# Identification of *N*-acetyl-D-glucosamine-specific lectins from rat liver cytosolic and nuclear compartments as heat-shock proteins

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Cytosolic and nuclear O-linked N-acetylglucosaminylation has been proposed to be involved in the nuclear transport of cytosolic proteins. We have isolated nuclear and cytosolic *N*-acetyl-D-glucosamine (GlcNAc)-specific lectins from adult rat liver by affinity chromatography on immobilized GlcNAc and identified these lectins, by a proteomic approach, as belonging to the heat-shock protein (HSP)-70 family (one of them being heat-shock cognate 70 stress protein). Two-dimensional electrophoresis indicated that the HSP-70 fraction contained three equally abundant proteins with molecular masses of 70, 65 and 55 kDa. The p70 and p65 proteins are phosphorylated and are themselves O-linked GlcNAc (O-GlcNAc)-modified. The HSP-70 associated into high molecular mass complexes that dissociated in the presence of reductive and chaotropic agents. The lectin(s) present in this complex was (were) able to recognize cytosolic and nuclear ligands, which have been isolated using wheat germ agglutinin affinity chromatography. These ligands are O-GlcNAc glycosylated as demonstrated by [$^3$H]galactose incorporation and analysis of the products released by reductive $\beta$-elimination. The isolated lectins specifically recognized ligands present in both the cytosol and the nucleus of human resting lymphocytes. These results demonstrated the existence of endogenous GlcNAc-specific lectins, identified as HSP-70 proteins, which could act as a shuttle for the nucleo-cytoplasmic transport of O-GlcNAc glycoproteins between the cytosol and the nucleus.

Key words: N-acetylglucosaminylation, cytosol, nucleus, phosphorylation, traffic.

## INTRODUCTION

For a long time, glycosylation was thought to be restricted to proteins confined to the cell surface or within the lumen of intracellular organelles. This view was governed by the general understanding of the biosynthetic pathways of N- and O-linked glycans. But, for fifteen years, the existence of a major form of glycosylation found within the cytosol and the nucleus has been well established, i.e. O-linked N-acetylglucosaminylation [1,2], the addition of a single monosaccharide [*N*-acetyl-D-glucosamine (GlcNAc)] on to serine or threonine residues of the peptide backbone. To date, approximately 100 nuclear and cytoplasmic proteins modified with O-linked GlcNAc (O-GlcNAc) have been identified. They include neurofilament proteins [3], nuclear pore proteins [4,5], and several transcription factors, such as Sp1 [6] and c-Myc [7,8]. Interestingly all of the O-GlcNAc-modified proteins undergo reversible phosphorylation/dephosphorylation and form heteromeric complexes with other proteins, and most of them are translocated between the cytosol and the nucleus [9].

O-linked N-acetylglucosaminylation was suggested to play a role in nucleo-cytoplasmic transport. It is well known that cytosolic proteins with molecular masses exceeding 40 kDa migrate into the nucleus only if they contain a specific nuclear localization sequence. Duverger et al. [10] suggested the existence of sugar-dependent nuclear import of proteins from the cytosol. In digitonin-permeabilized cells, they found that BSA substituted with either di-*N*-acetyl-chitobiose (GlcNAc$\beta$1-4GlcNAc) or $\alpha$-glucose residues was transported into the nucleus by a time- and ATP-dependent mechanism, indicating that GlcNAc could act as a nuclear localization signal. Nuclear pore proteins, being themselves O-linked N-acetylglucosaminylated and facing both sides of the nuclear envelope, are possibly involved in this cytosol-to-nucleus transport. Several reports favoured this possibility. Finlay et al. [11] reported a specific inhibition of the *in vitro* nuclear transport of fluorescein-labelled phycoerythrin by the GlcNAc-specific lectin wheat germ agglutinin (WGA). Miller and Hanover [12] demonstrated in the *Xenopus* egg that functional nuclei can be reconstituted after the addition of nuclear pore proteins from rat liver or *Xenopus* eggs. However, the nuclear transport was not recovered when the nuclear pore proteins were depleted of their WGA-binding glycoproteins.

Six nuclear lectins have been characterized so far: carbohydrate-binding protein (CBP)-35 (galectin-3) and CBP-14 (galectin-1) specific for galactose residues; and CBP-67, CBP-33 and CBP-70 specific for glucose residues. CBP-70 was shown to also be specific for GlcNAc residues, as was CBP-22, which was co-isolated with CBP-70 from HL60 cell nuclei [13]. CBP-70 also localized in the cytosol and one 82 kDa ligand was characterized for the nuclear CBP-70 [14]. Rousseau et al. [15] demonstrated, using electron microscopy, immunofluorescence analysis and subcellular fractionation, that CBP-70 is a pluri-localized lectin that is also found in the endoplasmic reticulum, Golgi apparatus and mitochondria. The nuclear lectins have mainly been found in ribonucleoprotein complexes [16]. The role of these nuclear

Abbreviations used: BiP, immunoglobulin heavy-chain binding protein; CBP, carbohydrate-binding protein; DTT, dithiothreitol; GlcNAc, *N*-acetyl-D-glucosamine; O-GlcNAc, O-linked GlcNAc; GRP78, 78 kDa glucose-regulated protein; HPAEC, high-pH anion-exchange chromatography; HRP, horseradish peroxidase; Hsc-70, heat-shock cognate 70 stress protein; HSP, heat-shock protein; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; TBS, Tris-buffered saline; WGA, wheat germ agglutinin.

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lectins remains to be determined, but data suggested that CBP-14 and CBP-67 might be involved in the nucleo-cytoplasmic transport of mRNA [17]. Hubert et al. [18] have shown that galectin-3 (CBP-35) is in higher concentration in the nucleus than in the cytoplasm and that it is required for spliceosome formation and mRNA processing. Kuchler et al. [19,20] described the existence of GlcNAc-binding sites during cerebellar ontogenesis in the nucleolus, in specialized regions of the nucleus and in the cytosol using biotinylated neoglycoproteins. Although these previous studies suggest a role for lectins in nuclear transport, their precise function remains to be determined.

The aim of the present study was to characterize GlcNAc-specific lectins in the cytosol and the nucleus of rat liver and to see whether O-GlcNAc could represent a signal for nuclear transport of cytosolic and nuclear glycoprotein ligands. These different lectins were demonstrated to belong to the HSP-70 family and the others were similar to immunoglobulin heavy-chain binding protein (BiP).

