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CBP70, a Glycosylated Nuclear Lectin

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Abstract Some years ago, a lectin designated CBP70 that recognized glucose (Glc) but had a stronger affinity for N-acetylglucosamine (GlcNAc), was first isolated from HL60 cell nuclei. Recently, a cytoplasmic form of this lectin was described, and one 82 kDa nuclear ligand was characterized for the nuclear CBP70. In the present study, the use of Pronase digestion and the trifluoromethanesulphonic acid (TFMS) procedure strongly suggest that the nuclear and the cytoplasmic CBP70 have a same 23 kDa polypeptide backbone and, consequently, could be the same protein. In order to know the protein better and to obtain the best recombinant possible in the future, the post-translational modification of the nuclear and cytoplasmic CBP70 was analyzed in terms of glycosylation. Severals lines of evidence indicate that both forms of CBP70 are N- and O-glycosylated. Surprisingly, this glycosylation pattern differs between the two forms, as revealed by β-elimination, hydrazinolysis, peptide-N-glycosydase F (PNGase F), and TFMS reactions. The two preparations were analyzed by affinity chromatography on immobilized lectins [*Ricinus communis-I* agglutinin (RCA-I), *Arachis hypogaea* agglutinin (PNA), *Galanthus nivalis* agglutinin (GNA), and wheat germ agglutinin (WGA)] and by lectin-blotting analysis [*Sambucus nigra* agglutinin (SNA), *Maackia amurensis* agglutinin (MAA), *Lotus tetragonolobus* (Lotus), succinylated-WGA, and *Psathyrella velutina* agglutinin (PVA)]. Both forms of CBP70 have the following sugar moities: terminal βGal residues, Galβ1–3 GalNAc, Man α1–3 Man, sialic acid α2–6 linked to Gal or GalNAc; and sialic acid α2–3 linked to Gal. However, only nuclear CBP70 have terminal GlcNAc and α-L-fucose residues.

All these data are consistent with the fact that different glycosylation pattern found for each form of CBP70 might act as a complementary signal for cellular targeting. J. Cell. Biochem. 66:370–385, 1997.

Key words: nucleus; glycoprotein; lectin; HL60; affinity chromatography

The presence of nuclear lectins was demonstrated in different mammalian cell lines, such as BHK [Sève et al., 1985, 1986], HL60 [Facy et al., 1990], and 3T3 fibroblasts [Roff and Wang, 1983; Moutsatsos et al., 1986], as well as in reptilian [Hubert et al., 1985] and protozoan cells [Olins et al., 1988]. Six nuclear carbohydrate-binding proteins (CBP) have since been isolated: CBP35 [Roff and Wang, 1983], recently named galectin-3 [Barondes et al., 1994] and CBP14 [Cuperlovic et al., 1995], which recognize lactose; CBP67 [Schröder et al., 1992], CBP70 [Sève et al., 1993], and CBP33 [Lauc et al., 1994], which bind to glucose (Glc). CBP70 was also described as an N-acetylglucosamine (GlcNAc)-binding protein, as was CBP22 [Felin et al., 1994]. In addition, CBP70 is not a nuclear lectin stricto sensu. Indeed, a cytoplasmic form of CBP70 was recently described in several cell types, including HL60 cells [Hadj-Sahraoui et al., 1996; Felin et al., 1997]. Furthermore, an 82 kDa nuclear ligand for CBP70, which contains GlcNAc residues, was characterized, that showed for the first time, a nuclear glycoprotein–lectin interaction [Felin et al., 1997]. This result strengthens the postulate emitted some years ago concerning a possible involvement of such interactions in the nuclear physiology [Hubert et al., 1989]. To study the role of CBP70 and its ligand and/or in the near future, to obtain the best possible recombinant, it appeared very important to us to analyze the polypeptide in detail. For that purpose, the post-translational modifications of CBP70 were examined, with special attention being given to glycosylation, because it is now well accepted

Abbreviations: CBP, carbohydrate-binding protein; GlcNAc, N-acetylglucosamine; TFMS, trifluoromethanesulfonic acid; PNGase F, peptide-N-glycosydase F; RCA-I, *Ricinus communis-I* agglutinin; PNA, *Arachis hypogaea* Agglutinin; GNA, *Galanthus nivalis* agglutinin; Lotus, *Lotus tetragonolobus;* WGA, *Wheat germ* agglutinin; SNA, *Sambucus nigra* agglutinin; MAA, *Maackia amurensis* agglutinin; PVA, *Psathyrella velutina* agglutinin.

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that glycosylation can play important biological roles in the development, growth, function, and/or survival of an organism. The more specific biological roles of oligosaccharides are often mediated by unusual oligosaccharide sequences, by unusual presentations of common terminal sequences, or by further modifications of the sugars themselves [reviewed in Varki, 1993]. All these elements were available for cytoplasmic glycoproteins. However, despite the accumulation of evidence demonstrating that glycoproteins are present in animal cell nuclei [reviewed in Hart et al., 1989; Hubert et al., 1989; Stein et al., 1975, 1981], the biosynthesis, structure, and sugar composition of nuclear glycoproteins remain poorly documented. Most of the data currently available derive from cytological studies using lectin histochemistry [Roth, 1983; Sève et al., 1984; Kan and Pinto da Silva, 1986; Kinoshita et al., 1988], labeled glycosidases [Londono and Bendayan, 1987], or autoradiography after incorporation of radiolabeled sugars into cells [Bennet and Hemming, 1986]. However, biochemical analysis of purified high-mobility group proteins 14 and 17 indicated that these proteins are N-glycosylated [Reeves et al., 1981]. Furthermore, our group demonstrated that N-glycosylated proteins containing fucose exist in the nucleolar ribonucleoprotein complexes of HeLa cells [Codogno et al., 1992]. Ten years ago, Hart and coworkers described an unusual glycosylation form which was mainly present in the nucleus [Holt and Hart, 1986; reviewed in Haltiwanger et al., 1990, and in Hayes and Hart, 1994]. In that case, there was an O-linkage between the protein and an GlcNAc residue. Many nuclear proteins that play important roles in nuclear physiology have such a glycosylation profile, for example, Sp1 [Jackson and Tjian, 1988], RNA polymerase II [Kelly et al., 1993], serum response transcription factor [Reason et al., 1992], c-Myc [Chou et al., 1995], p53 [Shaw et al., 1996], and some nuclear pore proteins [Davis and Blobel, 1987]. More recently, the presence of sialic acids was described in addition to O-GlcNAc residues on the nucleoporin p62 [Emig et al., 1995]. Because the transcriptional activation function of Sp1 [Jackson and Tjian, 1988], the active nucleocytoplasmic exchanges [Jackson and Tjian, 1988], and the nuclear import of proteins in vitro [Finlay et al., 1987] were inhibited by exogenous vegetables lectins as wheat germ agglutinin (WGA), which binds to O-GlcNAc residues, or *Sambuccus nigra* agglutinin (SNA), which binds to sialic acids α2–6 linked to galactose (Gal) or N-acetylgalactosamine (GalNAc), it appears that glycosylation of nuclear proteins is involved in crucial nuclear functions.

This paper describes the combination of different techniques, such as affinity chromatography, labeling with a radioactive sugar, chemical reactions, and enzymatic analysis, which led us to conclude that (1) CBP70 is a glycosylated lectin; (2) this glycosylation is composed of N- and O-linked glycans, never reported so far for an internal nuclear protein; and (3) the cytoplasmic glycosylation of CBP70 differs from that of the nuclear one; however, both forms share the same polypeptide backbone.

To the best of our knowledge, this is the first time that a lectin located in both cytoplasmic and nuclear compartments has been studied from the point of view of its glycosylation. Furthermore, these results, which are in agreement with the recent data concerning a possible involvement of saccharides, as supplementary directives orienting proteins into the nucleus [Duverger et al., 1993, 1995, 1996], argue for an active role of glycosylation in CBP70 localization.

MATERIALS AND METHODS

Cell Culture and Metabolic Labeling of Cells

HL60 cells (provided by Dr. Chomienne, Hôpital Saint-Louis, Paris, France) were grown in suspension in RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco), 2 mM L-glutamine, 50 IU/ml penicillin, and 50 mg/ml 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 them in fresh culture medium every 3 days. When required, 0.25×10^8 cells grown in RPMI 1640 were transferred in glucose-free RPMI medium supplemented as described above. Then, [2-^{3}H]mannose (spec act 777 GBq/mmol, New England Nuclear, Dupont-de-Nemours, Boston, MA) was added to the culture medium (15,44 MBq/ml final). Cells were allowed to grow for 24 h in the presence of radiolabeled mannose. Cells were used between passages 15 and 40.

