anti-CBP70 Ab (1/500), iii) anti-P53 Ab (1/1,000) or preincubated with anti-CBP70 Ab (1/500) and then incubated with anti-PrP or anti-P53 Ab both diluted 1/1,000. After washing in $PBS^+$, the nuclei were incubated for 30 min at 4°C in the dark with FITC-conjugated goat anti-mouse Ig Ab (1/100) or FITC-conjugated swine anti-rabbit Ig Ab (1/500). Nuclei were then washed twice in $PBS^+$ and the fluorescent intensity of 5,000 nuclei from each experimental incubation was recorded using a FACScan analyzer (Becton-Dickinson, San Jose, CA). For control experiments, nuclei were incubated with: i) the buffer alone, ii) the secondary Ab alone.

### Affinity-Chromatography Procedures

*N*-acetyl-β-D-glucosamine phenylisothiocyanate and α-D-glucopyranosyl phenylisothiocyanate (Sigma) were immobilized on acetone-dehydrated Trisacryl GF 2000 M (IBF, Villeneuve-La-Garenne, France). The nuclear protein extract was adjusted to 0.5 mmol/L $CaCl_2$ and 0.5 mmol/L MgCl2 ($PBS^+$), and affinity chromatography was performed as previously described [Sève et al., 1993]. To obtain purified nuclear CBP70, the proteins, eluted from GlcNAc columns with 0.2 mol/L GlcNAc, were dialyzed overnight against $PBS^+$ at 4°C, then incubated with glucose columns and eluted with 0.2 mol/L glucose. The eluted fraction was concentrated on Centricon-10 filters. The protein concentration was determined using the Micro BCA Protein Assay Reagent Kit (Pierce, Chemical CO., Rockford, IL, USA). Affinity chromatography was also performed with wheat germ agglutinin (WGA) immobilized on Sepharose-6 MB (Sigma). The nuclear extract was incubated with 1 ml of immobilized WGA, overnight at 4°C with gentle shaking. After column packing, the proteins specifically retained on WGA-Sepharose were eluted with 0.2 mol/L GlcNAc and concentrated on Centricon-10 filters. The protein concentration was determined using the Micro BCA Protein Assay Reagent Kit.

### Biotinylation of CBP70, Anti-CBP70, and Anti-PrP Ab

The purified nuclear CBP70 was labeled with Sulfo-NHS-biotin (Pierce), according to the manufacturer's protocol. Briefly, CBP70 was dialyzed against 100 mmol/L sodium borate buffer pH 8.5 (conjugation buffer). Seventy-four micrograms of Sulfo-NHS-biotin, dissolved in water, were slowly added to 7.4 μg of CBP70. The reaction proceeded for 4 h at room temperature in the dark with stirring. Labeled CBP70 was dialyzed against $PBS^+$ in order to separate the unreacted Sulfo-NHS-biotin from the labeled CBP70. The same procedures were carried out for the antibodies raised against CBP70 and PrP.

### Preparation of Glycopeptides From NB4 Cells

The nuclear extract of $10^7$ NB4 cells was incubated at 60°C overnight under a toluene layer with Pronase (4 U, 76,600 PUK/g) from *Streptomyces griseus* (Calbiochem, San Diego, CA) in 2 ml of pronase buffer (50 mmol/L Tris-HCl pH 8.0, containing 10 mmol/L $CaCl_2$), as previously reported [Codogno et al., 1985]. The samples were boiled for 10 min to denature the residual enzyme. Then, the digested NB4 nuclear extract was chromatographied on a Sephadex G50 column (Pharmacia, Orsay, France) in order to exclude Pronase enzymes.

### Electrophoretic and Immunoblotting Analyses

The nuclear protein extract or the proteins eluted from the WGA column were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) on 12% acrylamide mini-slab running gels under denaturing conditions, according to Laemmli's procedure [Laemmli, 1970]. Molecular mass markers were purchased from Bio-Rad (Hercules, CA). For immunoblotting or lectinoblotting analyses, the resolved polypeptides were transferred electrophoretically onto Immobilon-P paper (Millipore, Bedford, MA) at 60 V for 1 h at room temperature, in 10 mmol/L (cyclohexylamino)-1-propane sulfonic acid, pH 10.7 (Caps buffer). The Immobilon paper was incubated overnight in saturating PBS buffer containing 5% non-fat milk powder. The membrane was then washed three times in PBS containing 0.5% Tween-20 (PBS-Tween) and incubated for 2 h with anti-PrP Ab (1/10,000) or with anti-CBP70 Ab (1/1,000). After incubation, the blot was washed

three times in PBS-Tween, incubated for 45 min at room temperature with peroxidase-conjugated goat anti-mouse or with peroxidase-conjugated goat anti-rabbit Ig Ab, each diluted 1/10,000 in PBS-Tween. After three washes in PBS-Tween, the blot was finally developed using the enhanced chemiluminescence (ECL) reagents (Amersham, Les Ulis, France). Controls were run to ascertain that background levels of non-specific binding of peroxidase-conjugated goat anti-mouse and peroxidase-conjugated goat anti-rabbit Ig polyclonal Ab were acceptable. The same experiments were performed by using biotinylated anti-PrP Ab (1/10,000) or biotinylated anti-CBP70 Ab (1/4,000) and then streptavidin-labeled peroxidase (1/10,000). In parallel, two identical blots were incubated for 2 h with biotinylated purified nuclear CBP70 with or without Pronase digested glycopeptides. The blots were then washed three times in PBS-Tween, incubated for 45 min at room temperature with streptavidin-coupled peroxidase (1/10,000) and washed three times in PBS-Tween before visualization with ECL.