## MATERIALS AND METHODS

### Biochemicals and apparatus

WGA immobilized on Sepharose was generously provided by Professor H. Debray (Unité Mixte de Recherches 8576 du CNRS). Centricon cell and exclusion membranes were obtained from Pall Filtron (St Germain-en-Laye, France). Nitrocellulose membranes were from Schleicher & Schuell (CERALABO; Ecquevilly, France). Anti-phosphothreonine and anti-phosphoserine antibodies, horseradish peroxidase (HRP)-labelled WGA, alkaline phosphatase (from bovine intestinal mucosa), $\beta$-*N*-acetylglucosaminidase (from *Diplococcus pneumoniae*), GlcNAc $\beta$-1,4-galactosyltransferase, PMSF, leupeptin, pepstatin, biotin-$\epsilon$-aminocaproic acid *N*-hydrosuccinimide ester, ampholytes and PMA were purchased from Sigma–Aldrich (St Quentin-Fallavier, France). The Superose 6 column, P-500 pump, LCC-500 liquid chromatography controller, dual path monitor UV-2, molecular-mass markers, ECL® Western blotting detection reagents and UDP-[6-$^3$H]galactose (9.70 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). CHAPS was from ICN Pharmaceuticals (Orsay, France), silica gel was purchased from Merck (Nogent-sur Marne, France), and silica gel for TLC was also obtained from Merck (Darmstadt, Germany). The gas phase apparatus was a GC-14A from the Shimadzu Corporation (Kyoto, Japan) and the column used was a BP-70 column from SGE International Pty Ltd (Les Ulis, France). The liquid scintillation counter was a 6000 TA from Beckman Instruments Inc. (Palo Alto, CA, U.S.A.). High-pH anion-exchange chromatography (HPAEC) apparatus was purchased from Dionex (Voisins Le Bretonneux, France) and the MS apparatuses [matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) and Q-STAR] were from Applied Biosystems (Les Ulis, France).

### Rat liver nuclei and cytosol preparation

Adult Wistar albino rats were starved overnight prior to decapitation. After perfusion, livers were removed and placed into ice-cold 20 mM Tris/HCl and 0.25 M sucrose (pH 7.4), containing a protease inhibitor cocktail (1 mM PMSF, 1 $\mu$g/ml leupeptin and 1 $\mu$g/ml pepstatin). From this step onwards, all operations were performed between 0 and 4 °C. Livers were ground in a Fischer apparatus and homogenized in 5 vol. of the same medium in a Potter–Elvehjem homogenizer by 3 strokes at 600 rev./min. The homogenate was centrifuged at 1500 ***g*** for 15 min. The supernatant (post-nuclear) was then centrifuged at 100000 ***g*** for 60 min, and the resulting supernatant was considered as the cytosolic fraction. The resulting pellet of the first centrifugation was carefully recovered and resuspended in 2.4 M sucrose, 1 mM $MgCl_2$ and 10 mM potassium phosphate buffer (pH 6.8), containing the protease inhibitor cocktail. The suspension was centrifuged at 100000 ***g*** for 1 h. The pellet corresponding to the nuclear fraction was resuspended in 0.25 M sucrose, 0.5 mM $MgCl_2$ and 20 mM Tris/HCl (pH 7.5) and centrifuged at 1000 ***g*** for 10 min. The washing was repeated twice in order to obtain a white nuclear pellet. The pellet was finally homogenized in 10 vol. of 200 mM KCl, 1 mM dithiothreitol (DTT), 1 mM PMSF, 1 $\mu$g/ml leupeptin, 1 $\mu$g/ml pepstatin, 1 % (v/v) Triton X-100, 0.1 % sodium deoxycholate and 0.2 M GlcNAc in 20 mM Tris/HCl (pH 7.8), and centrifuged at 100000 ***g*** for 1 h. The supernatant corresponding to membrane-depleted nuclei was collected and dialysed extensively against the same buffer without detergent or GlcNAc.

### Affinity chromatography on immobilized GlcNAc and WGA

For the isolation of GlcNAc-binding lectins, the soluble and nuclear extracts were passed through a column (17 cm × 1.4 cm) made of GlcNAc immobilized on 6 % (w/v) agarose through a 6-carbon-atom spacer-arm equilibrated in binding buffer (20 mM Tris/HCl, 200 mM KCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.8). The column was washed with 200 ml of the binding buffer, followed by 50 ml of the same buffer containing 0.1 M GalNAc and, finally, eluted with 50 ml of the buffer containing 0.1 M GlcNAc.

For the isolation of cytosolic and nuclear GlcNAc-containing glycoproteins, the material not adsorbed on to the immobilized GlcNAc column was submitted to chromatography on a column of WGA immobilized on Sepharose at 4 °C. The binding buffer was as described above. After washing overnight, elution was performed with the binding buffer containing 0.2 M GlcNAc. The different fractions were concentrated in a Centricon cell with a 10 kDa exclusion size filter at 4 °C followed by extensive dialysis against the binding buffer.

### PAGE and Western-blot analysis

Proteins were separated by SDS/PAGE (10 % gels) [21] and stained with Coomassie Brilliant Blue and/or silver stain, or electrophoretically transferred on to nitrocellulose. Nitrocellulose membranes were first saturated for 45 min in Tris-buffered saline (TBS)/Tween [15 mM Tris, 140 mM NaCl and 0.05 % Tween (v/v), pH 8.0] containing 3 % (w/v) BSA. Membranes were then incubated in TBS/Tween containing anti-phosphothreonine or anti-phosphoserine antibodies [diluted 1:500 (v/v)] or with HRP-labelled WGA [diluted 1:10000 (v/v)] for 1 h. Blots were washed three times with TBS/Tween (for 10 min each) and detection was carried out using the ECL® Western blotting detection reagents. In order to control the specificity of the WGA binding, experiments were performed in the presence of 0.2 M GlcNAc.

### Isoelectric focusing and PAGE

For two-dimensional electrophoresis, samples were diluted in 20 % (v/v) IsoA (0.34 M SDS/0.15 M DTT) and 80 % (v/v) IsoE [0.108 M DTT, 0.108 M CHAPS, 15 M urea and 8.33 % (v/v) ampholytes (pH 3–10)]. The samples were run on a 4 % (w/v) polyacrylamide gel containing 9.5 M urea, 1 % (v/v) ampholytes (pH 5–7), 4 % (v/v) ampholytes (pH 3–10) and a 5 %

(v/v) detergent solution (0.3 g of CHAPS, 100 $\mu$l of Nonidet P40 and 900 $\mu$l of water) at 200 V for 1 h, followed by 500 V for 1 h and, finally, 800 V for 16 h. Phosphoric acid (20 mM) was used as the lower buffer and NaOH (20 mM) was used as the upper buffer. After isoelectrofocusing, gels were extracted from the capillaries, placed in transfer solution [0.5 M Tris/HCl, 10 % (w/v) SDS and 0.005 % Bromophenol Blue, pH 8.8] for 10 min, and proteins were resolved by SDS/PAGE (10 % gels).

