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Glycosylated Nuclear Lectin CBP70 Also Associated With Endoplasmic Reticulum and the Golgi Apparatus: Does the "Classic Pathway" of Glycosylation Also Apply to Nuclear Glycoproteins?

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Abstract The subcellular plurilocalization of some lectins (galectin-1, galectin-3, galectin-10, calreticulin, etc.) is an intriguing problem, implying different partners according to their localization, and involvement in a variety of cellular activities. For example, the well-known lectin, galectin-3, a lactose-binding protein, can act inside the nucleus in splicing events, and at the plasma membrane in adhesion, and it was demonstrated that galectin-3 interacts in the cytoplasm with Bcl-2, an antiapoptotic protein. Some years ago, our group isolated a nuclear lectin CBP70, capable of recognizing *N*-acetylglucosamine residues. This lectin, first isolated from the nucleus of HL60 cells, was also localized in the cytoplasm. It has been demonstrated that CBP70 is a glycosylated lectin, with different types of glycosylation, comparing cytoplasmic and nuclear forms. In this article, we have studied the localization of CBP70 in undifferentiated HL60 cells by electron microscopy, immunofluorescence analysis, and subcellular fractionation. The results obtained clearly demonstrated that CBP70 is a plurilocalized lectin that is found in the nucleus, at the endoplasmic reticulum, the Golgi apparatus, and mitochondria, but not at the plasma membrane. Because CBP70, a nuclear glycoprotein, was found to be associated also with the endoplasmic reticulum and the Golgi apparatus where the glycosylation take place, it raised the question: where does the glycosylation of nuclear proteins occur? J. Cell. Biochem. 78:638–649, 2000.

Key words: lectin; glycosylation; O-GlcNAc; plurilocalization

Lectins are ubiquitous proteins that are bona fide elements of mammalian, reptilian, sponge, and protozoan cells [Drickamer and Taylor, 1993]. There is great interest in these proteins because of their ability to recognize sugars linked to glycoproteins. The glycoprotein–lectin interactions are labile. Lectins can also interact with proteins via protein–protein interactions. Data strongly suggest that these protein–protein interactions can be modulated, when the lectin interacts with glycosidic moieties of a glycoconjugate [Hinek et al., 1988, Sève et al., 1993, 1994, Yang et al., 1996]. This phenomenon opens a new field of investigation for exploring the mechanisms involved in the cascade of formation and disruption of protein complexes that constitute essential steps in many biological processes.

In animals cells, lectins were first described at the plasma membrane level [Ashwell and Harford, 1982]. Since this discovery, the presence of lectins has been reported in many subcellular compartments of animals cells, such as: the ER, the Golgi apparatus, the cytosol,

Abbreviations used: Ab, antibod(y/ies); BSA, bovine serum albumin; CBP, carbohydrate-binding protein; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; Ig, immunoglobulins; PBS, phosphate-buffered saline with (+) or without (−) Ca^{2+} and Mg^{2+}; PDI, protein disulfide isomerase.

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#Our colleague and friend Dr. Annie-Pierre Sève passed away (January 7, 2000), while this work was under review. We are grateful to her for the generous advice she provided us during these last years. We wish here to bear testimony to her invaluable scientific contribution and her exceptional enthusiasm in managing this project.

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and the nucleus [Roche and Monsigny, 1996]. Among all the animal lectins characterized to date, some are especially interesting, because they: 1) are plurilocalized, and 2) have different partners, according to their localization. Consequently, such lectins are also plurifunctional. An increasing number of plurilocalized lectins have been described so far: galectin-1 [Perillo et al., 1995; Cooper, 1997; Lutomski et al., 1997; Vyakarnam et al., 1997]; galectin-3 [Liu et al., 1995; Ochieng and Warfield, 1995; Van den Brûle et al., 1995; Wang et al., 1995; Akahani et al., 1997; Cooper, 1997; Huflejt et al., 1997]; galectin-10 [Dvorak et al., 1996]; calreticulin [Krause and Michalak, 1997]; and CBP70 [Hadj-Sahraoui et al., 1996; Felin et al., 1997]. Galectin-3, the most well-known plurilocalized lectin, has been described 1) in the nucleus, where it plays a role in the splicing of RNA [Dagher et al., 1995, Vyakarnam et al., 1997]; 2) in the cytosol, where it inhibits apoptosis by interacting with Bcl-2 [Yang et al., 1996; Akahani et al., 1997]; and 3) at the plasma membrane level, where it plays a role in cell–cell [Inohara and Raz, 1994, 1995; Bresalier et al., 1996; Inohara et al., 1996] or extracellular matrix–cell interactions [Hughes, 1994; Van den Brûle et al., 1995].

CBP70 was first isolated from HL60 cell nuclei [Sève et al., 1993]. In this cellular compartment, CBP70 interacts with galectin-3 via a protein–protein interaction, mediated by the addition of lactose, probably resulting in modification of the galectin-3 conformational structure [Sève et al., 1993]. This result was also corroborated by in vitro experiments [Sève et al., 1994]. In keeping with these findings, we proposed a possible involvement of such interactions in nuclear physiology [Hubert et al., 1989]. The presence of CBP70 in the cytoplasm has also been described [Hadj-Sahraoui et al., 1996; Felin et al., 1997]. Recent data strongly suggest that there is a correlation between the presence of CBP70 at the membrane and the capacity of the differentiated cells to adhere.

Another interesting point is the capacity of these isoforms of CBP70 lectins to recognize *N*-acetyl-glucosamine (GlcNAc) [Felin et al., 1994]. Because most of the nuclear and many of the cytosolic glycoproteins are modified with O-GlcNAc residues [Hart et al., 1996; Haltiwanger et al., 1997], these glycoproteins are good candidates for interacting with CBP70. In this context, it is noteworthy that an 82-kDa nuclear ligand for CBP70, containing GlcNAc residues has been characterized [Felin et al., 1997].