### Immunoprecipitation

Nuclear extracts were resuspended in PBS-supplemented with 0.5% Triton X-100, 0.25% BSA, 0.5 mmol/L $MgCl_2$, and 0.5 mmol/L $CaCl_2$. The pre-clearing of nuclear extracts was performed by incubation with 200 μl of a 50% (w/v) suspension of protein-A Sepharose-4 Fast Flow (Pharmacia, Orsay, France) for 1 h at 4°C in immunoprecipitation buffer (0.5% Triton X-100, 0.25% BSA, 1 mmol/L PMSF in $PBS^+$). Immunoprecipitation was initiated by adding 10 μg of anti-CBP70 Ab to the pre-cleared supernatant and left to incubate overnight at 4°C. Then, 100 μl of a 50% suspension of protein-A Sepharose-4 Fast Flow were added. The reaction mixture was incubated for 1 h at 4°C, after which the supernatant was discarded, and the beads were washed five times with immunoprecipitation wash buffer (1% Triton X-100, 0.5% BSA, 1 mmol/L PMSF in $PBS^+$), then washed twice in $PBS^+$. The beads were eluted in Laemmli's buffer and boiled for 5 min. Protein immunoprecipitates were separated by SDS–PAGE 12% gels under denaturating conditions and transferred onto Immobilon-P paper. Bands were revealed by biotinylated anti-PrP or biotinylated anti-CBP70 Ab. Control experiments were performed using preimmune serum.

## RESULTS

### A) Colocalized Expression of PrPc and CBP70 in NB4 Cell Nuclei

**1) Nuclear expression of PrPc and CBP70.** The regulation of prion mRNA could be performed by PrPc via interaction with galectin-3 [Schröder et al., 1994]. Following our observation that a protein–protein interaction between CBP70 and galectin-3 also occurs in cell nucleus [Sève et al., 1993, 1994], we hypothesized that CBP70 could be a nuclear partner for PrPc. Nuclear protein extracts of NB4 cells were subjected to SDS–PAGE 12% gels, electroblotted and PrPc (35 kDa) or CBP70 (70 kDa) were specifically identified with anti-PrP (Fig. 1A, lane A) or anti-CBP70 Ab (Fig. 1A, lane B). Only one polypeptide was detected in the nuclear fraction with anti-PrP Ab, whereas several bands were identified in total cellular and membrane extracts (C. Rybner, personal communication). This experiment shows PrPc expression in the nuclei of hematopoietic cells, not infected by the scrapie agent. In order to check the absence of contamination of NB4 nuclear protein extracts by either cytoplasmic proteins or nuclear membrane proteins, Western-blot controls were performed using Ab raised against calnexin, a resident rough endoplasmic reticulum protein [Hammond and Helenius, 1994] and LAP2, an inner membrane nuclear protein [Buendia and Courvalin, 1997]. We also used an Ab raised against the skeletal nuclear protein lamin B [Gerace et al., 1978], to verify the integrity of the nuclei. Neither calnexin nor LAP were revealed in the nuclear extracts (Fig. 1B) whereas lamin B was revealed (Fig. 1B).

**2) Nuclear localization of PrPc and CBP70 in NB4 cells.** Confocal microscopy analyses were performed to determine the subcellular localization(s) of PrPc and CBP70 in permeabilized cells with Ab raised against PrP and CBP70. Figure 2 shows that PrPc (Fig. 2b) and CBP70 (Fig. 2c), revealed by anti-PrP and Cy5-conjugated goat anti-mouse Ig Ab and anti-CBP70 and FITC-conjugated swine anti-rabbit Ig Ab, respectively, are localized in the cytoplasm and the nucleus of NB4 cells. The pattern of fluorescence of both proteins is particularly intense in the nucleolus (Fig. 2b,c) that is not stained by DAPI (Fig. 2a). Double-labeling experiments (Fig. 2d) show an overlapping of the two colors in some areas of the