Cell Fractionation and Protein Extraction

Very clean membrane-depleted nuclei were isolated from HL60 cells according to a previ-

ously reported method [Facy et al., 1990]. Briefly, exponentially growing HL60 cells (1×10^9 cells) were washed twice in 10 mM Tris–HCl (pH 7.5) containing 20 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.2 mM spermidine (TKCM buffer), and collected by low-speed centrifugation (800*g* for 10 min). The cell pellets were resuspended in 20 ml of TKCM buffer containing 1 mM phenylmethylsulfonylfluoride (PMSF), 10 mg/ml each of antipain, leupeptin, pepstatin A, and chymostatin, 0.17 IU/ml aprotinin and 20 mM benzamidine as protease inhibitors (Sigma, St. Louis, MO). Triton X-100 was added at a final concentration of 0.5%. Cells were homogenized, and membrane-depleted nuclei were pelleted by centrifugation at 1,000*g* for 10 min. The supernatant was centrifuged at 100,000*g* for 1 h at 4°C, using a Beckman rotor type 55.2 Ti. The supernatant containing cytoplasmic proteins was then dialyzed against Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS^-) at 4°C overnight. This dialyzed supernatant is referred to as cytoplasmic extract throughout this paper. Nuclear proteins were extracted from membrane-depleted nuclei with NaCl adjusted to 2 M (final concentration) in TKCM buffer for 1 h at room temperature. Excess NaCl was removed by overnight dialysis against 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. The same procedure was applied with the [2-^{3}H]mannose radiolabeled cells.

CBP70 Purification

N-Acetyl-β-D-glucosamine phenylisothiocyanate and α-D-glucopyranosylisothiocyanate (Sigma) were immobilized on acetone-dehydrated Trisacryl GF 2000 M (IBF, Villeneuve-La-Garenne, France). The nuclear protein solution or the cytoplasmic extract, unlabeled or radiolabeled with [2-^{3}H]mannose, was adjusted to 0.7 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS^+) and affinity chromatography was performed as previously described [Sève et al., 1993]. To obtain purified nuclear or cytoplasmic CBP70, the proteins, eluted from GlcNAc columns with 0.2 M GlcNAc, were dialyzed overnight against PBS^+ at 4°C and then incubated with glucose columns and eluted with 0.2 M glucose. The eluted fraction was concentrated on Centricon-10 filters and, when required, nuclear and cytoplasmic CBP70 were lyophilized. The protein concentration was determined using the Micro BCA Protein Assay Reagent Kit (Pierce, Chemical Co., Rockford, IL).

Electrophoresis Analysis

The polypeptides were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% or 15% acrylamide minislab running gels under denaturing conditions, according to Laemmli's procedure [Laemmli, 1970]. The polypeptide bands were visualized by silver staining. The radiolabeled polypeptides were visualized by autoradiography.

Immunoblotting Analysis

Polypeptides, resolved by SDS-PAGE on 12% or 15% acrylamide minislab running gels, were transferred electrophoretically onto Immobilon-P paper (Millipore, Bedford, MA) (60 V at 4°C for 1 h) in 10 mM (cyclohexylamino)-1-propane sulfonic acid, pH 10.7 (Caps buffer). The Immobilon paper was incubated overnight in saturating TBS buffer (20 mM Tris–HCl (pH 7.4), 0.5 M NaCl) containing 5% milk powder. Then it was washed three times in TBS containing 0.5% Tween 20 (TBS-Tween) and incubated for 3 h with rabbit antiserum raised against CBP70 (anti-CBP70), obtained as previously described [Sève et al., 1994] and diluted 1:1,000 in TBS-Tween. After incubation in the presence of antibodies, the paper was washed three times in TBS-Tween, incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat antirabbit IgG (Sigma) at a dilution of 1:10,000, then washed three times in TBS-Tween and finally in Tris-buffer (0.1 M Tris–HCl (pH 9.5), 100 mM NaCl and 5 mM $MgCl_2$) before staining with the 5-bromo 4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (Sigma) substrate. Controls were run to ascertain that background levels of nonspecific binding of alkaline phosphatase-conjugated goat antirabbit IgG were acceptable and that endogenous alkaline phosphatase was absent.

Pronase Digestion

Pronase (3 mg, 76,000 PUK/g) from *Streptomyces griseus* (Calbiochem, San Diego, CA) was pre-incubated for 30 min at 37°C in 1 ml of Pronase buffer [0.1 M Tris–HCl buffer (pH 8.0) containing 2 mM $CaCl_2$] in order to remove any

possible glycosidase activities. One-half of this Pronase solution (0.5 ml) was added to 30 µg of each lyophilized form of CBP70 and incubated at 57°C overnight (16 h); then, the second part of the Pronase solution was added and incubated again at 57°C for 8 h. The samples were boiled for 2 min to denature the residual enzyme and subjected to SDS-PAGE. The polypeptide bands were visualized by silver staining or immunoblotting analysis. Controls were run to ensure the specificity of the reaction, by incubating both form of CBP70 without Pronase and the Pronase alone, under the same conditions.

β-Elimination Reaction

Each lyophilized form of CBP70 (20 µg) was resuspended in 2×10^{-3} M NaOH, 10 µl of $NaB[^3H]_4$ (185 MBq/50 µl, DuPont-New England Nuclear), and tubes were incubated in ice for 2 h. Then, 2 M $NaBH_4$ in 0.1 M NaOH was added to the mixture, and incubation in ice was continued for 1 h. The solution was heated in a sealed tube at 45°C for 16 h [Spiro and Bhoyroo, 1974]. The tube was cooled to room temperature, and the excess $NaBH_4$ was destroyed by drop-by-drop addition of 300 µl of glacial acetic acid. The reaction mixtures were then passed through a column of AG50 W-X12 (H^+ form) (BioRad, Richmond, CA), the eluates were collected in a round-bottomed flask kept in ice, 1.5 ml of 1 M pyridine was added, and this mixture was evaporated to dryness below 30°C. The released oligosaccharides were resuspended in 50 µl of water and then subjected to paper chromatography for 24 h (Schleicher et Schüll 2043B, Dassel, W. Germany) using 1-butanol–pyridine: 0.1 M HCl (5:3:2, v/v) as the mobile phase. The radioactive peaks were detected with an automatic TLC-linear analyzer (Berthold, Paris, France).

Hydrazinolysis

Each lyophilized form of CBP70 (20 µg) was subjected to hydrazinolysis as previously described [Takasaki et al., 1982]. Briefly, the samples were transferred into a Teflon-capped tube, dried under vacuum, resuspended in 1 ml of freshly distilled anhydrous hydrazine (Pierce Chemical Co.) and heated at 100°C for 16 h. After evaporation of hydrazine under nitrogen, the residues were dissolved in 1 ml of saturated sodium bicarbonate solution, and N-re-acetylation was carried out with acetic anhydride (500 µl). The reaction mixtures were passed through a column of AG50 W-X12 (H^+ form), using water as the eluant. The eluates and washes obtained were evaporated to dryness below 30°C, and the residues obtained were dissolved in 200 µl 0.05 M NaOH, labeled with 5 µl of the dimethylformamide solution of $NaB[^3H]_4$ (185 MBq/50 µl, DuPont-New England Nuclear) and incubated in ice for 4 h. Then, 100 µl of $NaBH_4$ (5 g/l) were added to each sample, and after 1 h of incubation in ice, the reaction was stopped by adding 100 µl 1 M acetic acid. Each reaction mixture was passed through a new column of AG50 W-X12 (H^+ form), the eluates and the wash fluids were evaporated as described above, and the residues obtained were dissolved in 1 ml methanol. After evaporation, the samples were resuspended in 50 µl water and then subjected to paper chromatography for 16 h as above, using pyridine–ethyl acetate–acetic acid–water (5:5:1:3, v/v) as the mobile phase. The radioactive peaks were detected with an automatic TLC-linear analyzer.