In addition, it has been demonstrated that CBP70 is a glycosylated lectin [Rousseau et al., 1997], with different types of glycosylation, comparing cytoplasmic and nuclear forms. Both nuclear and cytoplasmic forms of CBP70 are *N*- and *O*-glycosylated. However, only the nuclear CBP70 contains terminal *N*- or *O*-linked GlcNAc, as well as α-L-fucose [Rousseau et al., 1997]. Although numerous nuclear glycoproteins have been described [Stein et al., 1975, 1981; Hubert et al., 1985; Ferraro et al., 1991; Codogno et al., 1992], the cellular location of their glycosylation remains an intriguing issue.

This study focuses interest on the localization of CBP70 at the ultrastructural level to determine precisely its nuclear and cytoplasmic localization, and to determine whether CBP70 could be observed in the ER and/or in the Golgi where classic glycosylation takes place. Using a combination of immunoelectron microscopy, immunofluorescence localization, and subcellular fractionation of HL60 cells, it was concluded that CBP70 is present in both the nucleus and the cytoplasm, but not at the plasma membrane. Within the cytoplasm, CBP70 was observed associated with the mitochondria, and the ER, and the Golgi apparatus, where the glycosylation takes place.

MATERIALS AND METHODS

Cell Culture

HL60 cells were grown in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Gibco, Cergy-Pontoise, France), 2 mmol/l L-glutamine, 50 IU/ml of penicillin, and 50 mg/ml of streptomycin. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere and maintained at a density of 2×10^5 to 1×10^6 cells/ml, by resuspending the cells in fresh culture medium every 3 days.

Antibodies

Anti-CBP70 polyclonal antibody were obtained as previously reported [Sève et al., 1993]. Swine anti-rabbit Ig conjugated to FITC were purchased from Dako A/S (Glostrup, Denmark). Monoclonal mouse anti-human mitochondria antibody was from Calbiochem-Novabiochem Corporation (La Jolla, CA, U.S.A.). Cy5-conjugated goat anti-mouse Ig

and peroxidase-conjugated goat anti-mouse polyclonal antibody were furnished by Jackson ImmunoResearch Laboratories (West Grove, PA). Monoclonal mouse anti-Golgi-β Cop antibody (clone M3A5), monoclonal mouse anti-Golgi 58K protein antibody (clone 58K-9), polyclonal rabbit anti-glucose-6-phosphate dehydrogenase antibody, peroxidase-conjugated goat anti-rabbit polyclonal antibody, and mouse anti-splicing factor SC-35 antibody (clone SC-35) were purchased from Sigma (St. Louis, MO). Monoclonal mouse anti-PDI antibody was from StressGen Biotechnologies (Victoria, B.C., Canada). Gold-conjugated goat anti-rabbit Ig (15-nm-diameter gold particles) was from Bio Cell (Cardiff, U.K.).

Fixation and Embedding Procedures

HL60 cells were pelleted at low-speed centrifugation and fixed in 2.5% glutaraldehyde in 0.1 mol/l cacodylate buffer (pH 7.4) for 2½ h at room temperature, postfixed with 1% osmium tetroxide in the same buffer, dehydrated in ethanol, and embedded in Epon 812. In a second approach, pelleted cells were fixed in 4% paraformaldehyde in 0.1 mol/l cacodylate buffer (pH 7.4) for 2 ½ h at room temperature. After fixation, cells were quickly rinsed with cacodylate buffer containing 0.1 mol/l of sucrose, washed 30 min in cacodylate buffer containing 0.1 mol/l of sucrose and 0.1 mol/l of glycine and finally, rinsed with cacodylate buffer containing 0.1 mol/l of sucrose. To eliminate free aldehyde groups, the specimens were treated with 0.5 mol/l NH_4Cl in the buffer for 20 min and washed again. Specimens were then partially dehydrated in ethanol series up to 95% ethanol, infiltrated with a London Resin (LR) White-ethanol mixture (1/2, 1/1, 2/1) 10 min for every change, followed by two changes of undiluted LR White for 15 min. After an additional change of undiluted resin, cells were embedded in gelatin capsules, sealed, and polymerized at 4°C for 24 h.

Immunoelectron Microscopy

Ultrathin sections were either 1) contrasted with uranyl acetate and lead citrate, or 2) collected on gold grids, floated for 10 min on Tris-buffered saline (TBS: 20 mmol/l Tris-HCl, 0.15 mol/l NaCl pH 7.5) containing 0.5% BSA and 3% of heat-inactivated normal goat serum (TBS_1).

The gold grids were transferred onto 25 μl of a 1:25 dilution of antiserum against CBP70 in TBS_1. Incubation was allowed to proceed for 24 h at 4°C. Grids were successively washed on five 25-μl droplets of TBS_1 (5 min on each), and a final jet of TBS_1, before incubation for 1 h with gold-conjugated goat anti-rabbit Ig diluted 1:10 in TBS_1, containing 0.2% of fish gelatin from cold-water fish skin. Sections were then washed on three 50-μl droplets (5 min on each), followed by staining with aqueous uranyl acetate for 15 min. All steps were carried out at room temperature.

Two controls were performed: 1) replacing the primary antibody with preimmune serum, at the same dilution as the rabbit anti-CBP70 serum; and 2) omitting the primary antibody.

Immunohistochemical Labeling and Confocal Analyses

Cells were pelleted onto glass slides in a cytocentrifuge at 400 rpm for 5 min, fixed and permeabilized in acetone at 4°C for 10 min, and then air dried. Slides were incubated with anti-CBP70 Ab (1:200, [Sève et al., 1993]), monoclonal mouse anti-human mitochondria Ab (1/100), monoclonal mouse anti-Golgi-β Cop Ab (clone M3A5, 1/20), monoclonal mouse anti-splicing factor SC-35 Ab (clone SC-35, 1/1,000), or monoclonal mouse anti-protein disulfide isomerase Ab (1/20) for 1 h at room temperature. After five washes in Ca^{2+}- and Mg^{2+}-containing PBS^+ supplemented with 1% BSA, the cells were incubated for 30 min at room temperature with FITC-conjugated swine anti-rabbit Ig (1:20) and Cy5-conjugated goat anti-mouse Ig (1:100). Finally, slides were washed five times in PBS^+ supplemented with 1% BSA, followed by two washes in PBS^+. All incubations were performed in a humidified chamber at room temperature.