#### Electrophoresis under non-denaturing conditions

Samples were placed in 20 mM Tris/HCl, 50 % (v/v) glycerol and 0.005 % Bromophenol Blue (pH 8.8) and were run in Tris/glycine buffer (pH 8.8) on a 5–15 % (w/v) polyacrylamide gradient gel without SDS or 2-mercaptoethanol at 50 V overnight at 4 °C. Proteins were then silver stained.

#### Separation of GlcNAc-specific lectins by FPLC

FPLC was performed using a Superose 6 column (29 cm × 1.4 cm) equilibrated with 20 mM Tris/HCl, 200 mM KCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH 7.8) and eluted with the same buffer. Absorption was measured at 280 nm. Fractions of 1 ml were collected, pooled according to the absorbance profile and analysed by SDS/PAGE.

#### Identification of WGA–Sepharose-bound proteins by MS

The WGA–Sepharose-bound proteins were separated by SDS/PAGE (10 % gels) and stained with Coomassie Brilliant Blue. The different protein bands were excised individually and in-gel digested with modified trypsin as described previously [22,23]. The tryptic peptides from each protein were analysed by MALDI–TOF MS on a Voyager instrument DE STR and/or sequenced by nanospray tandem MS (Q-STAR). Finally, proteins were identified using MS-Fit (http://prospector.ucsf.edu/htmlucsf3.0/msfit.htm).

#### Detection of phosphorylation on the GlcNAc-specific lectins

GlcNAc-specific lectins were submitted to acidic hydrolysis in 100 $\mu$l of 1 M HCl for 5 h at 95 °C. Following evaporation, water was added to the sample and the released phosphate groups were analysed by HPAEC, using an AS4A-SC column (Ion Pac®; 4 mm × 250 mm) and an AG4A-SC pre-column. The eluent was 40 mM NaOH at a flow rate of 1.5 ml/min. Phosphate ions were detected by conductimetry with a pulsed electrochemical detector.

#### Detection of O-GlcNAc on the GlcNAc-specific lectins

GlcNAc-specific lectins were submitted to reductive $\beta$-elimination in 0.1 M NaOH and 1 M sodium borohydride at 65 °C overnight. The reaction was stopped by drop-wise addition of ice-cold acetic acid under vigorous stirring until a pH value of 5.0 was reached. The $\beta$-eliminated material was dried several times under vacuum with anhydrous methanol in order to remove borate as methyl ester. The released saccharides were peracetylated in acetic anhydride for 4 h at 95 °C, dried and finally extracted in chloroform. After drying under nitrogen, the peracetylated saccharides were taken up in 100 $\mu$l of chloroform and 2 $\mu$l was injected into GLC and analysed on a BP-70 column (30 m × 0.32 mm) at a initial temperature of 150 °C, with a gradient of 3 °C/min to 230 °C, then with a gradient of 5 °C/min to 250 °C and finally with a plateau of 5 min at 250 °C.

#### Determination of the nature of the glycosylation of WGA-binding glycoproteins

Galactosyltransferase provides a specific and sensitive probe frequently used in the detection of O-GlcNAc residues on cytosolic and nuclear proteins [24,25]. Samples of glycoproteins isolated by affinity chromatography on WGA–Sepharose were supplemented with an equal volume of sample buffer (56.25 mM Hepes, 11.25 mM $MnCl_2$, 250 mM galactose and 12.5 mM AMP, pH 7.0) containing protease inhibitors (1 mM PMSF, 5 $\mu$g/ml leupeptin and 5 $\mu$g/ml pepstatin). Bovine milk GlcNAc$\beta$1,4-galactosyltransferase (0.025 unit) and 5 $\mu$Ci of UDP-[6-$^3$H]-galactose were finally added to initiate the reaction. The samples were subsequently incubated at 37 °C for 2 h.

Sugar chains were released from the precipitated labelled glycoproteins using reductive alkaline treatment as described above. The $\beta$-eliminated material was dried several times under vacuum with anhydrous methanol and then desalted by descending paper chromatography on Whatman 3 MM paper using 1-butanol/ethanol/water (4:1:1, by vol.) as the solvent. Radioactivity was measured after cutting horizontal strips (1 cm in width). The radioactive fractions were eluted from corresponding regions with water, freeze-dried and finally analysed by silica gel 60 TLC with 1-butanol/acetic acid/water (4:2:3, by vol.) as the solvent. Bands (0.5 cm in width) were recovered and measured for their radioactivity in a liquid scintillation counter.

#### Preparation of the biotinylated nuclear GlcNAc-specific lectins

Nuclear GlcNAc-specific lectins were incubated in a potassium phosphate buffer at pH 9.5, with biotin-$\epsilon$-aminocaproic acid *N*-hydrosuccinimide ester and 0.1 M free GlcNAc (in order to protect the carbohydrate-recognition domain) for 2 h at 4 °C. Excess biotin-$\epsilon$-aminocaproic acid *N*-hydrosuccinimide ester and GlcNAc were dialysed and biotinylated nuclear GlcNAc-specific lectins were precipitated with a saturated ammonium sulphate salt solution, centrifuged at 100000 ***g*** for 1 h, and finally dialysed against cold PBS [25 mM sodium phosphate buffer at pH 7.2 containing 150 mM NaCl].

#### Staining of WGA-selected proteins with biotinylated nuclear lectins

Blots of the two pools of nuclear and cytoplasmic WGA-binding glycoproteins were saturated in the presence of a 3 % (w/v) solution of periodate-treated BSA [26], supplemented with 5 ng of biotinylated nuclear GlcNAc-specific lectins, followed by HRP-labelled avidin, and revealed with the ECL® kit.

#### Binding of biotinylated nuclear lectins to human lymphocytes

Blood was collected, diluted once in buffer [20 mM phosphate, 150 mm NaCl and 1 mM EDTA (pH 7.2)] and loaded on to a 6 % Ficoll-Paque layer in 50 ml plastic centrifuge tubes (Falcon) and centrifuged for 50 min at 800 ***g*** (activation of lymphocytes was performed by incubation in 10 $\mu$g/ml PMA for three days). After washing in PBS (three times), the cells were fixed by addition of a mixture containing 4 % (w/v) paraformaldehyde and 0.1 % glutaraldehyde in PBS for 4 h with intermittent gentle agitation. After elimination of the fixative and washes in PBS by repetitive centrifugations, the fixed cells were incubated with a 3 % (w/v) solution of periodate-treated BSA in PBS for 30 min at 20 °C followed by the addition of biotinylated lectins (5 ng/ml final concentration in 0.01 % Triton X-100). Following incubation for 4 h at 20 °C, the cultures were washed in PBS over a

4 h period, followed by addition of HRP-labelled avidin (1 $\mu$g/ml in PBS), and incubated overnight at 4 °C. After rinsing, as above, the bound HRP-labelled avidin was revealed using the diaminobenzidine method [27]. Controls were made by adding 10 mM GlcNAc during incubation with biotinylated lectins and during the first washing.