Peptide N-Glycosidase F Procedure

Each lyophilized form of CBP70 (20 µg) was denatured by heating at 100°C for 2 min in 10 µl of phosphate EDTA buffer (pH 7.5–8.0) containing 0.5% SDS and 5% β-mercaptoethanol. After cooling, 2.5 µl 10% NP40 (v/v) was added prior to adding 2 units of peptide N-glycosidase F (also called PNGase F) derived from *Flavobacterium meningosepticum* (Oxford Glycosystems, Abingdon, UK). The reaction was allowed to continue for 24 or 48 h at 37°C. The samples were boiled for 2 min and the relative deglycosylation efficiency was monitored after silver staining of SDS-PAGE and immunoblotting analysis. Controls were run to assure the specificity of the reaction by incubating both form of CBP70 without PNGase F and the PNGase F alone, under the same conditions.

Trifluoromethanesulfonic Acid Treatment

Each lyophilizated form of CBP70 (20 µg) was deglycosyled by anhydrous trifluoromethanesulfonic acid (TFMS, Glycofree™ chemical deglycosylation kit, Oxford Glycosystems) for 4, 6, or 8 h at −20°C according to the manufacturer's protocol. The relative efficiency of deglycosylation of each form of CBP70 was monitored by silver staining of SDS-PAGE. Controls were run to ensure the specificity of the reaction by incubating serum albumin bovine or the recom-

binant galectin-3 with or without TFMS, under the same conditions for 8 h.

Affinity-Chromatography Analysis

Purified nuclear and cytoplamic CBP70 were independently subjected to affinity chromatography with different immobilized vegetable lectins, such as WGA insolubilized on Sepharose 6 MB (Sigma), RCA-I insolubilized on Agarose (EY Laboratories), PNA insolubilized on Agarose (Sigma), and GNA insolubilized on Agarose (Sigma). The nuclear proteins or cytoplasmic extracts were incubated with 1 ml of immobilized lectins overnight at 4°C under batch conditions. After packing in columns, the same procedure as that described above [Sève et al., 1993] was applied. The proteins specifically retained on WGA were eluted with 0.2 M GlcNAc, those retained to RCA-I were eluted with 0.2 M galactose, those bound to PNA were eluted with 0.2 M lactose, those linked to GNA were eluted with 0.3 M mannose, and then all the fractions were concentrated on Centricon-10 filters. Controls were run to assure the lack of nonspecific binding of proteins on each affinity chromatography by performing an elution with 0.2 M of an heterologous sugar, before the addition of the homologous sugar. The protein concentration was determined using the Micro BCA Protein Assay Reagent Kit (Pierce Chemical Co). The polypeptide bands were visualized by immunoblotting analysis.

Lectin-Blotting Analysis

The purified cytoplasmic or nuclear CBP70 electroblotted onto Immobilon paper was incubated overnight in saturating TBS buffer containing 2% bovine serum albumin (BSA) (Sigma). Then, it was washed three times in TBS-Tween and incubated for 2 h with the appropriate biotinylated lectins. The paper was washed three times in TBS-Tween, incubated for 1 h at 4°C with streptavidin-labeled peroxidase (Sigma) diluted 1:1,000, then washed three times in TBS-Tween before measuring epichemoluminescence reactions (Western blotting detection system (Amersham)). Controls were run to ensure the lack of non specific binding of each tested biotinylated lectins, by performing a preincubation either with 0.2 M of an heterologous sugar or with 0.2 M of the homologous sugar before the addition of each tested biotinylated lectins.

Biotinylated SNA, Lotus, and MAA were purchased from Oxford Glycosystems. Biotinylated succinylated-WGA were supplied by Sigma. Unlabeled PVA was a gift from Dr. Debray Henry (Laboratoire de Chimie Biologique, UMR 111, CNRS, Villeneuve d'Ascq, France) and was biotinylated according to Avellana-Adalid's procedure [Avellana-Adalid et al., 1990]. SNA (10 µg/ml), MAA (10 µg/ml), succinylated-WGA (10 µg/ml), Lotus (10 µg/ml) and PVA (15 µg/ml) were dissolved in TBS-Tween containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM $MnCl_2$.

RESULTS

Peptide Mapping of Nuclear and Cytoplasmic CBP70

To determine whether the nuclear and the cytoplasmic CBP70 had the same peptide backbone and consequently are the same protein, purified CBP70 from each cellular compartment were subjected to enzymatic digestion. The profiles of Pronase-digested nuclear (Fig. 1A,B, lane 1) and cytoplasmic CBP70 (Fig. 1A,B lane 3) were similar after silver staining (Fig. 1A) or immunoblotting analysis (Fig. 1B). The antibodies raised against CBP70 recognized residual CBP70 in both cases, as well as two polypeptides of 40 and 21 kDa (Fig. 1B, lanes 1 and 3). Pronase alone was subjected to electrophoretic migration, silver staining (Fig. 1A, lane 2), and immunoblotting analysis (Fig. 1B, lane 2) in order to follow the polypeptides due to the Pronase and to ensure that the polypeptides due to the pronase were not recognized by the antibodies raised against CBP70 (Fig. 1B, lane 2). In addition, to ensure that both form of CBP70 were not destroyed by the treatment, each lyophilized form of CBP70 was incubated under the same conditions without Pronase. As expected, the mobility of the nuclear (Fig. 1A, lane 4) and the cytoplasmic (Fig. 1A, lane 5) CBP70 was not affected. Therefore, these results strongly suggest that the peptidic sequence of the nuclear and the cytoplasmic CBP70 are identical.

Radiolabeling

To investigate the presence of carbohydrates on the two forms of CBP70, radioactive [2-^{3}H]mannose was metabolically incorporated into HL60 cells. With [2-^{3}H]mannose, interconversion reactions are restricted to GDP-fucose, and the radioactivity is essentially recovered in

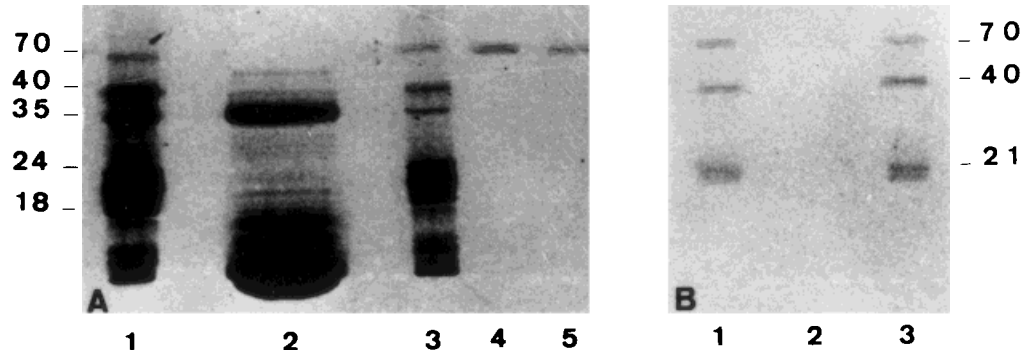

Fig. 1. Gel electrophoresis and Western blot analysis of purified nuclear and cytoplasmic CBP70 after Pronase treatment. Pronase-treated nuclear (*lane 1*) and cytoplasmic (*lane 3*) CBP70 were subjected to SDS-15% PAGE and bands were visualized by silver staining (**A**) or identified with anti-CBP70 after Western blotting (**B**). Pronase (*lane 2*), nuclear (*lane 4A*) and cytoplasmic (*lane 5A*) CBP70 were run in parallel.

N-glycans of glycoproteins. In this context, the nuclear (Fig. 2A) and the cytoplasmic CBP70 (Fig. 2B) were purified from the labeled cells after successive rounds of affinity chromatography (see Materials and Methods), analyzed on SDS-PAGE, and visualized by autofluorography after impregnation with En^{3}Hance (New England Nuclear) or identified with anti CBP70 after Western blotting (data not shown). The results obtained revealed that both the nuclear (Fig. 2C, lane 1) and the cytoplasmic (Fig. 2C, lane 2) CBP70 are glycosylated.

Specific Release of the O- and/or N-Linked Oligosaccharides Borne by CBP70

O-Glycans bound to Ser and Thr were released from the protein backbone by subjecting the glycoprotein to the β-elimination reaction with dilute alkali. The oligosaccharides released from purified nuclear or cytoplasmic CBP70 were subjected to paper chromatography. The scanning of the paper detected O-linked chains on CBP70, regardless of its cellular localization. However, the profiles obtained for both forms of CBP70 differed from one another. This result led us to conclude that a different O-glycosylation pattern exist for the two forms of CBP70 (Fig. 3A).

