Glycoproteins Present in the Fraction of Chromatin Proteins Loosely Bound to DNA from Hamster, Chicken and Frog Liver Cells

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Abstract. There are numerous glycoproteins recognized by Concanavalin A (ConA) and Galanthus nivalis agglutinin (GNA) in 0.35 mol/l NaCl soluble fraction of chromatin proteins loosely bound to DNA from hamster, chicken and frog liver cells. Results of our detailed comparative analysis show a marked similarity between liver chromatin glycoproteins from the examined animals. The presence of similar chromatin glycoproteins in different animal species may indicate that they play an important universal role in the liver cells.

Key words: Chromatin glycoproteins — Hamster liver — Chicken liver — Frog liver — Immunodetection — Digoxigenin-labelled lectins

Introduction

The dogma in glycobiology has been that glycoproteins do not exist in nonlumenal compartments of the cell. Now, the notion that glycoproteins occur in the nuclear and/or cytoplasmic portions of the cell has gained wide acceptance.

A number of nuclear proteins such as nuclear pore complex proteins (designated nucleoporins) (Panté and Aebi 1996), RNA polymerase II (Kelly et al. 1993), and some transcription factors (Jackson and Tjian 1988; Chou et al. 1995a,b) have been characterized as glycoproteins bearing single unmodified *N*-acetylglucosaminyl (Glc*N*Ac) moiety linked through an *O*-glycosidic bond to the hydroxyl group of serine and threonine residues, often occurring at multiple sites on the same protein. *O*-Glc*N*Ac is an abundant and reversible modification that is postulated to serve a regulatory role by mediating assembly of multimeric complexes and acting as an antagonist of phosphorylation (Hart et al. 1996; Haltiwanger et al. 1997; Hart 1997; Snow and Hart 1998). *O*-Glc*N*Ac-containing proteins appear to be glycosylated and deglycosylated in a dynamic fashion by Glc*N*Ac transferase(s) and specific *N*−acetyl-β-D-glucosaminidase(s), respectively (Haltiwanger et al. 1990,

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1992, Dong and Hart 1994) Glc*N*Ac transferase from rat liver cytosol capable of adding Glc*N*Ac to serine and threonine residues of protein, and a neutral soluble *N*−acetyl-*β*-D glucosaminidase from rat spleen cytosol, which was highly efficient at removing Glc*N*Ac have been purified and characterized (Haltiwanger et al 1990, 1992, Dong and Hart 1994)

On the contrary, little knowledge is at present available about the nuclear *N*-glycosylated proteins (in which oligosaccharides are attached to asparagine residues) They have been found in the nuclear envelope (gp210) (Wozniak et al 1989, Greber et al 1990, Wozniak and Blobel 1992, Berrios et al 1995), the euchromatin fraction (Reeves and Chang 1983, Kan and Pinto da Silva 1986) and nuclear matrix (Burrus et al 1988, Ferraro et al 1991, 1994, Lipinska et al 1994)

The monosaccharide analysis of *N* glycosylated glycoprotein with a molecular mass 66 kDa from pig kidney chromatin revealed the presence of *N*-acetylglucosamine, galactose, mannose and fucose (Eufemi et al 1992)

The importance of *N*-bound oligosaccharide chains of nuclear matrix proteins for this structure was indicated by the results of enzymatic deglycosylation experiments, which showed solubilization of a significant fraction of matrix proteins (25%) upon treatment of the matrix with *N*-glycosidase F (Ferraro et al 1994) Thus, it can be suggested that carbohydrate-protein interactions are additional factors, apart from disulphide cross-linkages, responsible for stabilization of the native nuclear matrix structure Indeed, Reeves and Chang (1983) found that the glycosylated high mobility group proteins, i e HMG14 and HMG17 bind preferentially to the nuclear protein matrix of mammalian cells Furthermore, this association appears to be mediated by the glycosyl chains since enzymatic removal of these modifications from the HMGs greatly reduced their binding to the nuclear matrix It is tempting to suggest that this carbohydrate modification of HMG proteins may have functional significance for the architectural organization of the active domains of chromatin in cells

Several authors demonstrated the presence of glycoproteins in chromatin many years ago (Stein et al 1975, Rizzo and Bustin 1977, Kan and Pinto da Silva 1986) Glycoproteins binding ConA were found to be present in the fractions of non-histone chromatin proteins which are loosely and tightly bound to DNA In studies of chromatin glycoproteins from mammalian tissues evidence has been provided for their marked tissue-specificity (Ferraro et al 1988, 1989, Cervoni et al 1990, Eufemi et al 1991) The tissue specificity of glycoproteins together with their preferential distribution in the actively transcribing chromatin suggest that they play a role in the regulation of genome expression On the contrary, little knowledge is at present available about the species-specificity of nuclear glycoproteins

In this study we undertook a comparative analysis of glycoproteins recognized by ConA and GNA present in 0 35 mol/l NaCl soluble fraction of liver chromatin proteins loosely bound to DNA from three animal species widely separated on the evolutionary scale, i e hamster, chicken and frog

Materials and Methods

Livers of three animal species: hamster (*Mesocricetus auratus*), chicken (*Gallus gallus*) and frog (*Rana esculenta*) were used for experiments.