### Assays for phosphatase and glucosaminidase activities

Phosphatase activity was assayed by incubation of nuclear and cytosolic GlcNAc-specific lectins with *p*-nitrophenyl phosphate as substrate, in 100 mM Tris/HCl, 50 mM $MgCl_2$ and 100 mM NaCl, pH 9.5. Glucosaminidase activity was assayed using *p*-nitrophenyl-2-acetamido-2-deoxy-$\beta$-D-glucopyranoside in 50 mM acetate, pH 6.0. Positive controls of the reactions were obtained using alkaline phosphatase from bovine intestinal mucosa and $\beta$-*N*-acetylglucosaminidase from *D. pneumoniae*.

## RESULTS

### Cytosolic and nuclear GlcNAc-specific lectins have identical molecular masses, but differ in their pIs

Cytosolic and nuclear GlcNAc-specific lectin fractions were isolated by affinity chromatography on a GlcNAc-immobilized column. The binding of these compounds was considered as specific since the adsorbed compounds could not be eluted with *N*-acetylgalactosamine, but only with 0.2 M GlcNAc. As shown in Figures 1(a) and 1(b), the GlcNAc lectin fractions showed a relatively simple electrophoretic profile with three constituents in equivalent proportions of 70, 65 and 55 kDa. This pattern was identical in the cytosol and in the nucleus.

When the same samples were submitted to two-dimensional electrophoresis (Figures 1c and 1d, for cytosolic and nuclear GlcNAc-specific lectins respectively), differences in the pI and in the number of pI isoforms were found between the cytosolic and nuclear GlcNAc-specific lectins. The 70 kDa cytosolic protein showed three spots, whereas the nuclear protein migrated as six spots. A similar observation was made for the 65 kDa protein, for which only one cytosolic form was observed whereas three nuclear forms occurred. These pI shifts suggested differences in the phosphorylation status of the different proteins. Molecular mass and pI were determined by comparison with standard proteins.

### GlcNAc-specific lectins are phosphoproteins and are modified by O-GlcNAc glycosylation

The phosphorylation status of the 70 and 65 kDa proteins was investigated using anti-phosphoserine and anti-phosphothreonine antibodies. While no binding was observed using the anti-phosphothreonine antibody (Figure 2A), the 70 and 65 kDa proteins were specifically stained using the anti-phosphoserine antibody (Figure 2A) in both the cytosolic and nuclear lectin fractions. The presence of phosphate was also confirmed using acidic hydrolysis of these lectins followed by HPAEC analysis of the released products (Figure 2B). We estimated, using standard phosphate dilutions, the presence of 3–5 mol of phosphate/mol of GlcNAc-specific lectin. When these proteins were stained using the WGA–HRP technique (WGA is a lectin that recognizes all terminal non-reducing GlcNAc residues, especially O-GlcNAc) staining was observed for both cytosolic and nuclear p70 and p65 proteins (Figure 2C). The specificity of the latter binding was verified by incubating the same material in the presence of 0.1 M GlcNAc (Figure 2C). The carbohydrates released from the lectins by $\beta$-elimination were analysed by GLC (Figure 2D). The unique presence of peracetyl glucosaminitol confirmed the O-linked N-acetylglucosaminylation of the nuclear and cytosolic lectins.

### GlcNAc-specific lectins are part of high molecular mass complexes

In order to evaluate the molecular mass of the native GlcNAc-specific lectins, non-denaturing electrophoresis was performed

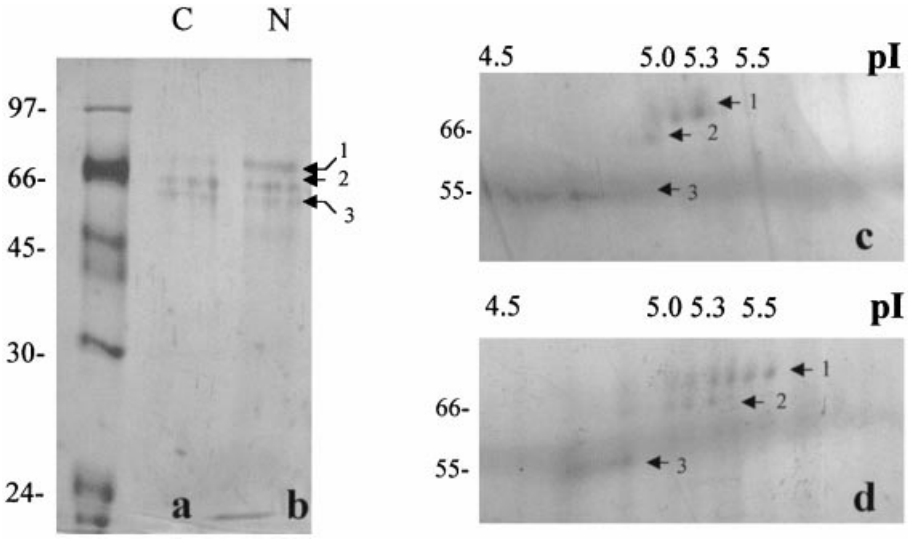


**Figure 1 SDS/PAGE and two-dimensional electrophoresis analysis of the GlcNAc-specific lectin fractions**

Electrophoretic profiles (silver staining) of the GlcNAc-specific lectin fractions isolated from cytosolic (C; **a** and **c**) and nuclear (N; **b** and **d**) fractions on a column of immobilized GlcNAc. (**a** and **b**) SDS/PAGE (10% gels). The quantity of protein loaded was estimated at 0.5 $\mu$g in each well. (**c** and **d**) Two-dimensional electrophoresis. Arrows indicate the position of the 70, 65 and 55 kDa proteins (labelled 1, 2 and 3 respectively). Molecular-mass markers are shown (in kDa) on the left.