Two different techniques were used to generate the specific release of N-linked oligosaccharides. Hydrazynolysis was the first method applied; this chemical procedure is applicable to the study of N-linked oligosaccharides without any limitations and the best chemical method for liberating N-linked oligosaccharides. Purified nuclear and cytoplasmic CBP70 were individually hydrazinolyzed, and the finals products were subjected to paper chromatography. The scanning of the paper identified N-linked chains on both forms of CBP70 but, again, the patterns obtained for the two preparations of CBP70 were different from one each other, indicating that a difference of N-glycosylation exist between the two forms (Fig. 3B). The differences obtained with both techniques—β-elimination and hydrazinolysis—were reproducible, and the experiments were run five times. Because hydrazinolysis is known to be a drastic treatment, the release of N-linked oligosaccharides was also induced by peptide-N-glycosydase F (PNGase F), a glycosidase that, under very gentle conditions, highly specifically engenders the release of this kind of oligosaccharide, without destroying the polypeptide part of the glycoprotein. However, the weakest element of this method is the inaccessibility of the sugar chains on glycoproteins to the glycosidase, when the proteins are heavily glycosylated. Each form of the CBP70 was deglycosylated with the PNGase F. This glycosidase removes the N-linked oligosaccharides by cleaving the bond between the Asn and the innermost GlcNAc of the glycan. Moreover, all the O-glycans, including those with the unusual single GlcNAc residue linkage to Ser or Thr, remained unaffected. After silver staining, the profiles of PNGase F-digested nuclear (Fig. 4A, lane 2) and cytoplasmic (Fig. 4A, lane 4) revealed three polypeptides of 41, 31, and 27 kDa, as well as some residual 70 kDa polypeptide. However, in the nuclear fraction, an additional polypeptide of 25 kDa was visible (Fig. 4A, lane 2). In both cases, the 35 kDa polypeptides generated by PNGase F were seen after silver staining (Fig. 4A, lanes 2,

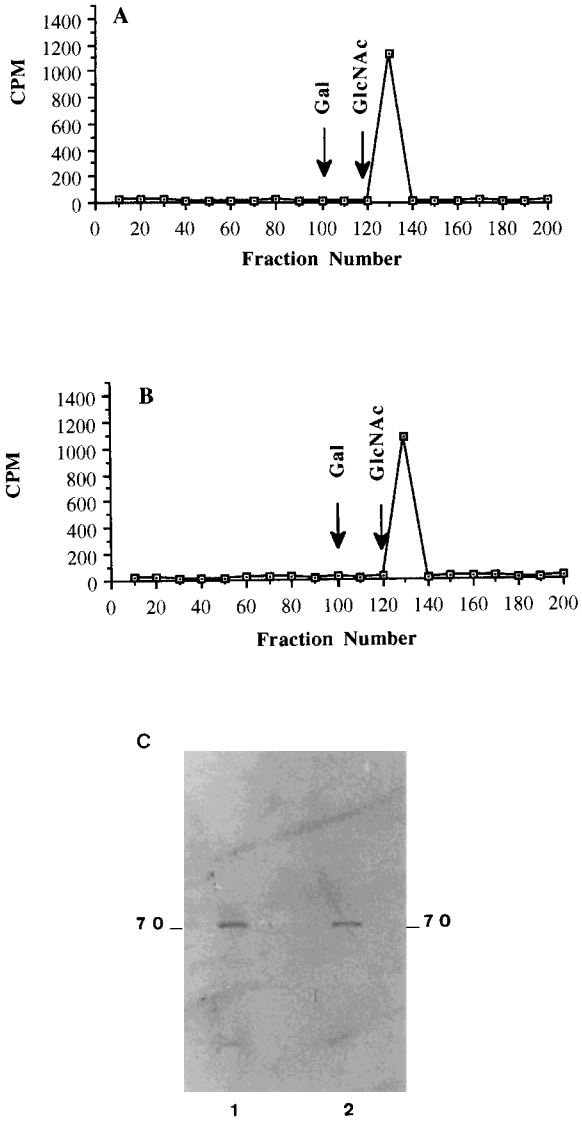

Fig. 2. Metabolic labeling of the nuclear and the cytoplasmic CBP70 by incorporation of [2-^{3}H]mannose. Elution profil of radiolabeled nuclear (**A**) and cytoplasmic (**B**) CBP70 after the second round of affinity chromatography (Glc affinity chromatography). The specific Glc elution obtained in both cases were subjected to SDS–12% PAGE and were visualized by autoradiography (**C**). *Lane 1,* nuclear CBP70; *lane 2,* cytoplasmic CBP70.

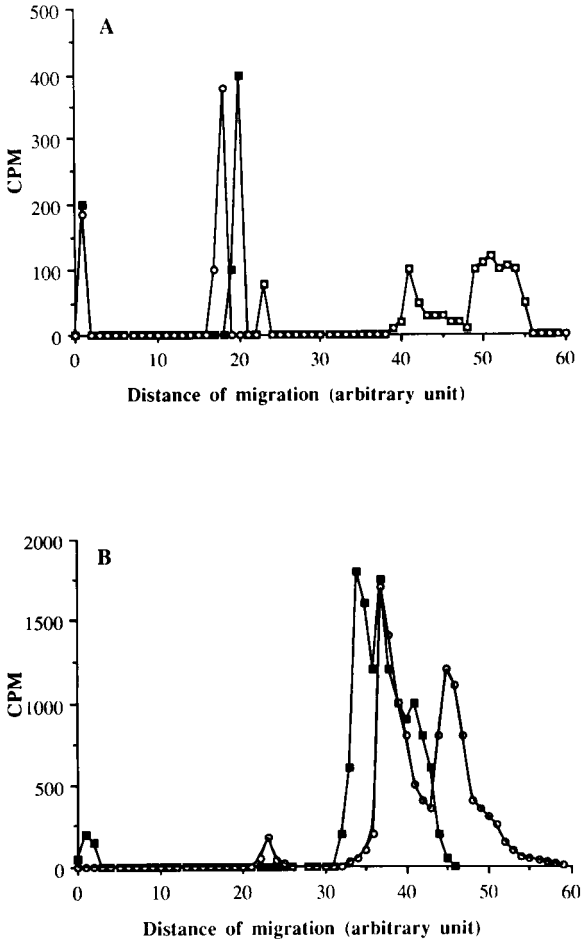

Fig. 3. β-Elimination and hydraznolysis reactions of purified nuclear and cytoplasmic CBP70. **A:** radiolabeled O-linked oligosaccharides were released from nuclear (-○-) or cytoplasmic (-■-) CBP70 after β-elimination. **B:** Radiolabeled N-linked oligosaccharides were released from nuclear (-○-) or cytoplasmic (-■-) CBP70 after hydrazynolysis. In both cases, -□- correspond to a similar pattern between the nuclear and the cytoplasmic oligosaccharides released.

4). Immunoblotting analysis detected two polypeptides of 70 and 41 kDa in each fraction, but the polypeptides of 31, 27, and 25 kDa were not recognized by the antibodies raised against CBP70 (Fig. 4B, lanes 2, 4). Therefore, it appeared that the anti-CBP70 bound to the carbohydrate part of the protein. To be sure that the shift in mobility was due to the removal of N-linked oligosaccharides by the enzyme and not, for example, to proteolytic cleavage by an endogenous protease, both lyophilized forms of CBP70 were incubated under the same conditions without PNGase F. As expected, the mobility of the nuclear (Fig. 4A,B, lane 1) and the cytoplasmic (Fig. 4A,B, lane 3) CBP70 was not affected. Moreover, to confirm that the bands of 27 and 25 kDa were not derived from the PNGase F preparation, the PNGase F was submitted to the same protocol. In this case, only the 35 kDa polypeptide due to the PNGase F could be visualized after silver staining (Fig. 4C). This finding allowed us to conclude that only both 25 and 27 kDa bands were derived from the CBP70 preparations.