Samples were examined by confocal laser scanning microscopy using a Bio-Rad MRC-1024 confocal imaging system (Bio-Rad Microscience Ltd., Hertfordshire, U.K.) and an inverted Diaphot 300 Nikon microscope. Images were collected using an oil immersion lens (60×, NA I.4 plan Apochromat). For FITC and Cy5 excitation, a krypton/argon ion laser (Ion Laser Technology Inc., Salt Lake City, UT, U.S.A.) operating with the 488 nm line was used. For DAPI excitation, an enterprise ion laser (Coherent Laser Group, Santa Clara, CA, U.S.A.) operating with a 353-nm nonline was used. FITC, Cy5, and DAPI images were digitalized to obtain pseudocolored green for FITC, red for Cy5, and blue for DAPI; yellow colora-

tion 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 ~0.8 μm.

Subcellular Fractionation

Subcellular fractionation of HL60 cells was performed with modifications from a previously described method [Tang et al., 1998]. Briefly, HL60 cells were harvested by centrifugation at 800*g* for 10 min at 4°C. The cell pellets were washed twice with ice-cold 10 mmol/l Tris-HCl pH 7.4, 20 mmol/l KCl, 2 mmol/l $CaCl_2$, 2 mmol/l $MgCl_2$, 1 mmol/l EDTA, 1 mmol/l EGTA, 0.25 mol/l sucrose, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mmol/l phenylmethylsulfonyl fluoride. The cell suspension was homogenized in the same buffer with a glass Pyrex homogenizer using a type B pestle, and the resultant crude homogenate was subjected to sequential centrifugations. Each successive supernatant was used for the next centrifugation. The first centrifugation was at 200*g* for 5 min at 4°C to remove unbroken cells as a pellet. Nuclei pellet was obtained by centrifugation at 1,000*g* for 10 min. The mitochondrial pellet was obtained after centrifugation at 10,000*g* for 15 min at 4°C. The resulting supernatant was then centrifuged at 100,000*g* for 1 h at 4°C. The pellet obtained was considered as the microsomal fraction and the resulting supernatant was considered as the cytosolic fraction. The protein concentration of all the fractions obtained was determined using the Micro BCA protein Assay Reagent Kit (Pierce Chemical Co., Rockford, IL, U.S.A.).

Electrophoresis and Immunoblotting Analysis

Subcellular fractions (10 μg of each) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (gel 12%), according to Laemmli's procedure [Laemmli, 1970]. The resolved polypeptides were electrotransferred onto Immobilon-P (60 V at 4°C for 1 h) in 10 mmol/l (cyclohexylamino)-1-propane sulfonic acid, pH 10.7 (Caps buffer). The blots were incubated overnight in saturating TBS buffer (20 mmol/l Tris-HCl [pH 7.4], 0.5 mol/l NaCl) containing 5% BSA, and then were washed three times in TBS containing 0.5% Tween 20 (TBS-Tween) and incubated for 2 h with one of the following Ab: anti-CBP70 Ab (1:300 in TBS-Tween), monoclonal mouse anti-splicing factor SC-35 Ab (1:500), monoclonal mouse anti-human mitochondria Ab (1:100), monoclonal mouse anti-Golgi 58K protein Ab (1:500), monoclonal mouse anti-protein disulfide isomerase Ab (1:250), or polyclonal rabbit anti-glucose-6-phosphate dehydrogenase Ab (1:2,000). After incubation, the immunoblots were washed three times in TBS-Tween, incubated for 1 h at room temperature with the appropriate second Ab: peroxidase-conjugated polyclonal goat anti-mouse Ig or peroxidase-conjugated polyclonal goat anti-rabbit Ig, each diluted 1:10,000 in TBS-Tween, then washed three times in TBS-Tween, and finally developed using the Enhance Chemiluminescence (ECL) reagents (Amersham, Les Ulis, France).

RESULTS AND DISCUSSION

As previously described by affinity chromatography and immunofluorescence analysis, CBP70 is a plurilocalized lectin [Hadj-Sahraoui et al., 1996; Felin et al., 1997]. To achieve higher resolution localization, ultrastructural analysis of the intracellular distribution of CBP70 was performed. Figure 1 shows that undifferentiated HL60 cells possess a large nucleus containing a slightly condensed chromatin network and one to three nucleoli. In the cytoplasm, endoplasmic reticulum, mitochondria, and free ribosomes are readily observed (Fig. 1). Compared to the control sections incubated with preimmune serum (Fig. 2A,B), the immunogold labeling of anti-CBP70 reveals localization of the protein both in the nucleus and the cytoplasm (Fig. 3 and Fig. 4 A–C).

Within the nucleus, gold particles were localized throughout the interchromatin spaces, as well as in the nucleolus (Fig. 3). Such observations of random sections through the nucleus allow us to conclude that there is an accumulation of gold particles inside some nuclear areas that did not seem to correspond to the perichromatin fibrils and interchromatin granules [Puvion-Dutilleul and Puvion, 1995; Puvion and Puvion-Dutilleul, 1996]. The nuclear envelope is also labeled (Fig. 3). In the nucleolus, the dense fibrillar and granular parts are labeled with the anti-CBP70 (Fig. 3).