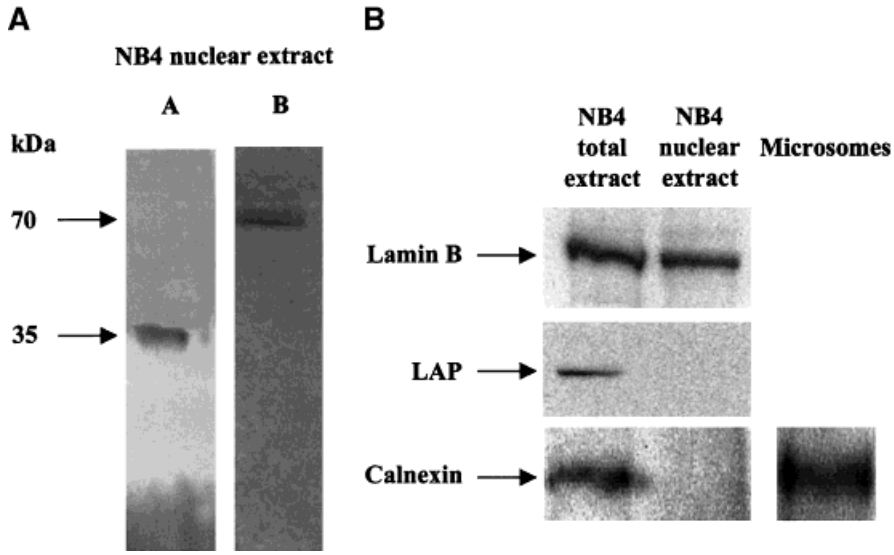


**Fig. 1.** Expression of PrPc and CBP70 in NB4 nuclei. **Panel A**: A nuclear protein extract of NB4 cells was subjected to SDS–PAGE 12% gels, electrotransferred, and immunoblotted with the anti-PrP 3F4 monoclonal Ab (**lane A**) or the anti-CBP70 polyclonal Ab (**lane B**). **Panel B**: Total extract, nuclear extract from NB4 cells, and microsomes from AHH1 cells were subjected to SDS–PAGE 12% gels, electrotransferred, and immunoblotted with anti-lamin B, anti-LAP, or anti-calnexin Ab.

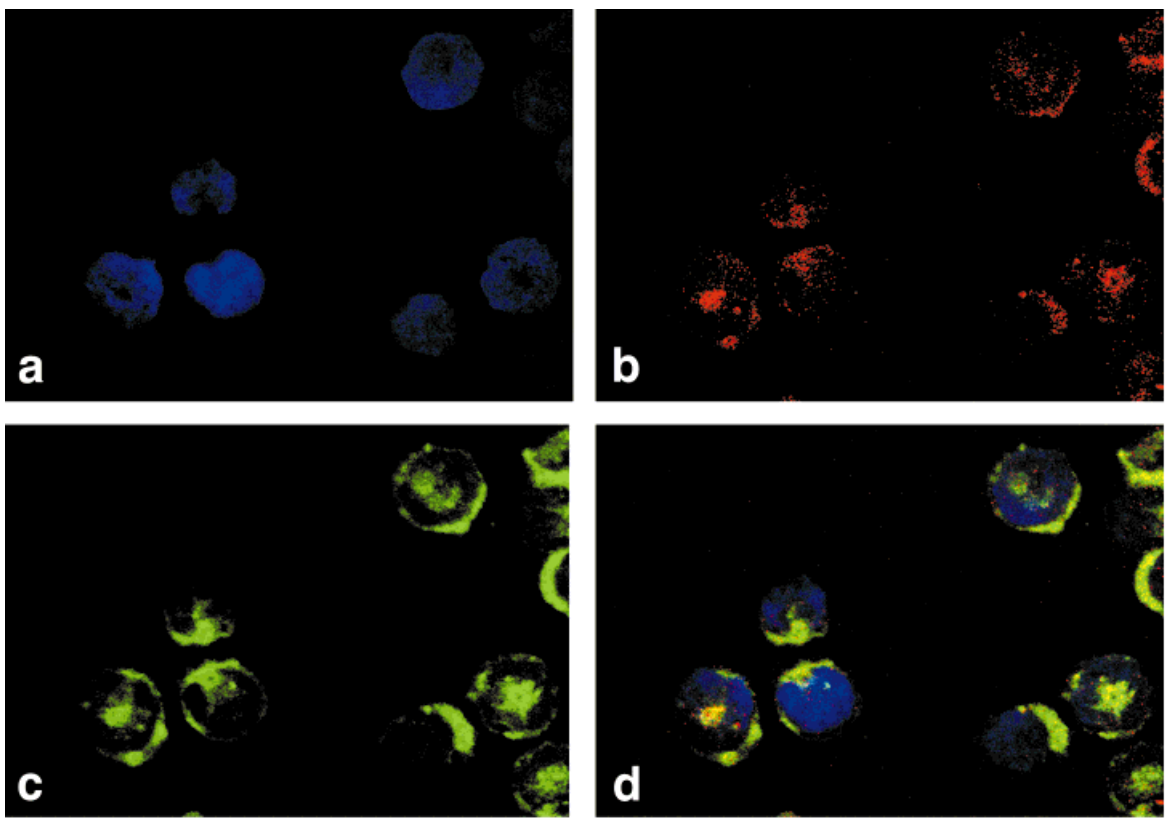


**Fig. 2.** Immunofluorescent subcellular localization(s) of PrPc and CBP70 in permeabilized NB4 cells. NB4 cells were permeabilized in acetone, labeled with 1) anti-prion and Cy5-conjugated goat anti-mouse Ig Ab yielding the red fluorescence pattern (**b**); 2) anti-CBP70 and FITC-conjugated swine anti-rabbit Ig Ab yielding the green fluorescence pattern (**c**); 3) simultaneously labeled with anti-PrP and anti-CBP70 Ab. In **panel d**, red (PrPc) and green (CBP70) markers overlap in some area of the nucleus to form yellow fluorescence indicating that these proteins are colocalized. Cell nuclei were labeled with DAPI and represented in blue (**a**).

nucleus (yellow fluorescence) which suggests a colocalization of both proteins.