According to the results previously reported, the use of PNGase F confirmed (1) the presence of N-linked chains on both forms of CBP70, and

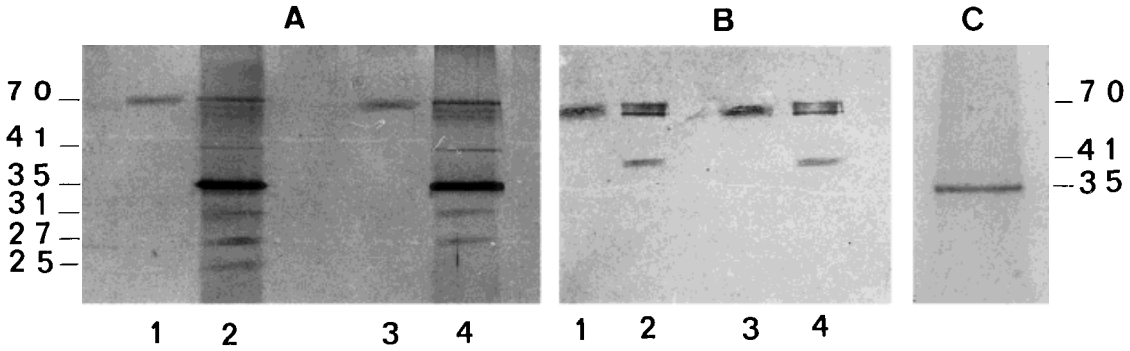

Fig. 4. Gel electrophoresis and Western blot analysis of purified nuclear and cytoplasmic CBP70 treated with peptide N-glycosidase. Nuclear (*lane 2*) and cytoplasmic (*lane 4*) CBP70 treated with peptide N-glycosidase were subjected to SDS–12% PAGE and bands were visualized by silver staining (**A**) or identified with anti-CBP70 after Western blotting (**B**). Nuclear (A, B, *lane 1*) and cytoplasmic (A, B, *lane 3*) CBP70 incubated under the same conditions without PNGase F, and the PNGase F alone (**C**) were run in parallel.

(2) a different N-glycosylation pattern between the two forms of CBP70.

Trifluoromethanesulfonic acid (TFMS) was used to totally deglycosylase both forms of CBP70. In this context, a time course of the deglycosylation reaction was done on both forms of CBP70. After 4 h of TFMS treatment, no indication of deglycosylation could be visualized on the nuclear and the cytoplasmic CBP70 (data not shown). However, after 6 h of TMSF treatment, only slight deglycosylation of the nuclear CBP70 could be visualized with the appearance of a 66 kDa polypeptide (Fig. 5A, lane 2), while the deglycosylation of the cytoplasmic CBP70 treated in parallel was greater, as revealed by the presence of the three polypeptides of 41, 36, and 34 kDa (Fig. 5A, lane 4). After 8 h of treatment, deglycosylation was more extensive for the nuclear CBP70, as revealed by the appearance of a series of new polypeptides ranging from 66 kDa to 23 kDa (Fig. 5B, lane 2). For, the cytoplasmic CBP70, the deglycosylation pattern obtained was similar to the one obtained after 6 h of TFMS treatment with, in addition, the appearance of two polypeptides of 25 kDa and 23 kDa (Fig. 5B, lane 4). The increase of the density of polypeptides with a range of 41 to 23 kDa, when cytoplasmic CBP70 was treated for 8 h, can be attributed to the better accessibility of the silver ions to the carboxyl and sulfhydryl groups of CBP70 during staining, after the carbohydrate moities had been attacked by TFMS. To ensure that the shift in mobility was not due to proteolytic cleavage by a contaminating protease, both forms of CBP70 were incubated under the same conditions without TFMS. As expected, the mobility of the nuclear (Fig. 5A,B, lane 1) and the cytoplasmic (Fig. 5A,B, lane 3) CBP70 was not affected. Moreover, the absence of polypeptide cleavage by the TFMS was verified by subjecting BSA, a non-glycosylated protein and the recombinant galectin-3, a nonglycosylated nuclear lectin, to the same protocol. As expected, after 8 h of TFMS treatment, the mobility of the bovine serum albumin (Fig. 5C, lane 3), as well as the mobility of the recombinant galectin-3 (Fig. 5C, lane 4) was not affected. When the control reaction was performed without TFMS, the mobility of BSA (Fig. 5C, lane 1), as well as the mobility of the recombinant galectin-3 (Fig. 5C, lane 2), was not affected. It should be noted that only polypeptides within the range of 70–56 kDa were obtained after immunoblotting analysis of both fractions after 8 h of TFMS treatment (data not shown). These results are similar to those obtained after PNGase F digestion and indicate that the anti-CBP70 recognize the carbohydrate part of the protein. Thus, the different patterns of deglycosylation observed between the nuclear and the cytoplasmic CBP70 after 6 or 8 h of TFMS attack confirmed (1) the presence of glycans moieties on both forms of CBP70, (2) the difference of glycosylation between the nuclear and the cytoplasmic CBP70, and (3) that the two forms have a similar 23 kDa polypeptide. Interestingly, it can be noted that, in nuclear and cytoplasmic preparations, regardless of the deglycosylation technique (PNGase F and TFMS), a 70 kDa polypeptide remained. Consequently, it appears that the deglycosylation of CBP70 was not complete (Figs. 4A,B, lanes 2, 4, 5A,B, lanes 2, 4) probably due to the

multimerization of CBP70 after its purification (data not shown). Indeed, it must be kept in mind that this protein which is a lectin is also a glycoprotein that allows the purified protein to form multimeric complexes. This process of multimerization is increased by the reaction conditions necessary for the deglycosylation procedure and then, all the CBP70 molecules are not accessible for the different reagents used.

The incorporation of [2-^{3}H]mannose and the different deglycosylation techniques used led us to conclude that both forms of CBP70 are glycosylated. In both cases, this glycosylation consists of O- and N-linked oligosaccharides. Most surprisingly, the data obtained revealed a difference between the glycosylation patterns of the nuclear and the cytoplasmic forms of the protein.

Affinity Chromatography and Lectin-Blotting Analysis

Because of the different CBP70 glycosylations observed between the nuclear and cytoplasmic forms, the next obvious step was to determine which sugars are involved in such differences. Within this context, purified nuclear and cytoplasmic CBP70 were subjected to *Ricinus communis* agglutinin-1 (RCA-1, specific to terminal βGal residues), *Arachis hypogaea* (peanut) agglutinin (PNA, specific to Gal β1–3 GalNAc α1-Ser/Thr), and *Galanthus nivalis* agglutinin (GNA, specific to terminal Man α1–3 Man residues) affinity chromatography. The unbound and specifically eluted materials were electrophoresed and immunoblotted. The results obtained with the RCA-I, PNA, and GNA (Fig. 6A,B,C, respectively) columns showed that the nuclear (lane 3) and cytoplasmic (lane 6) forms were found in the eluted fraction. This indicated, respectively, the presence of the following sugar residues: (1) terminal βGal residues, (2) Gal β1–3 GalNAc α1-Ser/Thr (which confirm the presence of O-linked residues on both forms of CBP70), and (3) terminal Man α1–3 Man residues on both forms of CBP70 that strongly indicate the presence of high-mannose oligosaccharides on both forms of CBP70.

It should be noted that a part of the nuclear (lanes 1) and cytoplasmic (lanes 4) CBP70 are also found in the unbound fraction. This result might be explained by the saturation of the columns or by the modification of the 3D structure of the protein probably due to the degradation of the protein.