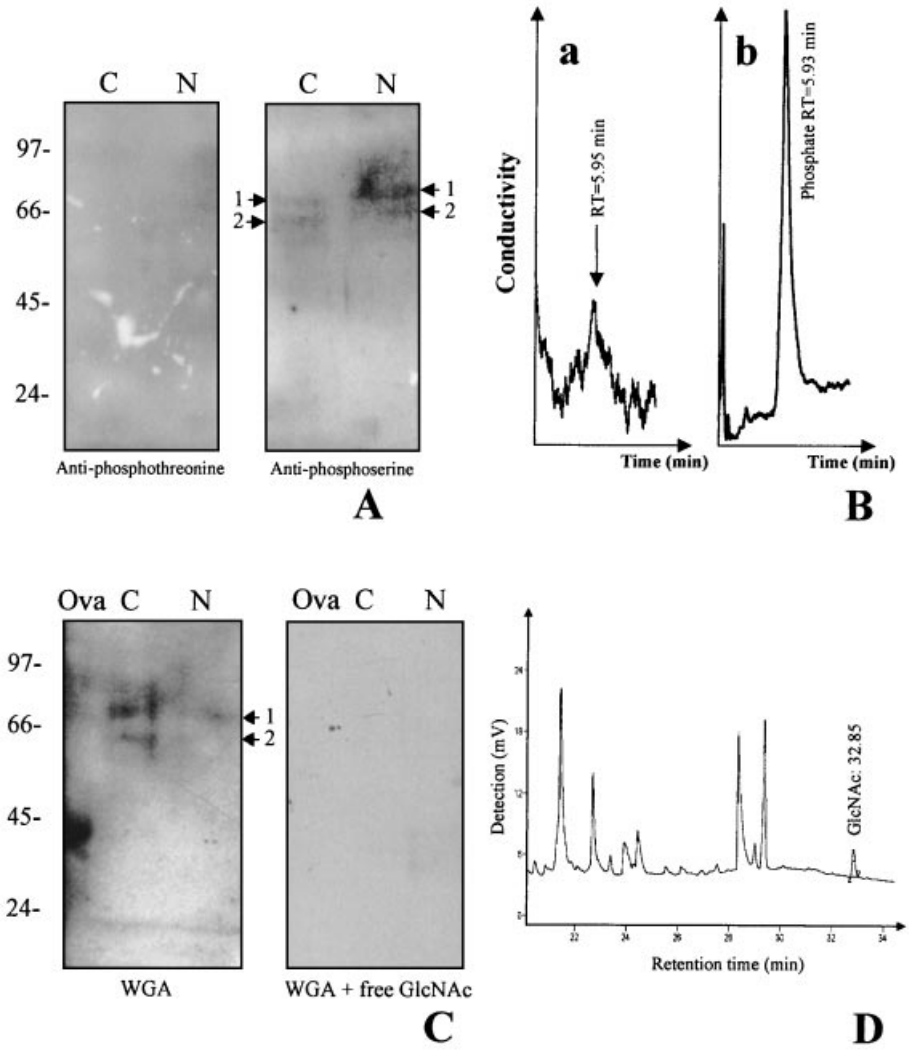


**Figure 2 Studies of the phosphorylation and GlcNAc glycosylation of the 70 and 65 kDa proteins**

(**A**) Phosphorylation of the cytosolic (C) and nuclear (N) GlcNAc-specific lectins was assessed using anti-phosphothreonine and anti-phosphoserine antibodies. The quantity of protein loaded was estimated at 0.5 $\mu$g in each well. Molecular-mass markers are shown (in kDa) on the left. (**B**) Characterization of the lectins phosphate content by mild acid hydrolysis followed by HPAEC analysis. a, GlcNAc lectins; and b, phosphate standard. RT, retention time. (**C**) WGA lectin staining of cytosolic and nuclear lectins. A control chase experiment using 0.1 M GlcNAc in the incubation medium was performed. Ovalbumin (Ova; 45 kDa) was used as a positive control. Molecular-mass markers are shown (in kDa) on the left. (**D**) Characterization of the GlcNAc content of isolated lectins by GLC. Glycans were released by reductive $\beta$-elimination and analysed in the peracetylated form by GLC. The peak at retention time 32.85 min corresponds to peracetylated glucosaminitol. In (**A** and **C**) arrows indicate the reactivity of the positive bands (1, 70 kDa protein; 2, 65 kDa protein).

on both nuclear and cytosolic GlcNAc-specific lectins. As shown in Figure 3, GlcNAc-specific lectins exist as very high molecular mass complexes, some of them having molecular masses that exceed $10^6$ Da. In addition to these major forms, lower molecular mass bands of approx. 600, 800 and 900 kDa could be distinguished.

In order to determine the monomer composition of each native isoform, these high molecular mass complexes were separated by FPLC on a Superose 6 column (Figure 4a) and each collected peak was subjected to SDS/PAGE (10% gels). As shown in Figure 4(b), it could be concluded that each native complex was composed of stoichiometric amounts of the 70, 65 and 55 kDa proteins. Since the three subunits were found in equivalent proportions in all native complexes, it was suggested that the high molecular mass complexes were associations of three (570 kDa), four (760 kDa), five (950 kDa) and six (1140 kDa) triplets.

### Cytosolic and nuclear GlcNAc-specific lectins are associated with a phosphatase activity

The high molecular masses of the complexes found within the cytosolic and nuclear lectin fractions suggested either a co-association between several GlcNAc-specific lectins or an association of one GlcNAc-specific lectin with other proteins.

The hypothesis that the complexes were made of GlcNAc-specific lectins and GlcNAc-containing glycoproteins associated by lectin–ligand interactions (which were dissociated upon SDS/PAGE) was unlikely since the complexes were not dissociated

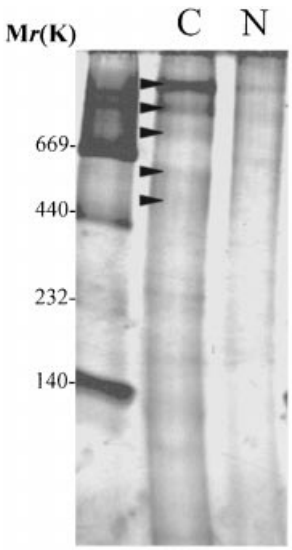


**Figure 3 Non-denaturing PAGE [5–15% (w/v) polyacrylamide gradient] of the GlcNAc-specific lectin fractions**

C, cytosolic fraction; N, nuclear fraction.

upon non-denaturing electrophoresis in the presence of 0.2 M GlcNAc in the sample and in the electrophoretic gel (results not shown).

The alternative hypothesis was that the complex was made of GlcNAc-specific lectins associated with enzymes functionally associated with these lectins. Therefore the cytosolic and nuclear GlcNAc-binding lectin-containing fractions were checked for the presence of phosphatase and *N*-acetylglucosaminidase activities. As shown in Table 1, the cytosolic and nuclear GlcNAc-specific lectin-containing fractions were only associated with phosphatase activity. The phosphatase activity could also be detected using Nitro Blue Tetrazolium and 5-bromo-4-chloroindol-3-yl phosphate after electrophoresis of the cytosolic and nuclear GlcNAc-specific lectin fractions on a non-denaturing gel (results not shown).