Some years ago, CBP70 was isolated from the nucleus of HL60 cells [Sève et al., 1993]. During affinity chromatography, this lectin was coisolated with galectin-3 (previously named CBP35) via protein–protein interactions. We demonstrated that this interaction

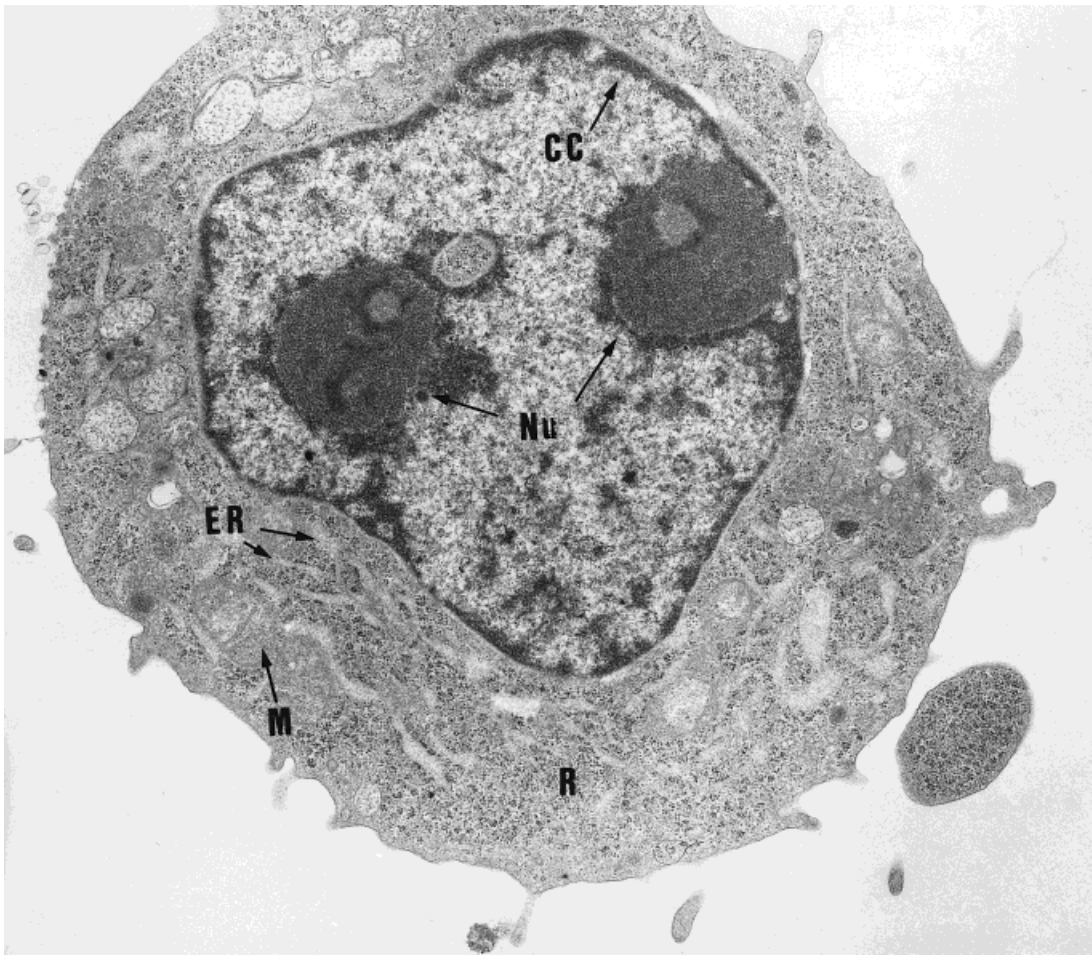

Fig. 1. Ultrastructure of an undifferentiated HL60 cell. Undifferentiated HL60 cell exhibiting a large nucleus containing some condensed chromatin (CC) and nucleoli (Nu). In the cytoplasm, endoplasmic reticulum (ER), mitochondria (M), and free ribosomes (R) are readily observed (×15,000).

was disrupted by the addition of lactose [Sève et al., 1993, 1994]. Galectin-3 has been described in various nuclear territories [Hubert et al., 1995]. Because galectin-3 was demonstrated to be colocalized with spliceosome assembly and especially with the splicing component SC35 [Dagher et al., 1995; Hubert et al., 1995], an exploration of the colocalization of CBP70 and the splicing component SC35 was performed. The binding of anti-CBP70 was revealed with FITC anti-rabbit Ig (Fig. 5D). The binding of anti-SC35 was revealed with Cy5-conjugated goat anti-mouse Ig (Fig. 5E). In the composite of double-immunofluorescence patterns, the yellow areas represent regions of coincidence of the green (FITC) and red (Cy5) stains. Using confocal analysis, no yellow fluorescence was observed (Fig. 5F,G), implying no colocalization between CBP70 and SC35 in the spliceosome assembly. These data strongly suggest that the interaction between the two partners (CBP70 and galectin-3), previously detected by affinity chromatography [Sève et al., 1993], probably takes place in the dense fibrillar component of nucleoli [Hubert et al., 1995].

Gold conjugated anti-CBP70 does not label the plasma membrane (Fig. 4A). This observation corroborates the fluorescence data of intact HL60 cells labeled with FITC-anti-CBP70 (data not shown). Within the cytoplasm, it was possible to observe a low labeling of CBP70 in the ER (Fig. 4B). However, probably because of the technique used, it was difficult to detect any association between CBP70 and the Golgi apparatus (Fig. 4). More gold particles were associated with the mitochondria (Fig. 4C). To explore the possible presence of CBP70 associated with the ER, Golgi, and mitochondria (as observed by electron microscopy), a double la-