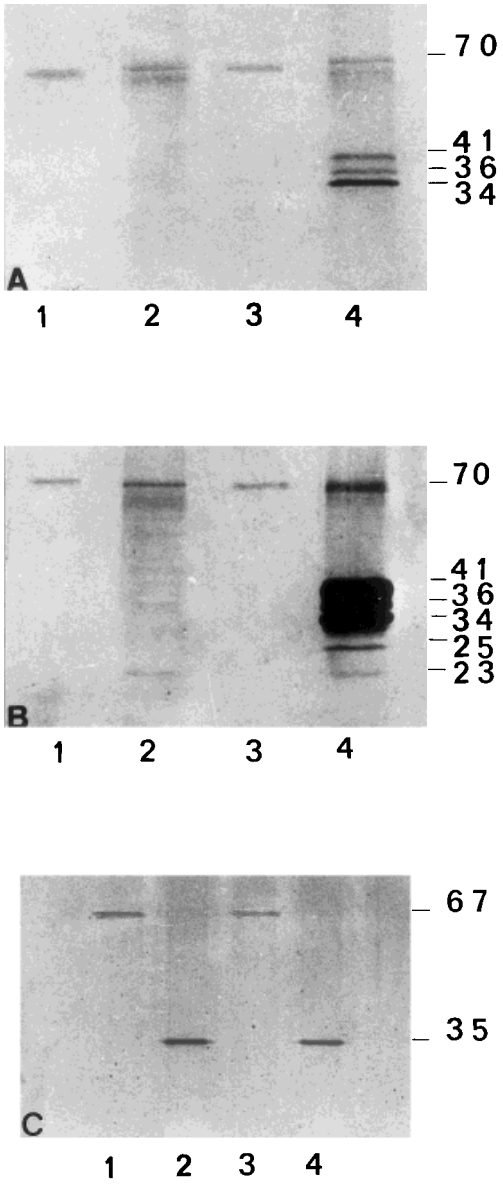

Fig. 5. Gel electrophoresis and Western blot analysis of purified nuclear and cytoplasmic CBP70 after TFMS treatment. Nuclear (*lane 2*) and cytoplasmic (*lane 4*) CBP70 were subjected to TFMS reaction for 6 h (**A**) or 8 h (**B**). The extracts obtained were subjected to SDS–12% PAGE and bands were visualized by silver staining. Nuclear (A, B, *lane 1*) and cytoplasmic (A, B, *lane 3*) CBP70 incubated under the same conditions without TFMS were run in parallel. BSA (C, *lane 3*) and galectin-3 (C, *lane 4*) were subjected to TFMS treatment for 8 h. BSA (C, *lane 1*) and galectin-3 (C, *lane 2*) incubated under the same conditions without TFMS were run in parallel.

Moreover, various biotin-labeled lectins were used to identify other terminal sugars borne by each form of the CBP70. Biotinylated *Sambucus nigra* agglutinin (SNA, specific to sialic acid α2–6 linked to Gal or GalNAc) and *Maackia amurensis* agglutinin (MAA, specific to sialic

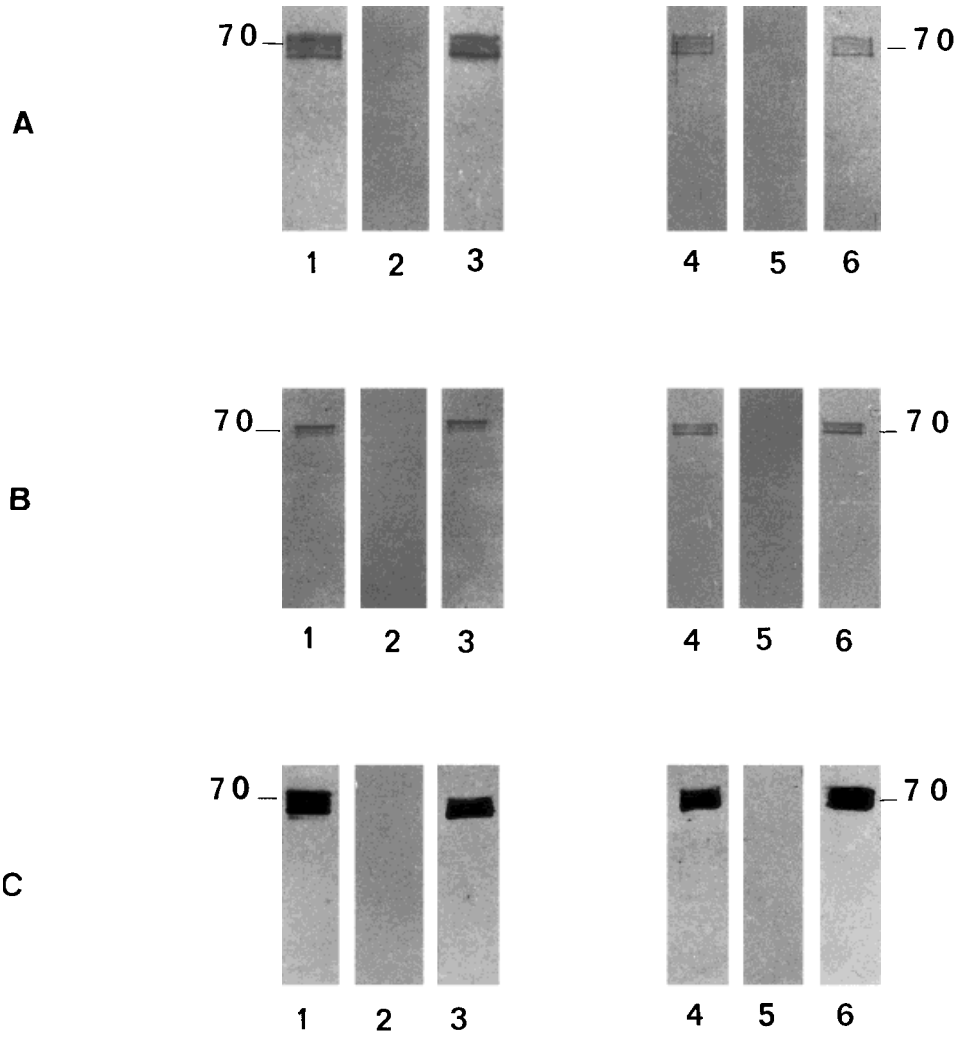

Fig. 6. Characterization of terminal saccharides residues of purified nuclear and cytoplasmic CBP70 by RCA-I (**A**), PNA (**B**), and GNA (**C**) affinity chromatography. The polypeptides were labeled with anti-CBP70 after Western blotting. *Lanes 1–3,* nuclear CBP70; *lanes 4–6,* cytoplasmic CBP70; *lanes 1, 4,* unbound fraction; *lanes 2, 5,* elution with the appropriate heterologous sugar; *lanes 3, 6,* elution with the appropriate homologous sugar.

acid α2–3 linked to Gal) blot analyses of both forms of CBP70 gave positive labeling (Fig. 7A,B lanes 1, 4, respectively), indicating that the two forms of CBP70 contain sialic acid α2–6 linked to Gal or GalNAc and sialic acid α2–3 linked to Gal. Otherwise, the use of *Lotus tetragonolobus* (Lotus, specific for α-L-fucose) revealed that only the nuclear CBP70 contain α-L-fucose residues (Fig. 7C, lanes 1, 4).

Because the results obtained by Hart and coworkers [Haltiwanger et al., 1990] indicated preferential O-GlcNAc glycosylation in the nucleus, WGA affinity chromatography was performed. Both forms of CBP70 were found in the eluted fraction (Fig. 8A, lanes 3, 6). Note the presence of nuclear (Fig. 8A, lane 1) and cytoplasmic (Fig. 8A, lane 4) CBP70 in the unbound fraction. WGA is known to have broad specificity for different sugar structures (GlcNAc β1–4 GlcNAc > GlcNAc > sialic acid). Consequently, CBP70 glycosylation was also assessed with succinylated WGA, which recognizes only the terminal GlcNAc residues. As could be expected, the succinylated WGA blot reacted positively only with nuclear CBP70 (Fig. 8B, lanes 1, 4). This result was corroborated by the use of *Psathyrella velutina* agglutinin (PVA, specific to terminal GlcNAc) (Fig. 8C, lanes 1, 4).