The presence of PrPc in NB4 nuclei was further confirmed by flow-cytofluorometry performed on isolated membrane-depleted nuclei with anti-PrP Ab (Fig. 3B1, gray line). When these nuclei were incubated with anti-PrP and with FITC-conjugated goat anti-mouse Ig Ab, there was an increase of fluorescence in comparison with the autofluorescence of the cells (Fig. 3B1, dark line). The presence of CBP70 in the nuclear compartment has already been demonstrated by this method in HL60 cells [Sève et al., 1994]. This lectin is also expressed in NB4 cells nuclei and can then be used as a nuclear marker (Fig. 3A1, gray line). When these nuclei were preincubated with anti-CBP70 Ab (without a secondary Ab) and then labeled with anti-PrP and FITC-conjugated goat anti-mouse Ig Ab, the labeling of PrPc was decreased (Fig. 3B2, gray bold line). This observation strongly suggests either competi-

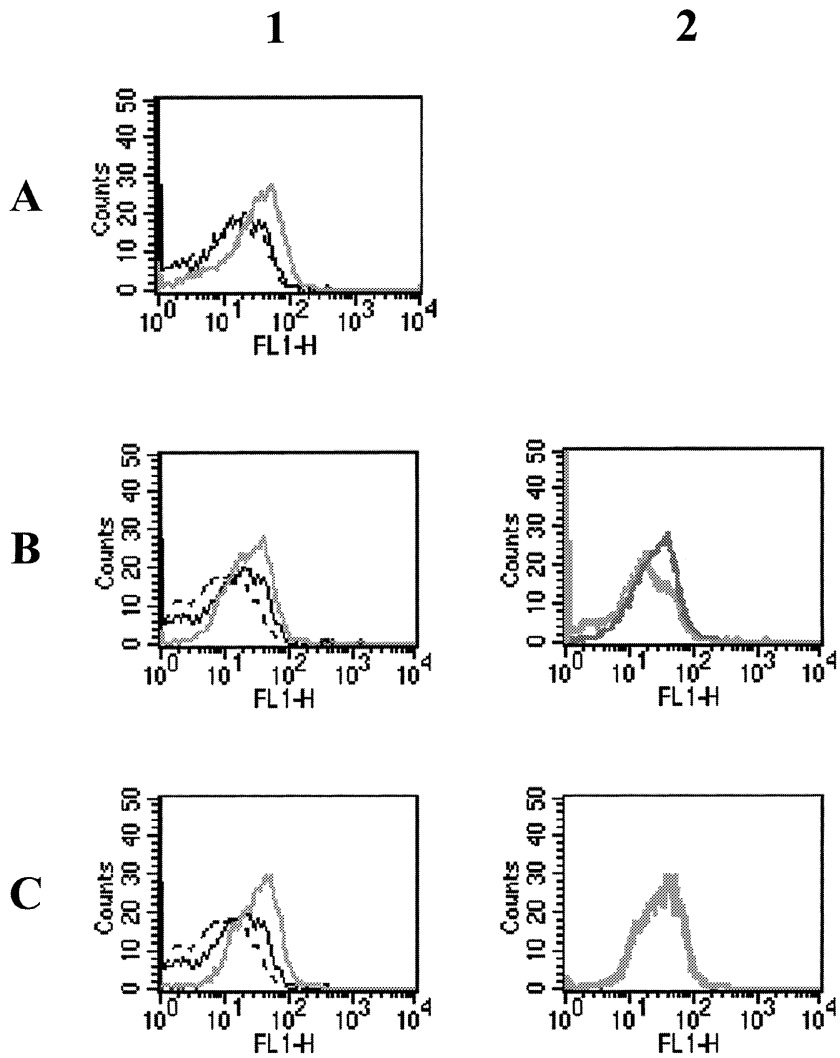


**Fig. 3.** Quantitative flow-cytofluorometry analysis of CBP70, PrPc, and P53 expression in NB4 cells nuclei. Counts = cell number, FL1-H = fluorescence intensity expressed in arbitrary units ($log^{10}$ scale). Peaks represent the mean fluorescence intensity of 5,000 nuclei. **A1**: anti-CBP70 and FITC-conjugated swine anti-rabbit Ig Ab; **B1**: anti-PrP and FITC-conjugated goat anti-mouse Ig Ab; **C1**: anti-P53 and FITC-conjugated goat anti-mouse Ig Ab; **B2**: preincubation with anti-CBP70 and then incubation with anti-PrP and FITC-conjugated goat anti-mouse Ig Ab; **C2**: preincubation with anti-CBP70 and then incubation with anti-P53 and FITC-conjugated goat anti-mouse Ig Ab. Dark line (▬): NB4 nuclei incubated with the buffer alone; dotted line (▬ ▬ ▬): NB4 nuclei incubated with the secondary Ab alone; gray line (▒): NB4 nuclei incubated with the first and secondary Ab; gray bold line (▓): NB4 nuclei preincubated with anti-CBP70 and then incubated with anti-PrP or anti-P53 and FITC-conjugated goat anti-mouse Ig Ab.

tion between anti-PrP and anti-CBP70 Ab for accession to the same site or the possibility of steric hindrance owing to the close proximity of the epitopes recognized by anti-PrP and anti-CBP70 Ab. In order to show that it was a specific effect, we carried out an experiment with another nuclear marker: the P53 protein which is expressed in NB4 cells nuclei (T. Sahraoui, personal communication). We confirmed the presence of P53 protein in the nucleus of NB4 cells (Fig. 3C1, gray line). When nuclei were preincubated with anti-CBP70 Ab in the same conditions as performed with the anti-PrP Ab (Fig. 3B2), we have not observed any shift in the P53-labeling (Fig. 3C2, gray line vs. gray bold line). The shift observed in the PrP-labeling after preincubation with the anti-CBP70 Ab was specific and suggests a possible interaction between both proteins.