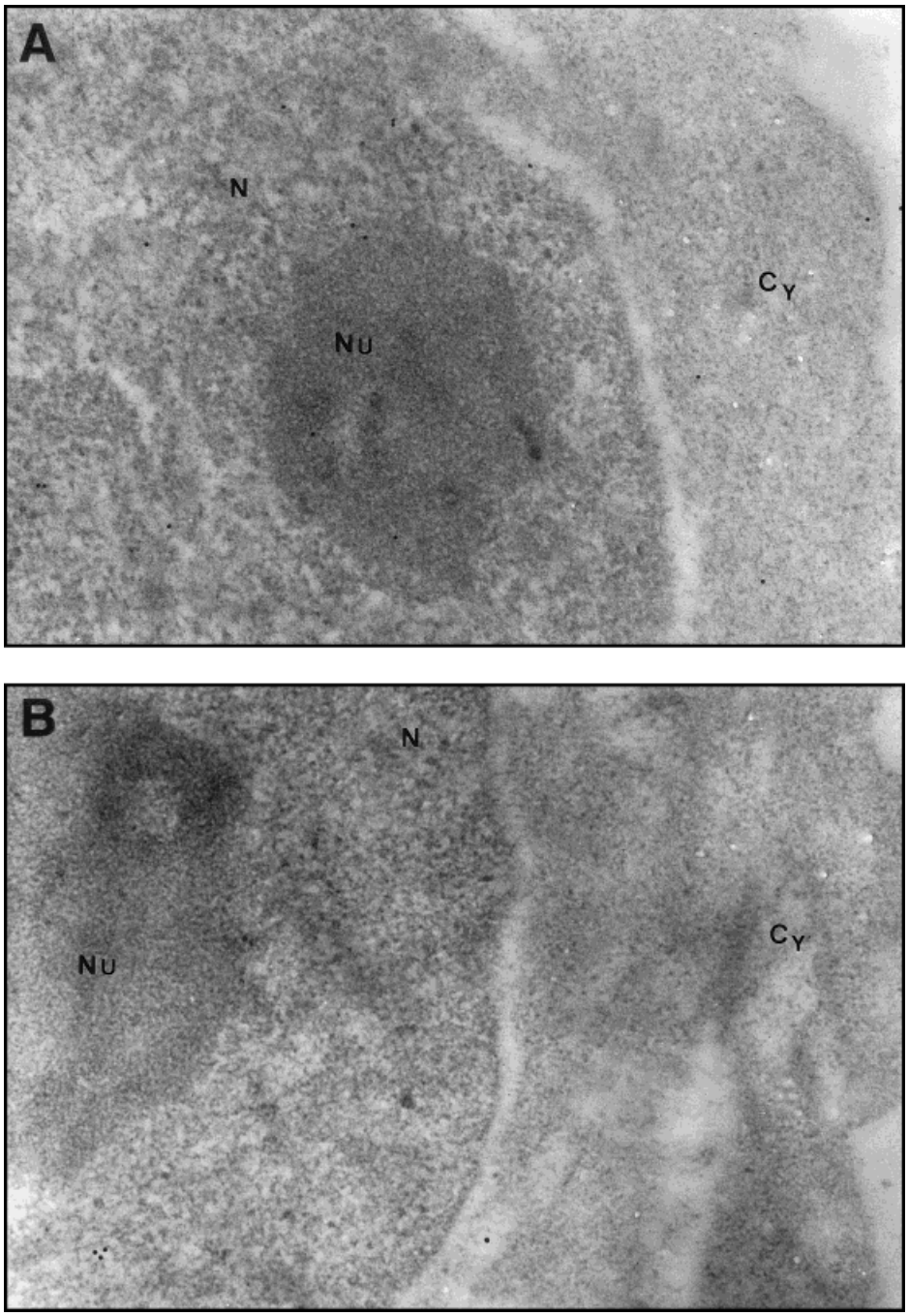

Fig. 2. Electron micrographs of sections of undifferentiated HL60 cells. Thin sections were reacted with (**A**) the preimmune serum of the rabbit anti-CBP70 (1:25) and gold-conjugated goat anti-rabbit Ig (1/10); or (**B**) the gold-conjugated goat anti-rabbit Ig alone (1:10). Note the specificity of gold particles in the nucleus and the cytoplasm. N, nucleus; Nu, nucleolus; Cy, cytoplasm (×55,000).

beling with specific antibodies of these cytoplasmic compartments was performed. Anti-CBP70 was still observed by FITC fluorescence (Fig. 5H,L,P). In all cases, the monoclonal mouse anti-PDI antibody, mouse anti-Golgi-β Cop (clone M3A5), and mouse anti-human mitochondria were revealed with Cy5-conjugated goat anti-mouse Ig (Fig. 5I,M,Q, respectively). Colocalization of anti-PDI and anti-CBP70 was visualized by an intense yellow fluorescence in the cytoplasmic area near the nucleus, allowing us to conclude that CBP70 is localized in