The degradation of cytoplasmic and nuclear CBP70 could probably be due to the three successive affinity chromatographies performed to obtain the purified CBP70 and then to isolate the protein according to its peripheral sugars. It was reported that some lectins could bind

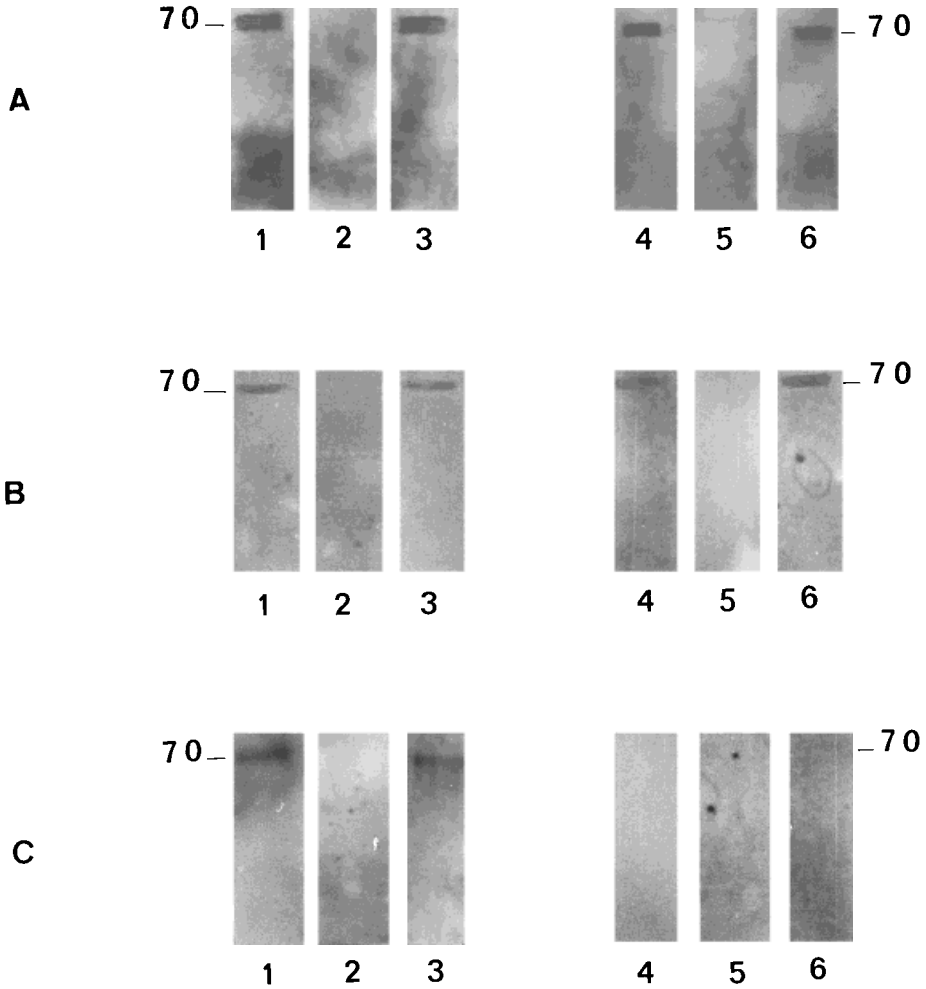

Fig. 7. Detection of sialic acids and α-L-fucose by lectin-blotting analysis with biotinylated SNA (**A**), MAA (**B**), or Lotus (**C**) lectins. *Lanes 1–3,* nuclear CBP70; *Lanes 4–6,* cytoplasmic CBP70; *Lanes 1, 4,* incubation with the appropriate lectin; *Lanes 2, 5,* competition with the appropriate homologous sugar. *Lanes 3, 6,* competition with the appropriate heterologous sugar.

with high affinity to noncarbohydrate epitopes on proteins. But in this case, this binding is through the carbohydrate recognition sites on the lectins and can be inhibited specifically with the appropriate monosaccharides [Scott et al., 1992]. Therefore, for all the different affinity chromatographies performed, the specificity of the binding was tested by doing an elution with 0.2 M of a heterologous sugar, before the addition of the homologous sugar. In such conditions, neither the nuclear nor the cytoplasmic CBP70 was eluted (respectively, Figs. 6A,B,C and 8A, lanes 2, 5). Moreover, the specificity of the lectin fixation on the blot was also tested. The binding of SNA, MAA, Lotus, succinylated WGA, and PVA was completely inhibited in the presence of 0.2 M of their competitive inhibitor sugar (Fig. 7A,B,C, lanes 2, 5) and (Fig. 8B,C lanes 2, 5), but was not inhibited in the presence of 0.2 M of an heterologous sugar (Fig. 7A,B,C, lanes 3, 6) and (Fig. 8B,C, lanes 3, 6). Consequently, these results strongly suggest that all the lectins data reported in this paper here are specifics, and confirm that a difference exists between the glycosylation of nuclear and cytoplasmic CBP70. Nuclear CBP70 has α-L-fucose and terminal GlcNAc residues, while the cytoplasmic form does not (Table I).

DISCUSSION

The major results obtained in this study demonstrated for the first time that a lectin, CBP70, which is present both in the nucleus and the cytoplasm, is a glycoprotein whose glycosylation differes depending on the subcellular compartment in which it is found. Initially, CBP70

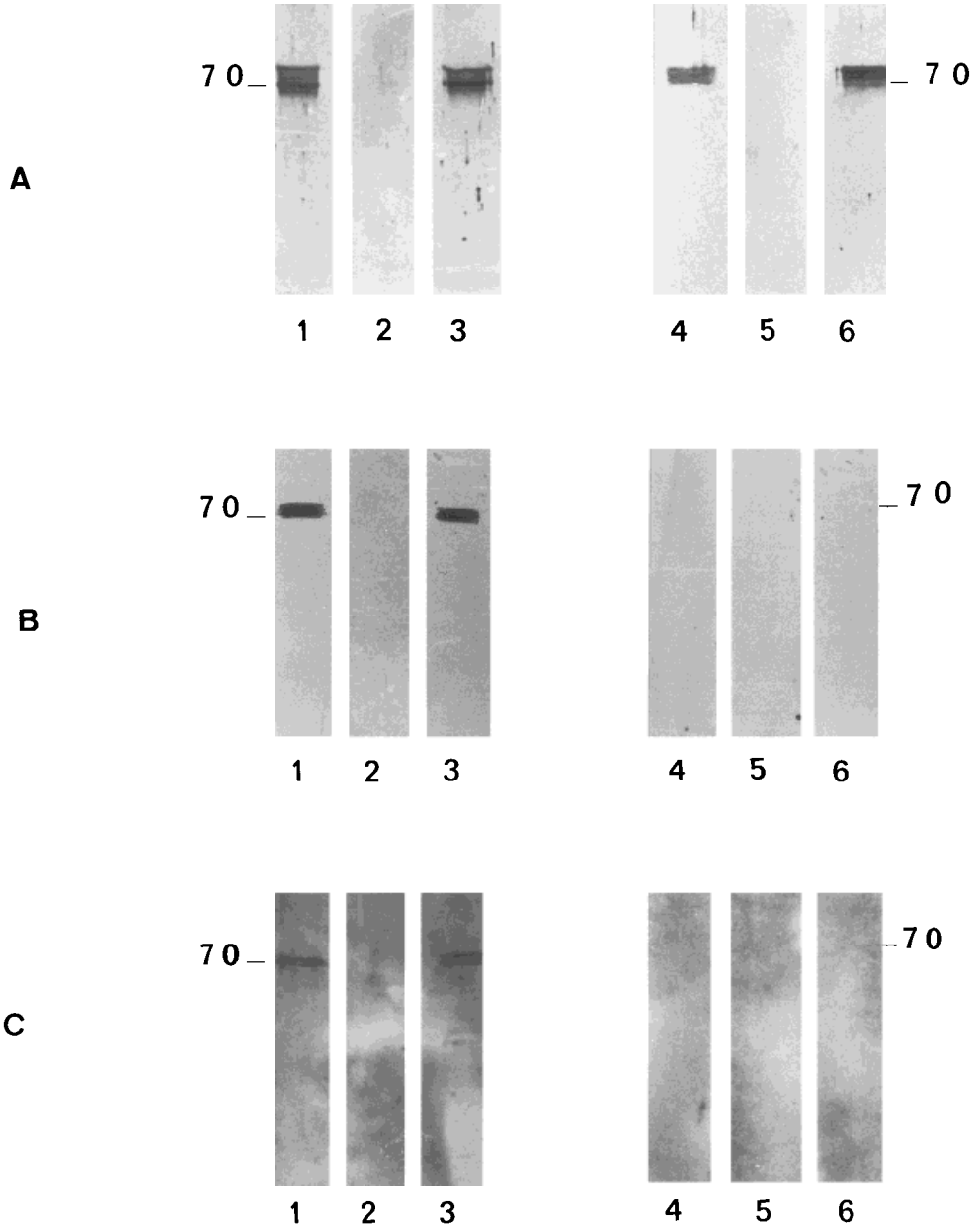

Fig. 8. Characterization of terminal GlcNAc residues by WGA-affinity chromatography (**A**) and lectin-blotting analysis with succinylated-biotinylated-WGA (**B**), and biotinylated-PVA (**C**) of purified nuclear and cytoplasmic CBP70. *Lanes 1–3,* nuclear CBP70; *lanes 4–6,* cytoplasmic CBP70. **A:** *Lanes 1, 4,* unbound fraction; *lanes 2, 5,* elution with the appropriate heterologous sugar; *lanes 3, 6,* elution with the appropriate homologous sugar. **B,C:** *Lanes 1, 4,* incubation with the appropriate lectin; *lanes 2, 5,* competition with the appropriate homologous sugar; *lanes 3, 6,* competition with the appropriate heterologous sugar.

was isolated from HL60 cell nuclei [Sève et al., 1993]; recently, a cytoplasmic form of this lectin was described, on the basis that the two forms have the same molecular mass, the same carbohydrate binding sites that recognize both Glc and GlcNAc, and are recognized by the same antibody raised against nuclear CBP70 [Hadj-Sahraoui et al., 1996; Felin et al., 1997].