### B) Interaction Between PrPc and CBP70

Because PrP is a glycoprotein bearing complex oligosaccharide chains and CBP70 is a lectin, we wanted to determine whether the interaction between these two proteins could be physiological. Sialoglycoproteins including PrP were concentrated by incubating nuclear protein extracts with WGA-Sepharose-6MB. WGA is known to recognize GlcNAc and sialic acid residues [Nagata and Burger, 1974]. The nuclear proteins eluted from the column with GlcNAc were subjected to SDS–PAGE 12% gels and electroblotted. The membrane was incubated with biotinylated nuclear CBP70. Polypeptides of 35 kDa molecular mass were identified among the sialoglycoproteins recognized by biotinylated nuclear CBP70 (Fig. 4, lane A). One polypeptide among those migrating at 35 kDa could correspond to PrP. In parallel, the same nuclear glycoprotein extract was analyzed by Western-blotting using anti-PrP Ab. In this case, only a 35 kDa polypeptide was revealed (Fig. 4, lane B). These results represented an additional evidence in favor of the interaction between PrPc and CBP70. To determine the nature of this interaction, the same experiment was performed in the presence of biotinylated nuclear CBP70 preincubated with competitor glycopeptides obtained after Pronase digestion of the NB4 nuclear extract. Glycopeptides as competitors were chosen rather than a simple sugar such as GlcNAc because *N*-glycans chains borne by PrP are highly complex [Endo et al., 1989]. In these

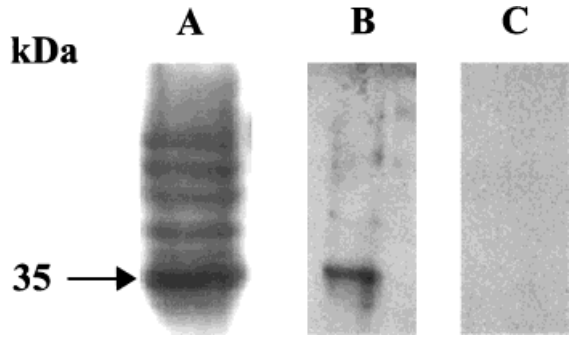


**Fig. 4.** Interaction of biotinylated CBP70 with NB4 nuclear glycoproteins. The nuclear glycoproteins eluted from WGA columns, were subjected to SDS–PAGE 12% gels, electroblotted, incubated with biotinylated purified nuclear CBP70 (**lane A**), anti-PrP Ab (**lane B**), or with biotinylated purified nuclear CBP70 preincubated with Pronase-digested glycopeptides prepared from NB4 nuclear extract (**lane C**).

conditions, no labeling was detected (Fig. 4, lane C). This absence of labeling is probably due to a competition between glycopeptides and oligosaccharide chains of PrPc. Indeed, in the presence of glycopeptides, all interactions between nuclear glycoproteins carrying GlcNAc and CBP70 are inhibited. This competition experiment argues for a sugar-dependent interaction and consequently a possible glycoprotein–lectin interaction between PrPc and CBP70.

To determine whether these two proteins interact directly or indirectly in NB4 cell nuclei, nuclear protein extracts were immunoprecipitated with anti-CBP70 Ab or preimmune serum, separated by SDS–PAGE 12% gels, electroblotted, and the membranes were incubated with biotinylated anti-PrP Ab and streptavidin-labeled peroxidase. PrPc was detected among the proteins immunoprecipitated with anti-CBP70 Ab (Fig. 5, lane Aa). When blots were incubated with biotinylated anti-CBP70 Ab, CBP70 was also detected among the polypeptides immunoprecipitated (Fig. 5, lane Ba). The IgG present in the preimmune serum were not revealed by streptavidin-peroxidase because they were not biotinylated (Fig. 5, lanes Ab and Bb). Nevertheless, it should be noted that a 50 kDa polypeptide was revealed in conditions using biotinylated anti-PrP and anti-CBP70 Ab (Fig. 5, lanes Aa and Ba). In our immunoprecipitation experiment conditions, this 50 kDa polypeptide could correspond to the heavy chains of Ig of the reacting Ab used at high concentration (10 μg of anti-CBP70 Ab).

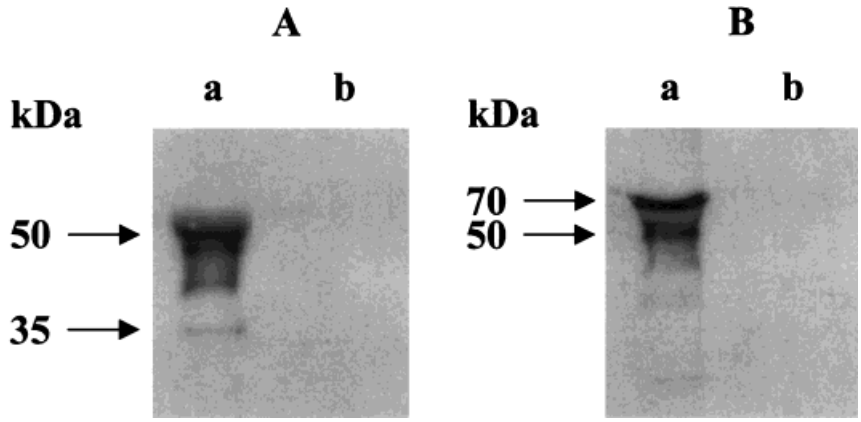


**Fig. 5.** PrPc co-immunoprecipitated with nuclear CBP70. NB4 nuclear extracts were immunoprecipitated either with anti-CBP70 Ab (**lanes Aa** and **Ba**) or the preimmune serum (**lanes Ab** and **Bb**), separated by SDS–PAGE 12% gels, electrotransferred, and revealed with biotinylated anti-PrP Ab (**lane A**) or biotinylated anti-CBP70 Ab (**lane B**) and steptavidin-labeled peroxidase.