**Table 1 Assays for the presence of enzymic activities associated with the cytosolic and nuclear GlcNAc-specific lectin fractions**

Commercially available enzymes (alkaline phosphatase and $\beta$-hexosaminidase) were used as positive controls, and water was used as a negative control.

| Assay | Absorbance (420 nm) |
|---|---|
| Phosphatase activity assay | |
| Positive control (alkaline phosphatase) | 0.269 |
| Negative control (water) | 0.002 |
| *p*-Nitrophenyl phosphate + GlcNAc cytosolic lectin fraction | 0.178 |
| *p*-Nitrophenyl phosphate + GlcNAc nuclear lectin fraction | 0.117 |
| Glucosaminidase activity assay | |
| Positive control ($\beta$-hexosaminidase) | 0.175 |
| Negative control (water) | 0.000 |
| *p*-Nitrophenyl GlcNAc + GlcNAc cytosolic lectin fraction | 0.002 |
| *p*-Nitrophenyl GlcNAc + GlcNAc nuclear lectin fraction | 0.004 |

## Cytosolic and nuclear GlcNAc-specific lectins belong to the HSP-70 family

To establish the identity of the different GlcNAc-specific lectins, these proteins were resolved by 10% (w/v) preparative SDS/PAGE and were stained with Coomassie Brilliant Blue. The three different bands were excised out of the gel, digested with trypsin and analysed by MALDI–TOF-MS. The combination of the molecular masses, the pIs of the proteins and the masses of the peptides allowed us to identify these proteins by the use of algorithms ProteinProspector/MS-FIT or TagIdent, for sequence tags, on the Expasy web site http://www.expasy.ch/tools/ (Table 2, the data presented only refers to the 70 kDa band). This strategy allowed us to identify the GlcNAc-specific proteins as members of the HSP-70 family. p70 was identified as heat-shock cognate 70 stress protein (Hsc-70; pI = 5.38; molecular mass = 70871.6 Da; accession number in SwissProt databank = P08109). The other bands have been identified using

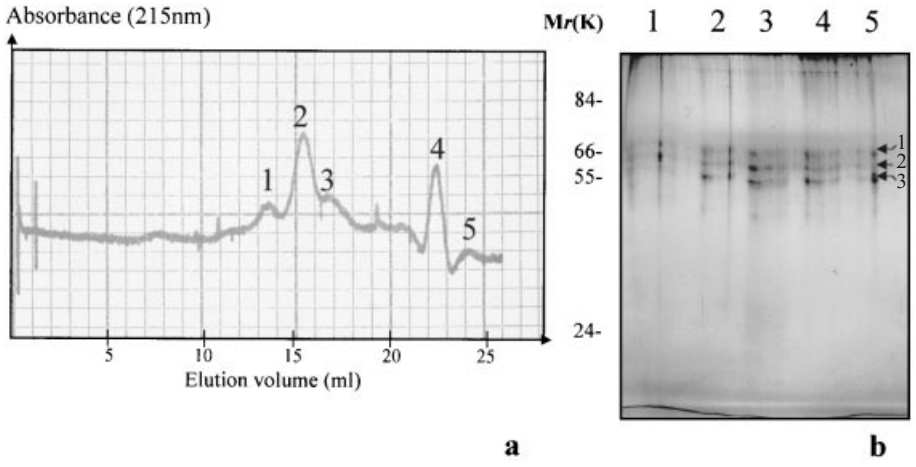


**Figure 4 Evidence for the formation of complexes between the 70, 65 and 55 kDa subunits**

The high molecular mass complexes were separated by FPLC on a Superose 6 column (**a**) and each collected peak was further analysed using SDS/PAGE (10% gels) (**b**). 1; 70 kDa protein, 2; 65 kDa protein; 3, 55 kDa protein. Peaks labelled 1, 2, 3, 4 and 5 correspond to the peaks that we collected by FPLC and that we subjected to SDS/PAGE. Molecular-mass markers are shown (in kDa) on the left.

**Table 2 Identification of the heat-shock cognate 70 stress protein (molecular mass = 70871.6 Da; pI = 5.38; accession number = P08109)**

| Masses submitted (*m/z*) | $M+H^+$ matched | Delta mass (p.p.m.) | Start amino acid | End amino acid | Peptide sequence |
|---|---|---|---|---|---|
| 861.445 | 861.442 | 3.48 | 252 | 258 | DISENKR |
| 1197.673 | 1197.663 | 5.69 | 459 | 469 | FELTGIPPAPR |
| 1199.686 | 1199.674 | 4.32 | 160 | 171 | DAGTIAGLNVLR |
| 1228.641 | 1228.628 | 9.28 | 26 | 36 | VEIIANDQVLR |
| 1253.638 | 1253.616 | 10.66 | 302 | 311 | FEELNADLFR |
| 1481.861 | 1481.807 | 35.35 | 329 | 342 | SQIHDIVLVGGSTR |
| 1487.766 | 1487.701 | 39.11 | 37 | 49 | TTPSYVAFTDTER |
| 1691.680 | 1691.726 | −27.26 | 221 | 236 | STAGDTHLGGEDFDNR |
| 1838.197 | 1838.013 | 98.09 | 326 | 342 | LDKSQIHDIVLVGGSTR |

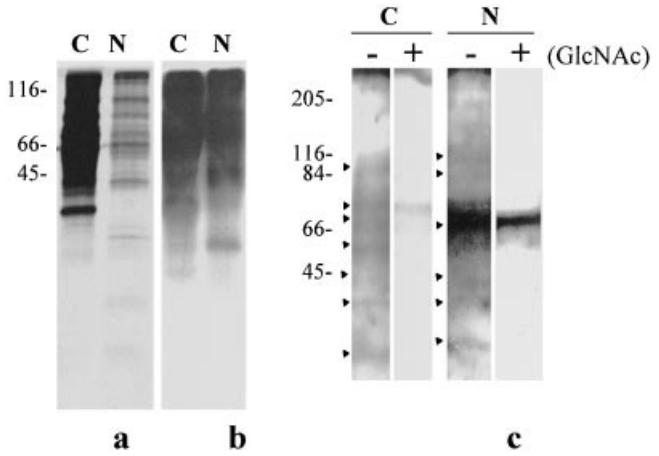


**Figure 5 Identification of the ligands of the GlcNAc-specific lectins**

Cytosolic (C) and nuclear (N) extracts were enriched on a WGA-immobilized column. The retained proteins were resolved by SDS/PAGE (10% gels) and silver stained (**a**) or electrotransferred on to nitrocellulose and tested for their affinity to the biotinylated GlcNAc-specific lectins (**c**). Cytosolic and nuclear extracts were labelled with [$^3$H]galactose, separated by SDS/PAGE (10% gels) and fluorographed (**b**). Molecular-mass markers are shown (in kDa) on the left.