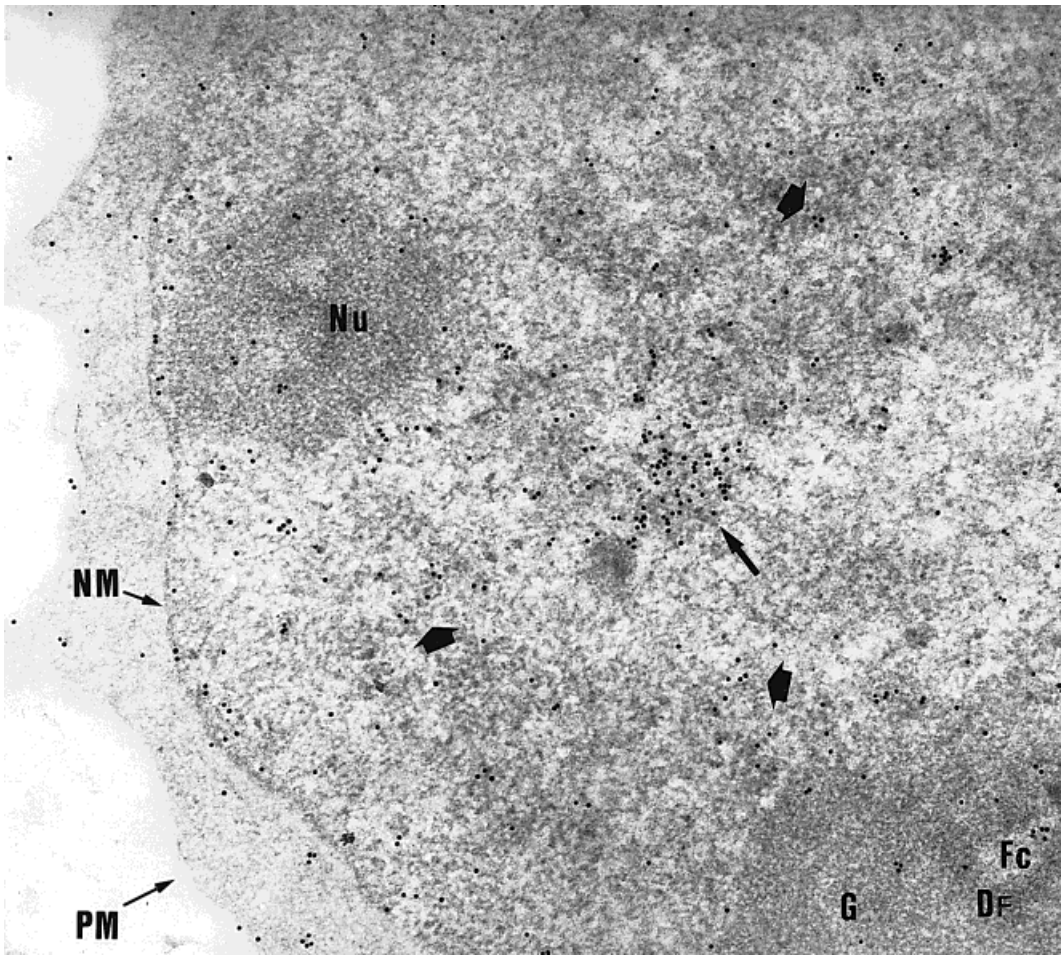

Fig. 3. Electron micrograph of undifferentiated HL60 cells illustrating specific localization of CBP70. Thin sections were reacted with rabbit anti-CBP70 (1:25) and gold-conjugated goat anti-rabbit Ig (1:10). Nu, nucleolus; Fc, fibrillar center; DF, dense fibrillar region; G, granular region; NM, nuclear membrane; PM, plasma membrane; thick arrows, interchromatin space; thin arrow, nuclear area with accumulation of gold particles (×55,000).

the ER (Fig. 5J,K). Similar results were obtained during immunostaining of the Golgi apparatus (Fig. 5N,O) and mitochondria (Fig. 5R,S). All these data argue for an association of CBP70 with the different cellular compartment (endoplasmic reticulum, Golgi apparatus, and mitochondria). However, it cannot be ruled out that a diffuse cytosolic location may lead to apparent colocalization with the other markers. The ultrastructural localization of CBP70 in mitochondria confirms previous results obtained with antibodies raised against Bcl-2 and anti-CBP70, strongly suggesting that these two proteins interact at the mitochondria membrane (unpublished observations). Nonspecific staining was not observed with FITC anti-rabbit Ig alone (Fig. 5A) and/or Cy5-conjugated goat anti-mouse Ig (Fig. 5B,C).

According to the results reported above, subcellular fractionation experiments confirm the presence of CBP70 in the 1) nuclear (Fig. 6A, lane 1); 2) cytosolic (Fig 6A, lane 2); 3) mitochondria (Fig. 6A, lane 3); and 4) microsomal fractions (Fig. 6A, lane 4). The verification of the purity of each subcellular fraction obtained was done by using the antibodies raised against different specific marker proteins previously described such as: monoclonal mouse anti-splicing factor SC-35 Ab for the nuclear fraction (Fig. 6B), polyclonal rabbit anti-glucose-6-phosphate dehydrogenase Ab for the cytosolic fraction (Fig. 6C), monoclonal mouse anti-human mitochondria Ab for the mitochondrial fraction (Fig. 6D), monoclonal mouse anti-Golgi 58K Ab (Fig. 6E), and monoclonal mouse anti-protein disulfide isomerase Ab (Fig. 6F)

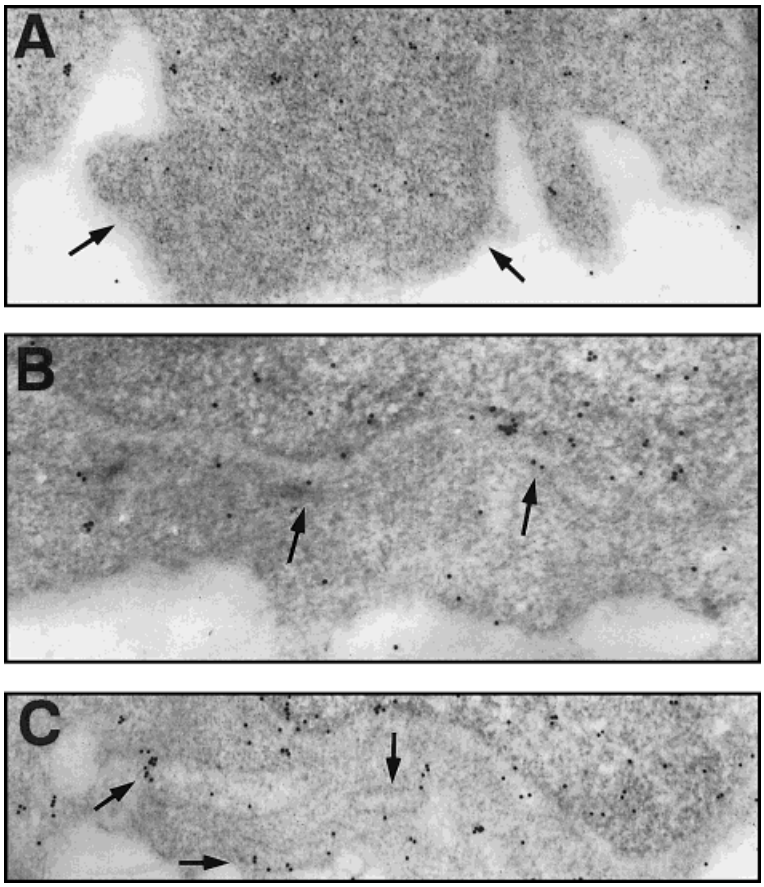

Fig. 4. Electron micrograph of cytoplasmic details in undifferentiated HL60 cells. Thin sections were labeled with rabbit anti-CBP70 (1:25) and gold-conjugated goat anti-rabbit Ig (1:10). **A:** Plasma membrane (arrows). **B:** Endoplasmic reticulum (arrows). **C:** Mitochondria (arrows). (×55,000.)