## DISCUSSION

The presence of the PrPc has already been shown at the membrane level in hematopoietic cells [Cashman et al., 1990; Dodelet and Cashman, 1998; Barclay et al., 1999; C. Rybner, personal communication] but PrPc has not been detected in nuclei of blood cells yet. For the first time, the results reported in this article demonstrate the presence of the PrPc in the nuclei of the human promyelocytic leukemia cell line NB4, particularly in the nucleolus. The scrapie form of the prion protein (PrPsc) has also been shown in nuclei of scrapie-infected neuroblastoma cells [Pfeifer et al., 1993]. The search for the nuclear localization signal (NLS) has been achieved and it has been demonstrated that PrP contains an inactive NLS sequence. It was proposed that PrP could interact with factor(s) to form a complex able to be targeted to the nucleus [Jaegly et al., 1998]. We have also identified the lectin CBP70 in the nucleus of the NB4 cells, as a possible PrPc partner.

According to its subcellular localization(s), PrPc can interact with different ligands. PrPc can bind to the anti-apoptotic protein Bcl-2 [Kurschner and Morgan, 1995]. Furthermore, it was previously reported that galectin-3 was able to interact with the prion mRNA and protein, which is unable to interact directly with its own mRNA [Schröder et al., 1994]. Our group has isolated the lectin CBP70 from HL60 cell nuclei [Sève et al., 1993]. This nuclear lectin is associated with galectin-3 by a protein–protein interaction in the nuclei of these cells [Sève et al., 1993, 1994]. The results of confocal and quantitative flow-cytofluorometry analyses strongly suggest that CBP70 and PrPc are colocalized in the nuclei and particularly in the nucleoli of NB4 cells. In addition, we showed that PrPc was co-immunoprecipitated with CBP70 from NB4 nuclear extracts. These findings support the idea that CBP70 could be a putative partner for PrPc. Both isoforms of PrP are *N*-linked glycoproteins [Endo et al., 1989]. The analysis of the glycoforms of scrapie prion protein PrPsc shows that it contains decreased levels of exposed GlcNAc residues compared with those of PrPc [Rudd et al., 1999], which could be a better ligand for the lectin CBP70. As CBP70 is a carbohydrate-binding protein which recognizes *N*-acetyl-β-D-glucosamine residues, a glycoprotein–lectin interaction between both proteins has been investigated. It was shown that biotinylated purified nuclear CBP70 recognized PrPc among the nuclear glycoproteins retained on a WGA column. In the same conditions, biotinylated nuclear CBP70 did not bind to PrPc when competition was performed with Pronase-digested glycopeptides prepared from NB4 nuclear extract. These data might indicate that a glycoprotein–lectin interaction occurs between PrPc and CBP70. Nevertheless, it cannot be excluded that a protein–protein interaction occurs between PrPc and CBP70. This type of interaction could be disrupted by a tri-dimensional modification of CBP70 after the binding of a glycopeptide to the carbohydrate-recognition domain of CBP70. We previously demonstrated such an interaction between

CBP70 and galectin-3, which was modulated by sugars [Sève et al., 1993, 1994].

The identification of CBP70 as a PrPc ligand was of a great interest because this interaction could explain the targeting of PrPc to the nucleus. Indeed, these data are consistent with the hypothesis that PrP can shuttle into the nucleus via a complex with other protein(s) targeted to this cellular compartment [Jaegly et al., 1998]. It was shown that CBP70, which is present in the nucleus and in the cytoplasm [Hadj-Sahraoui et al., 1996; Rousseau et al., 2000], is a glycoprotein whose glycosylation differs depending on its subcellular localization(s) [Rousseau et al., 1997]. It was suggested that sugar residues might be nuclear-targeting signals and could define a new nuclear import mechanism [Duverger et al., 1996]. Therefore, it was postulated that, if CBP70 sugar moieties could be a targeting signal to the nucleus [Rousseau et al., 1997], then CBP70 could act as a factor allowing PrPc entry into the nucleus. CBP70 could interact with PrPc and influences its biological function, or prevents its conversion into the pathological scrapie form PrPsc. Recent data argue for an essential role of the *N*-glycan chains borne by PrP. Indeed, it was suggested that PrP has an intrinsic tendency to acquire some PrPsc-like properties and that *N*-glycan chains protect PrPc against the conversion process [Lehmann and Harris, 1997]. It was proposed that PrPc could bind to a putative protein X that might act as a molecular chaperone in the formation of PrPsc [Telling et al., 1995]. Thus, it could be hypothesized that CBP70 might protect PrPc against its transformation into pathological PrPsc by preventing the binding of other proteins by sequestration in the nuclear compartment.

The analysis of cellular and subcellular localization(s) of PrPc is contributing to a better understanding of its biological function and the conversion process that leads to the formation of its pathological counterpart. These data may be helpful in the development of new therapeutic strategies that could specifically prevent the replication of the infectious agent in circulating blood elements and/or the diffusion to neuronal cells.