peptide sequences $^{63}$PSYVA$^{67}$ and $^{565}$LES$^{567}$ (where single-letter amino-acid notation has been used). These peptide sequences matched with different members of the HSP-70 family. Nevertheless, in view of the parent peptides masses (IT**PSYVA**FTPEGER, 1566.78 Da; and NE**LES**YAYSLK, 1316.63 Da) they are similar to 78 kDa glucose-regulated protein (GRP78; also termed BiP; pI = 5.07; molecular mass = 72437.6 Da; accession number = P06761) even if the apparent molecular masses of the 65 and 55 kDa proteins were smaller than that of BiP proteins (these proteins may correspond to a truncated BiP). No proteins of similar molecular mass besides HSPs/BiPs matched the two peptide sequences. In addition, this confirms that the proteins characterized in the present study are not contaminating keratin family members.

### Presence of cytosolic and nuclear ligands of GlcNAc-specific cytosolic and nuclear lectins

In order to look for the presence of endogenous ligands of the GlcNAc-specific lectins, cytosolic and nuclear extracts were passed through an immobilized WGA column. The bound proteins were resolved by SDS/PAGE (10% gels) and silver stained (Figure 5a). Cytosolic and nuclear extracts were also labelled in the presence of [$^3$H]galactose in the presence of bovine milk galactosyltranferase, resolved by SDS/PAGE and fluorographed (Figure 5b) to ensure the presence of terminal GlcNAc. The eluted proteins bound on WGA were transferred on to nitrocellulose and tested for their affinity to the biotinylated GlcNAc-specific cytosolic and nuclear lectins (Figure 5c). As shown in Figures 5(a) and 5(b), the electrophoretic profiles of the cytosolic and nuclear WGA-binding glycoproteins were different. The cytosolic fractions showed major high molecular mass constituents, which were not recognized by biotinylated GlcNAc-binding lectins, although they incorporated [$^3$H]galactose radioactivity.

The nature of the glycan ligands of the GlcNAc-binding lectins was confirmed after release of the O-linked glycans, previously labelled with [$^3$H]galactose, using the reductive $\beta$-elimination procedure. The released glycans were further desalted on paper chromatography and then analysed by TLC. As shown in Figure 6, a single peak co-migrating with standard *N*-acetyl-lactosaminitol was detected (no constituents with more than two monosaccharides were found). Consequently, the data demonstrated that in both the cytosolic and nuclear fractions, the unique ligand of the isolated GlcNAc-binding lectin fractions was O-GlcNAc.

### Cytochemical evidence of the presence of nuclear and cytosolic ligands in human lymphocytes

The binding of biotinylated nuclear GlcNAc-specific lectins to human resting and activated (cultured for 3 days after treatment with PMA) lymphocytes fixed with aldehyde mixtures and permeabilized in the presence of 0.01% Triton X-100 was also tested using HRP-labelled avidin and 3,3′-diaminobenzidine detection. As shown in Figure 7, most isolated cells (for resting lymphocytes) and cells at the surface of aggregates (for activated lymphocytes) were stained both in the cytosol and in the nucleus, although the relative intensities of the cytosolic and nuclear staining differed from one cell type to the other. The binding was considered to be specific, since it was completely inhibited using 10 mM GlcNAc during the incubation with biotinylated lectins and in the first washing after incubation.

## DISCUSSION

In the present study, we focused our work on the putative implication of O-GlcNAc in the transport of proteins between the cytosol and the nucleus. Such a role was previously suggested

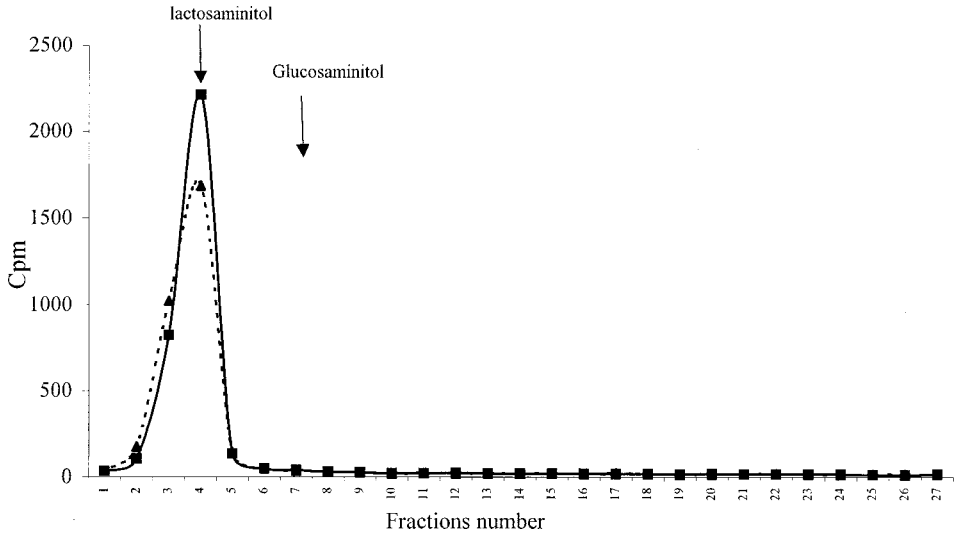


**Figure 6 Nature of the glycans recognized by nuclear GlcNAc-specific lectins**

The [$^3$H]galactose-labelled material was released by reductive $\beta$-elimination. After desalting on paper chromatography, the released glycans were further analysed by TLC (continuous line, rat cytosolic fraction; broken line, nuclear fraction).