for the microsomal fraction. It can be noted that a labeling is also detected in the nuclear and the cytosolic fraction with the monoclonal mouse anti-protein disulfide isomerase Ab (Fig. 6F, lanes 1 and 2). This is because the monoclonal mouse anti-protein disulfide isomerase Ab also recognized calreticulin as reported by Vaux and collaborators [Vaux et al., 1990] (Fig. 6F, upper band in lanes 1, 2, and 4) calreticulin is a plurilocalized protein that is found in the nuclear, the cytosolic, and the endoplasmic reticulum [Hammond and Helenius, 1995; Krause and Michalak, 1997]. Altogether, the confocal analyses and these data did not allow confirmation of whether the CBP70 is located either inside or at the surface of the compartments.

We reported previously that the nuclear and cytoplasmic forms of CBP70 were glycosylated by the addition of *N*- and *O*-linked oligosaccharides chains [Rousseau et al., 1997]. Despite the growing number of nuclear glycoproteins described [Stein et al., 1975, 1981; Hubert et al., 1985; Ferraro et al., 1991; Codogno et al., 1992], the mechanism of glycosylation of nuclear proteins remains unknown. It is generally admitted that these nuclear glycoproteins do not pass through the endoplasmic reticulum and the Golgi apparatus, the two compartments where classic *N*- and *O*-glycosylation occur. In this context, two hypotheses were presented in our previous article, to explain the glycosylation of CBP70: 1) CBP70 could be glycosylated by glycosyltransferases present in the cytosol; or 2) CBP70 could enter in the ER and the Golgi apparatus, despite the absence of a signal peptide, become glycosylated and emerge into the cytosol, targeted toward different localizations (e.g., nucleus, mitochondria, etc.). Interestingly, the results obtained in this article with anti-ER and anti-Golgi might support the second hypothesis [Rousseau et al., 1997]. Because of the association of CBP70 found with the ER and the Golgi apparatus, it