## ACKNOWLEDGMENTS

The authors thank Mrs. C. Doliger and Mr. M. Schmid from the Service Commun d'Imageries Cellulaires et Moléculaires of the Institut Universitaire d'Hématologie (Hôpital Saint-Louis) for the analyses of confocal immunofluorescence stainings and Mrs. E. Savariau from the Service Commun de Photographie of the Institut Universitaire d'Hématologie (Hôpital Saint-Louis) for the artwork. C. Rybner and S. Finel-Szermanski are recipients of a fellowship from the Ligue Nationale contre le Cancer and the Ministère de l'Enseignement Supérieur, de la Recherche Scientifique et Technique, respectively.

## REFERENCES

Akahani S, Nangia-Makker P, Inohara H, Kim HRC, Raz A. 1997. Galectin-3: a novel antiapoptotic molecule with a functional BH1 (NWGR) domain of Bcl-2 family. Cancer Res 57:5272–5276.

Barclay GR, Hope J, Birkett CR, Turner ML. 1999. Distribution of cell-associated prion protein in normal adult blood determined by flow cytometry. Br J Haematol 107:804–814.

Bendheim PE, Brown HR, Rudelli RD, Scala LJ, Goller NL, Wen GY, Kascsak RJ, Cashman NR, Bolton DC. 1992. Nearly ubiquitous tissue distribution of the scrapie agent precursor protein. Neurology 42:149–156.

Borchelt DR, Scott M, Taraboulos A, Stahl N, Prusiner SB. 1990. Scrapie and cellular prion proteins differ in their kinetics of synthesis and topology in cultured cells. J Cell Biol 110:743–752.

Buendia B, Courvalin JC. 1997. Domain-specific disassembly and reassembly of nuclear membranes during mitosis. Exp Cell Res 230:133–144.

Cashman NR, Loertscher R, Nalbantoglu J, Shaw I, Kascsak RJ, Bolton DC, Bendheim PE. 1990. Cellular isoform of the scrapie agent protein participates in lymphocyte activation. Cell 61:185–192.

Caughey B, Raymond GJ. 1991. The scrapie-associated form of PrP is made from a cell surface precursor that is both protease- and phospholipase-sensitive. J Biol Chem 266:18217–18223.

Caughey B, Race RE, Ernst D, Buchmeier MJ, Chesebro B. 1989. Prion protein biosynthesis in scrapie-infected and uninfected neuroblastoma cells. J Virol 63:175–181.

Codogno P, Botti J, Font J, Aubery M. 1985. Modification of the N-linked oligosaccharides in cell surface glycoproteins during chick embryo development. A using lectin affinity and a high resolution chromatography study. Eur J Biochem 149:453–460.

Dagher SF, Wang JL, Patterson RJ. 1995. Identification of galectin-3 as a factor in pre-mRNA splicing. Proc Natl Acad Sci USA 92:1213–1217.

Dodelet VC, Cashman NR. 1998. Prion protein expression in human leukocyte differentiation. Blood 91:1556–1561.

Duverger E, Roche AC, Monsigny M. 1996. N-acetylglucosamine-dependent nuclear import of neoglycoproteins. Glycobiology 6:381–386.

Edenhofer F, Rieger R, Famulok M, Wendler W, Weiss S, Winnacker EL. 1996. Prion protein PrPc interacts with molecular chaperones of the Hsp60 family. J Virol 70:4724–4728.

Endo T, Groth D, Prusiner SB, Kobata A. 1989. Diversity of oligosaccharide structures linked to asparagines of the scrapie prion protein. Biochemistry 28:8380–8388.

Facy P, Sève AP, Hubert M, Monsigny M, Hubert J. 1990. Analysis of nuclear sugar-binding components in undifferentiated and in vitro differentiated human promyelocytic leukemia cells (HL60). Exp Cell Res 190: 151–160.

Felin M, Doyennette-Moyne MA, Hadj-Sahraoui Y, Aubery M, Hubert J, Sève AP. 1994. Identification of two nuclear N-acetylglucosamine-binding proteins. J Cell Biochem 56:527–535.

Fournier JG, Escaig-Haye F, Billette de Villemeur T, Robain O. 1995. Ultrastructural localization of cellular prion protein (PrPc) in synaptic boutons of normal hamster hippocampus. C R Acad Sci 318:339–344.

Fournier JG, Escaig-Haye F, Billette de Villemeur T, Robain O, Lasmézas CI, Deslys JP, Dormont D, Brown P. 1998. Distribution and submicroscopic immunogold localization of cellular prion protein (PrPc) in extracerebral tissues. Cell Tissue Res 292:77–84.

Fournier JG, Escaig-Haye F, Grigoriev V. 2000. Ultrastructural localization of prion proteins: physiological and pathological implications. Microsc Res Tech 50:76–88.

Gerace L, Blum A, Blobel G. 1978. Immunocytochemical localization of the major polypeptides of the nuclear pore complex-lamina fraction. Interphase and mitotic distribution. J Cell Biol 79:546–566.

Gohel C, Grigoriev V, Escaig-Haye F, Lasmézas CI, Deslys JP, Langeveld J, Akaaboune M, Hantaï D, Fournier JG. 1999. Ultrastructural localization of cellular prion protein (PrPc) at the neuromuscular junction. J Neurosci Res 55:261–267.