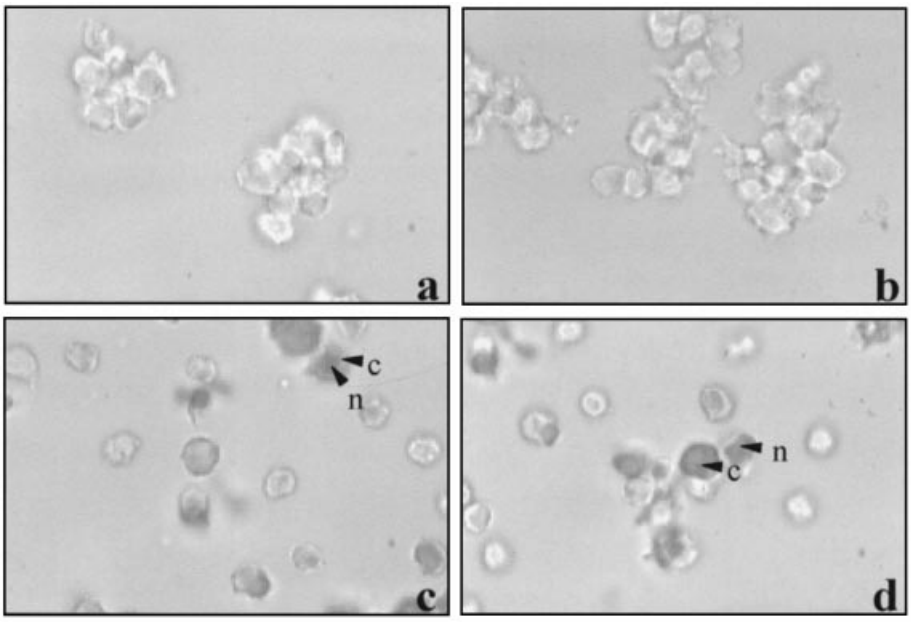


**Figure 7 Localization of the ligands of biotinylated nuclear GlcNAc-specific lectins in human lymphocytes using HRP-labelled avidin**

(**a** and **c**) Resting cells. (**b** and **d**) Activated cells. The specificity of the staining (c, cytosol; n, nucleus) was demonstrated using co-incubation with free 10 mM GlcNAc (**a** and **b**). Magnification, $\times$ 375.

by Duverger et al. [10], demonstrating that BSA substituted with $\beta$-di-*N*-acetyl-chitobiose was transported into the nucleus, whereas BSA was not. Similarly, Finlay et al. [11] showed specific inhibition of nuclear transport by WGA.

If O-GlcNAc is actually a signal for nuclear transport of cytosolic glycoproteins, then GlcNAc-specific lectins should exist. Such lectin activities have already been detected in the developing rat cerebellum by Kuchler et al. [19,20] using neoglycoproteins, and by Felin et al. [13] in the nucleus of HL60 cultured cells.

In the present work, we demonstrated that both the cytosolic and nuclear compartments of rat liver contain a similar pattern of proteins with apparent molecular masses of 70, 65 and 55 kDa. These compounds specifically bind to immobilized GlcNAc. Each of these proteins comprised several isoforms, which may reflect differences in phosphorylation, as demonstrated by two-dimensional electrophoresis. This hypothesis is reinforced by the specific staining of p70 and p65 using anti-phosphoserine antibodies (no staining was detected for p55).

Based on the comparisons of the pIs of these isoforms in each compartment, it was concluded that the nuclear subunits were less phosphorylated than the cytosolic ones. In the same way, we have demonstrated that these GlcNAc-binding lectins were themselves O-linked N-acetylglucosaminylated (it is noteworthy that the GlcNAc-specific lectins that are phosphorylated are also O-linked N-acetylglucosaminylated, i.e. p70 and p65). In addition, we have shown that aggregation into high molecular mass complexes (minimal observable molecular mass of 190 kDa in non-denaturing gels) was independent from GlcNAc self-interactions, or from ligand–lectin interactions, since the complexes between the 70, 65 and 55 kDa subunits were not dissociated using 0.2 M GlcNAc in the non-denaturing polyacrylamide gels.

The constituents of the GlcNAc-specific lectin complexes were identified, using an MS-based approach, as members of the HSP-70 family. p70 was identified as Hsc-70, while p65 and p55 present sequence similarities with GRP78, the endoplasmic-reticulum-localized HSP-70.

The HSP-70s were originally identified in response to stress and to heat shock [28], but they play many other roles, including protein folding, protein translocation, oligomeric assembly and protein degradation. The evidence that an HSP-70-like protein could specifically bind GlcNAc was first demonstrated by Minic et al. [29]. Using affinity chromatography, with either GlcNAc or chitotriose linked to agarose beads, Minic et al. [29] purified an additional protein similar to HSP-70. From a structural point of view, HSP-70 proteins are organized into three different domains among which the 44 kDa N-terminal domain possesses an ATPase activity [30]: the detection of a phosphatase activity associated with the GlcNAc-specific lectins could be related to this ATPase activity of HSPs.

Several parallels can be drawn between the identification of p70, described in the present study as being Hsc-70 and as CBP-70 by Felin et al. [13]. Indeed, Rousseau et al. [15] demonstrated that CBP-70, initially shown to be localized in the cytosol and the nucleus, is also associated with endoplasmic reticulum and the Golgi apparatus. Interestingly HSPs are ubiquitous and are found in numerous cellular compartments, from the cytosolic to the endoplasmic reticulum compartments (Hsc-70, the cytosolic HSP-70 form, and BiP, the endoplasmic reticulum HSP-70 form, have a similarity of 61.2 %). Then, the same authors observed the persistence of CBP-70 and of its GlcNAc binding sites after heat-shock treatments [31]. This observation reinforces the notion that the p70 protein, described in the present study as being Hsc-70, could be CBP-70.

Whether the GlcNAc-specific lectins are actually involved in the shuttling of O-N-acetylglucosaminylated glycoproteins between the cytosol and the nucleus remains unanswered. Yet, HSPs have been shown to play a role in many intracellular protein trafficking systems. Especially, Hsc-70 has been demonstrated to be implicated in nuclear transport. Shi and Thomas [32] examined cytosolic factors involved in nuclear import using ATP-affinity chromatography to remove ATP-binding proteins from a cytosolic extract. This depletion reduced transport of nucleoplasmin by 5–10-fold. The transport was restored by back addition of proteins eluted from the ATP–agarose with free ATP, this fraction containing Hsc-70 and HSP-70. Other studies [33] showed that Hsc-70 depletion of a cytosolic extract using antibodies reduced nuclear transport significantly, whereas the addition of purified Hsc-70 restored this transport. The karyophilic protein-dependent accumulation of Hsc-70 was dependent on the cytosolic extract, the temperature and ATP. Interestingly, this transport was sensitive to WGA, reinforcing the implication of a GlcNAc residue.

Based on our experiments (WGA–Sepharose enrichment, galactosyltranferase specific reaction and carbohydrate nature identification), it seems clear that the major ligand of the GlcNAc-specific lectins concerned O-GlcNAc glycoproteins. The use of the biotinylated lectin fractions for detecting endogenous ligands in human lymphocytes and the demonstration of a dual localization of the ligands both in the nucleus and in the cytosol suggests that the presence of O-GlcNAc glycoproteins is neither tissue- nor cell-specific, as already reported [9].

The finding that these GlcNAc-specific lectins belong to the HSP-70 family provides new insights in the field of HSP function.

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