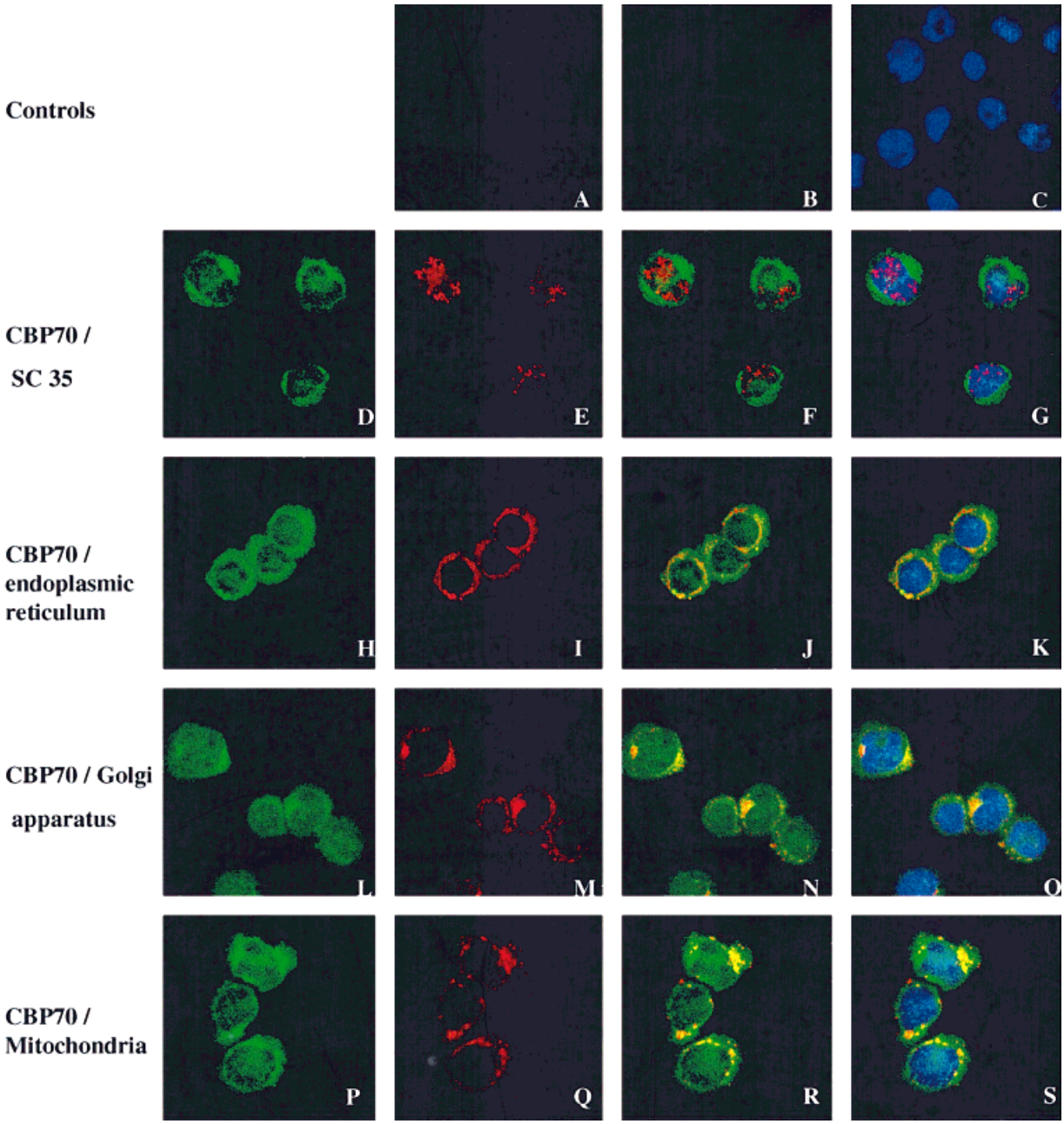

Fig. 5. Immunofluorescent subcellular localization of CBP70 in permeabilized HL60 cells. Undifferentiated HL60 cells were permeabilized with acetone, labeled with: 1) anti-CBP70 (1/200) and FITC-conjugated swine anti-rabbit Ig (1/20) (green fluorescence; **D,H L,P**); 2) anti-splicing factor SC-35 (1/1,000) and Cy5-conjugated goat anti-mouse Ig (1/100) (red fluorescence; **E**); 3) antiprotein disulfide isomerase (1/20) (**I**); 4) anti-Golgi-β Cop (1/20) (**M**) and 5) anti-human mitochondria (1/100), revealed with Cy5-conjugated goat anti-mouse Ig (1/100) (**Q**); 6) simultaneously, with anti-CBP70 and antisplicing factor SC-35 (**F,G**); 7) anti-CBP70 and antiprotein disulfide isomerase (**J,K**); 8) anti-CBP70 and anti-Golgi-β Cop (**N,O**); 9) anti-CBP70 and anti-human mitochondria (**R,S**). Note that in panel **J** or **K**, **N** or **O**, and **R** or **S**, green (CBP70) and red (protein disulfide isomerase, Golgi-β Cop or mitochondria) overlap in some area of the cytoplasm to form yellow, indicating that these proteins are colocalized. Cell nuclei were labeled with DAPI and represented in blue (**C**, **G**, **K**, **O**, and **S**). Controls were performed with: (**A**) FITC-conjugated swine anti-rabbit Ig alone (1/20); (**B**) Cy5-conjugated goat anti-mouse Ig alone (1/100); (**C**) FITC-conjugated swine anti-rabbit Ig, in presence of Cy5-conjugated goat anti-mouse Ig (1/100) and DAPI.

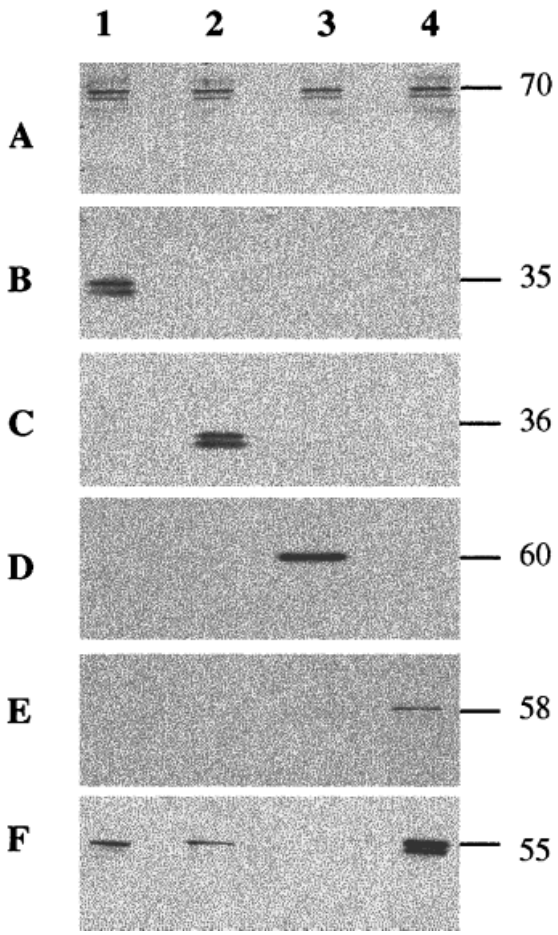

Fig. 6. Subcellular distribution of CBP70 in HL60 cells. The nuclear (**lanes 1**), the cytosolic (**lanes 2**), the mitochondrial (**lanes 3**), and the microsomal (**lanes 4**) subcellular fractions from HL60 cells were obtained according to the protocol described in Material and Methods. To detect any possible cross-contamination between subcellular compartments, the protein load in each lane has been adjusted so that the CBP70 amounts in each lane of (**A**) were roughly identical (identical signal intensity), irrespective of the amounts of CBP70 in each compartment, and allow a direct comparison with the intensity of a marker specific for a compartment (**C–F**). Each subcellular fraction was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (gel 12%), electrotransferred and subjected to immunoblotting analysis using either anti-CBP70 antibody (**A**), antisplicing factor SC-35 antibody (**B**), anti-glucose-6-phosphate dehydrogenase antibody (**C**), anti-human mitochondria antibody (**D**), anti-Golgi 58K protein antibody (**E**), or anti-protein disulfide isomerase antibody (**F**).

is now reasonable to postulate that after emerging from the ER and the Golgi apparatus into the cytosol, CBP70 might be directed toward its final subcellular localization by the addition of posttranslational modifications. At the present time, our study only shows a CBP70 distribution at a steady state. A molecular biology approach with the cloning of CBP70 cDNA and ectopic expression of CBP70 protein will be necessary for a precise analysis of posttranslational modifications involved in the intracellular traffic of CBP70. *O*-GlcNAc modification is a good candidate for this posttranslational signal, because 1) CBP70 glycosylation is different for the cytoplasmic form, compared to the nuclear form; 2) only the nuclear CBP70 contains terminal *N*- or *O*-linked GlcNAc, as well as α-L-fucose [Rousseau et al., 1997]; and 3) *O*-GlcNAc allows the targeting of neoglycoproteins inside the nucleus [Duverger et al., 1993, 1995, 1996].

In light of the results presented here with the CBP70, experiments are in progress to determine whether other nuclear glycoproteins exhibit the process of glycosylation as postulated for the CBP70.

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