To analyze both forms of CBP70, it was important to ensure negligible contamination of the nuclear or cytoplasmic extract by cytosolic or nuclear proteins, respectively. In this context, very clean nuclear and cytoplasmic fractions from HL60 cells were obtained according to a previously reported method [Sève et al., 1993; Felin et al., 1997]. Then, the combination of two

TABLE I. Summary of Different Results Obtained After Affinity Chromatography and Lectin-Blotting Analysis of Two Forms of CBP70

	Nuclear CBP70	Cytoplasmic CBP70
PNA		
Gal β1–3GalNAc	+	+
RCA		
Terminal βGal residues	+	+
GNA		
Man α1–3 Man	+	+
WGA		
GlcNAc β1–4 GlcNAc >GlcNAc >NeuAc	+	+
SNA		
NeuAc α2–6 Gal / Gal NAc	+	+
MAA		
NeuAc α2–3Gal	+	+
Succinylated WGA		
Terminal GlcNAc	+	−
PVA		
Terminal GlcNAc	+	−
Lotus		
Terminal α-L-fucose	+	−

successive rounds of affinity chromatography (GlcNAc and Glc) allowed us to obtain both purified forms of CBP70. To confirm the identity between the two forms of CBP70, the peptide sequencing was attempted. However, we failed to obtain the total peptide sequencing, probably on account of the presence of glycans moieties on both forms of CBP70. Glycosylation of CBP70 was evidenced by the incorporation of a metabolic labeling with [2-^{3}H]mannose, which revealed the presence of glycans in the nuclear and the cytoplasmic forms. After Pronase digestion and TFMS attack, both forms appeared to be constituted by a 23 kDa peptidic part. These results strongly suggest that the peptidic sequences of both nuclear and cytoplasmic CBP70 are identical. Nevertheless, it cannot be ruled out that the peptidic sequences of the nuclear and the cytoplasmic CBP70 are different. Indeed, it must be kept in mind that the two forms of CBP70 could be the product of (1) two closely related genes, or (2) an alternative splicing of the same gene. Whatever it may be, the TFMS analysis confirmed the metabolic labeling and the presence of glycan moieties on both forms of CBP70 and revealed that the oligosaccharide chains borne by the cytoplasmic and the nuclear CBP70 are different.

β-Elimination and hydrazynolysis strongly suggested that the two forms of CBP70 were both O- and N-glycosylated, but with different patterns of O- and N-linked glycans. In addition, PNGase F digestion revealed molecular mass decreases of 64% and 61% for the nuclear and cytoplasmic CBP70, respectively. However, it must be kept in mind that the molecular mass of CBP70 (70 kDa) was found after PAGE-SDS and did not correspond to the real molecular weight of the protein that migration was perturbed by the presence of saccharides moieties. Although these results confirmed the different glycosylation patterns between the nuclear and the cytoplasmic CBP70 forms, it remained to determine which glycan structures were different. In this context, the results of affinity chromatography and lectin-blotting analysis showed that both forms of CBP70 contained the common following sugar residues: (1) terminal βGal residues, (2) Galβ1–3 GalNAc α1-Ser/Thr, (3) terminal Man α1–3 Man residues, (4) sialic acids linked in α2–6 to Gal or GalNAc, and (5) sialic acids linked in α2–3 to Gal. Interestingly, it is the first time that the O-linked core structure Gal β1–3 GalNAcα1-Ser/Thr is found in a nuclear glycoprotein. However, a different glycosylation between both CBP70 was detected. Indeed, only nuclear CBP70 contains terminal N- or O-linked GlcNAc as well as α-L-fucose. Work is in progress to determine whether the terminal GlcNAc residues found in the nuclear CBP70 correspond to those described by Hart and coworkers [Haltiwanger et al., 1990].

This work raise two major questions concerning (1) the mechanism of glycosylation of nuclear glycoproteins, and (2) the significance of the difference of glycosylation observed between the nuclear and the cytoplasmic CBP70.

How and Where Can Such a Nuclear Protein Be Glycosylated?

Classically, it is widely accepted that the nuclear proteins do not pass through the endoplasmic reticulum and the Golgi apparatus, the two compartments where N- and O-glycosylation occur. Nevertheless, it is now well established that some nuclear proteins are glycosylated. Generally, most of these are substituted by one or more N-acetylglucosamine monosaccharide moieties, O-glycosidically linked to ser-

ine and threonine residues (termed O-GlcNAc) [Haltiwanger et al., 1990]. It is well known that this glycosylation takes place in the cytosolic compartment by the action of the O-GlcNAc-polypeptide N-acetylglucosaminyltransferase [Haltiwanger et al., 1990]. The presence of nuclear proteins bearing N-linked oligosaccharide chains has also been reported [Stein et al., 1975, 1981; Reeves et al., 1981; Ferraro et al., 1991; Codogno et al., 1992]. But, to the best of our knowledge, it is the first time that both N- and O-linked oligosaccharides chains are found on a same nuclear glycoprotein. Up to now, the glycosylation process of nuclear proteins bearing N-linked oligosaccharides chains or both N- and O-linked oligosaccharide chains, as reported here for CBP70, remains unknown. However, two hypotheses can be considered: (1) glycosyltransferases and glycosidases could be present in the cytosol and/or the nucleus and then act as an as yet unknown glycosylation pathway, for the proteins that do not pass through the endoplasmic reticulum and Golgi apparatus; and (2) the nuclear proteins could enter the endoplasmic reticulum and the Golgi apparatus, after being synthesized, to be glycosylated and then emerge in the cytosol for transport into the nucleus by an unknown mechanism. Whatever it may be, it remains to be explained how and where the glycosylation of CBP70 bearing at least classical N- and O-linked polysaccharides is accomplished.

What Might Be the Significance of These Differences in Glycosylation?

Because neoglycoproteins (BSA-free nuclear localization signal substituted by sugars) could be transported into the nucleus in an in vitro system, when it was substituted by specific sugars, especially N-acetylglucosamine [Duverger et al., 1993, 1995, 1996], the authors advanced two hypotheses to explain its entry into the nuclei: (1) the glycoprotein could be recognized and transported specifically into the nucleus by an NLS-bearing lectin, and (2) sugar residues as short basic amino acid sequences might be nuclear targeting signals and could therefore define a new nuclear import mechanism. Thus, on the basis of the hypotheses previously reported, it can be speculated that the intracellular localization of the CBP70 depends on it's glycosylation.

Moreover, the glycosylation (N- and/or O-linked) could modify the overall conformation of the lectin and consequently it's carbohydrate-recognition domain (CRD). Recently, we have found that the nuclear and the cytoplasmic CBP70 recognized different ligands [Felin et al., 1997]. Therefore, appropriate glycosylation, could confer to both forms of CBP70 a correct tridimensional structure that enable each of them to recognize, or be recognized, by their specific ligand (e.g., proteins, glycoproteins, or lectins) among all their different potentials ligands available.

Finally, the difference of glycosylation between the two forms could allow the organization of different complexes between the nucleus and the cytoplasm and might explain the plurilocalization, and consequently the plurifunctionality, of the lectin, depending on its localization.

For the first time, the results presented in this paper strongly suggest that the glycosylation of CBP70 could determine its localization and, in agreement with recent data, they strengthen the idea that sugar residues borne by nuclear glycoproteins might, directly or indirectly, be active in the nuclear physiology [Holt and Hart, 1986; Finlay et al., 1987; Haltiwanger et al., 1992; Hayes and Hart, 1994; Duverger et al., 1993, 1995, 1996]. Experiments under way to study more precisely the linkages and the sugars borne by the different forms of CBP70, as well as the signification of this difference in glycosylation between the nuclear and cytoplasmic CBP70.

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