Hadj-Sahraoui Y, Sève AP, Doyennette-Moyne MA, Saffar L, Felin M, Aubery M, Gattegno L, Hubert J. 1996. Nuclear and cytoplasmic expressions of the carbohydrate-binding protein CBP70 in tumoral or healthy cells of the macrophagic lineage. J Cell Biochem 62:529–542.

Hammond C, Helenius A. 1994. Quality control in the secretory pathway: retention of a misfolded viral membrane glycoprotein involves cycling between the ER, intermediate compartment, and Golgi apparatus. J Cell Biol 126:41–52.

Hubert J, Sève AP, Facy P, Monsigny M. 1989. Are nuclear lectins and nuclear glycoproteins involved in the modulation of nuclear functions? Cell Differ Dev 27:69–81.

Jaegly A, Mouthon F, Peyrin JM, Camugli B, Deslys JP, Dormont D. 1998. Search for a nuclear localization signal in the prion protein. Mol Cell Neurosci 11:127–133.

Kascsak RJ, Rubenstein R, Merz PA, Tonna-DeMasi M, Fersko R, Carp RI, Wisniewski HM, Diringer H. 1987. Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. J Virol 61:3688–3693.

Kurschner C, Morgan JI. 1995. The cellular prion protein (PrP) selectively binds to Bcl-2 in the yeast two-hybrid system. Brain Res Mol Brain Res 30:165–168.

Kuwabara I, Liu FT. 1996. Galectin-3 promotes adhesion of human neutrophils to laminin. J Immunol 156:3939–3944.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lanotte M, Martin-Thouvenin V, Najman S, Balerini P, Valensi F, Berger R. 1991. NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). Blood 77:1080–1086.

Lehmann S, Harris DA. 1997. Blockade of glycosylation promotes acquisition of scrapie-like properties by the prion protein in cultured cells. [published erratum appears in J Biol Chem 1998 Mar 6;273(10):5988] J Biol Chem 272:21479–21487.

MacGregor I, Hope J, Barnard G, Kirby L, Drummond O, Pepper D, Hornsey V, Barclay R, Bessos H, Turner M, Prowse C. 1999. Application of a time-resolved fluoroimmunoassay for the analysis of normal prion protein in human blood and its components. Vox Sang 77:88–96.

Nagata Y, Burger MM. 1974. Wheat germ agglutinin. Molecular characteristics and specificity for sugar binding. J Biol Chem 249:3116–3122.

Pfeifer K, Bachmann M, Schröder HC, Forrest J, Müller WEG. 1993. Kinetics of expression of prion protein in uninfected and scrapie-infected N2a mouse neuroblastoma cells. Cell Biochem Funct 11:1–11.

Rieger R, Edenhofer F, Lasmézas CI, Weiss S. 1997. The human 37-kDa laminin receptor precursor interacts with the prion protein in eukaryotic cells [see comments]. Nat Med 3:1383–1388.

Rousseau C, Felin M, Doyennette-Moyne MA, Sève AP. 1997. CBP70, a glycosylated nuclear lectin. J Cell Biochem 66:370–385.

Rousseau C, Muriel MP, Musset M, Botti J, Sève AP. 2000. Glycosylated nuclear lectin CBP70 also associated with endoplasmic reticulum and the Golgi apparatus: does the "classic pathway" of glycosylation also apply to nuclear glycoproteins? J Cell Biochem 78:638–649.

Rudd PM, Endo T, Colominas C, Groth D, Wheeler SF, Harvey DJ, Wormald MR, Serban H, Prusiner SB, Kobata A, Dwek RA. 1999. Glycosylation differences between the normal and pathogenic prion protein isoforms. Proc Natl Acad Sci USA 96:13044–13049.

Schröder HC, Scheffer U, Forrest JMS, Sève AP, Rytik PG, Müller WEG. 1994. Association of scrapie prion protein and prion protein-RNA stem-loop with nuclear carbohydrate-binding protein 35 and other RNA-binding proteins. Neurodegeneration 3:177–189.

Sève AP, Felin M, Doyennette-Moyne MA, Sahraoui T, Aubery M, Hubert J. 1993. Evidence for a lactose-mediated association between two nuclear carbohydrate-binding proteins. Glycobiology 3:23–30.

Sève AP, Hadj-Sahraoui Y, Felin M, Doyennette-Moyne MA, Aubery M, Hubert J. 1994. Evidence that lactose binding to CBP35 disrupts its interaction with CBP70 in isolated HL60 cell nuclei. Exp Cell Res 213:191–197.

Stahl N, Borchelt DR, Prusiner SB. 1990. Differential release of cellular and scrapie prion proteins from cellular membranes by phosphatidylinositol-specific phospholipase C. Biochemistry 29:5405–5412.

Taraboulos A, Serban D, Prusiner SB. 1990. Scrapie prion proteins accumulate in the cytoplasm of persistently infected cultured cells. J Cell Biol 110:2117–2132.

Telling GC, Scott M, Mastrianni J, Gabizon R, Torchia M, Cohen FE, DeArmond SJ, Prusiner SB. 1995. Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. Cell 83:79–90.

Woo HJ, Shaw LM, Messier JM, Mercurio AM. 1990. The major non-integrin laminin binding protein of macrophages is identical to carbohydrate binding protein 35 (Mac-2). J Biol Chem 265:7097–7099.

Yang RY, Hsu DK, Liu FT. 1996. Expression of galectin-3 modulates T-cell growth and apoptosis. Proc Natl Acad Sci USA 93:6737–6742.