Liver nuclei from the examined animal species were isolated by a modified sucrose method (Chauveau et al. 1956). All solutions for preparation were used at 4 °C and contained 1mmol/l phenylmethylsulphonyl fluoride (PMSF). The tissue was homogenized in the proportions of 1g to 10 ml of 0.25 mol/l sucrose, 5 mmol/l $MgCl_2$, and 0.8 mmol/l KH_2PO_4 at pH 6.7, filtered through four layers of gauze and spun down at 800 × *g* for 7 min. The pellet was resuspended in the above solution, and Triton X-100 was added to a final concentration of 0.5% and then the suspension was homogenized again and centrifuged at 800 × *g* for 7 min. The crude nuclear pellet was suspended in 10 vol. of 2.2 mol/l sucrose, 5 mmol/l $MgCl_2$ and centrifuged at 40 000 × *g* for 45 min. The purity and integrity of the nuclei were checked by light microscopy. Distinct non-nuclear contaminations were absent in purified nuclear preparations.

Liver chromatin of the three animal species was isolated from nuclei according to MacGillivray et al. (1972). The nuclear pellet was extracted twice with 10 vol. of 0.14 mol/l NaCl, 1 mmol/l PMSF for 10 min and centrifuged at 1000 × *g* for 10 min. Then, the pellet was extracted twice for 10 min with 0.1 mol/l Tris-HCl buffer (pH 7.5) containing 1 mmol/l PMSF and centrifuged at 10 000 × *g* for 10 min.

The fraction of chromatin proteins loosely bound to DNA was obtained from liver chromatin by three successive extractions using solution of 0.35 mol/l NaCl, 5% isopropanol, 1 mmol/l PMSF with centrifugation at 10 000 × *g* for 10 min. Finally, the three extracts were combined and centrifuged at 40 000 × *g* for 60 min (Durand and Pieri 1986).

Chromatin protein samples were mixed with 0.9 vol. of solubilizing buffer (20% glycerol, 4% sodium dodecyl sulphate /SDS/, 25 μg/ml PyronineY, 0.125 mol/l Tris-HCl buffer, pH 6.8) and with 0.1 vol. of 2-mercaptoethanol, and then heated in a boiling water bath for 5 min. One-dimensional electrophoresis was performed in slab polyacrylamide gels containing 0.1% SDS and 8% or 11.2% acrylamide (pH 8.8) with 3% stacking gel (pH 6.8) according to Laemmli (1970) at 25 mA/slab gel until the Pyronine Y marker reached the end of the stacking gel, and then at 35 mA/slab in the resolving gel until the marker dye reached the bottom of the gel. The slab gels were stained with Coomassie Brilliant Blue R-250 according to Fairbanks et al. (1971). Excess of stain was removed with 10% acetic acid containing 5% methanol. Relative molecular masses of protein bands were calculated by comparison with the standard proteins: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovoalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactoalbumin (14.4 kDa).

Proteins separated in SDS-polyacrylamide slab gels were transferred onto Immobilon-P transfer membranes (pore size 0.45 μm) by electrophoretic blotting in

20% methanol, 192 mmol/l glycine, and 25 mmol/l Tris (pH 8 3) for 15 h at 60 V and 4°C (Towbin et al 1979) Proteins immobilized on transfer membranes were stained by a 5 min incubation at room temperature with 0 2% Ponceau S in 3% acetic acid, followed by destaining in H_2O until the protein bands were visible, in order to control the efficiency of blotting and to mark the location of standard proteins The Ponceau S staining of protein bands disappeared during subsequent incubation of membrane in the blocking solution

The detection of lectin-binding glycoproteins immobilized on Immobilon-P sheets was accomplished by the method of Haselbeck et al (1990) The membranes were treated for 30 min at room temperature with 20 ml 0 5% blocking reagent (w/v) in Tris buffered saline (TBS 50 mmol/l Tris HCl, 150 mmol/l NaCl, pH 7 5) and were washed twice with TBS and once with buffer 1 consisting of TBS, 1 mmol/l $MnCl_2$, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, at pH 7 5 (50 ml each) The membranes were incubated for 1 h at room temperature with the lectin-digoxigenin (DIG) conjugate (ConA, 10 μg/ml, GNA, 1 μg/ml) in 20 ml of buffer 1

Then, the membranes were washed three times with 50 ml TBS, and polyclonal sheep anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (anti-DIG AP, 750 U/ml) were added in a 1 1000 dilution in 20 ml TBS and incubated for 1 h at room temperature The membranes were again washed three times with 50 ml TBS and the alkaline phosphatase reaction was carried out by incubating the membrane without shaking in 20 ml of the following freshly prepared solution 75 μl 5-bromo-4-chloro-3-indolyl phosphate 4-toluidine salt (BCIP 50 mg/ml in dimethylformamide) and 100 μl 4-nitro blue tetrazolium chloride (NBT 75 mg/ml, in 70% dimethylformamide) in 20 ml of buffer 2 (100 mmol/l Tris-HCl, 50 mmol/l $MgCl_2$, 100 mmol/l NaCl at pH 9 5) The reaction was completed within a few minutes The membranes were raised with H_2O to stop the reaction, and allowed to dry

In controls, preincubation of ConA and GNA with methyl-α-D mannopyranoside at a concentration of 0 2 mol/l was performed

The protein content was assayed using bovine serum albumin as a standard by the method of Lowry et al (1951)

All reagents used for the detection and characterization of glycoproteins on blots were from Boehringer Mannheim (Germany) Other reagents were of the highest purity available and were purchased from Sigma (St Louis, Missouri, U S A) The Immobilon-P transfer membranes were obtained from Millipore Corporation (Bedford, Massachusetts, U S A)

Results

This investigation focused on the comparative analysis of *N*-glycosylated proteins recognized by ConA and GNA present in 0 35 mol/l soluble fraction of liver chromatin proteins loosely bound to DNA from three animal species hamster, chicken and frog

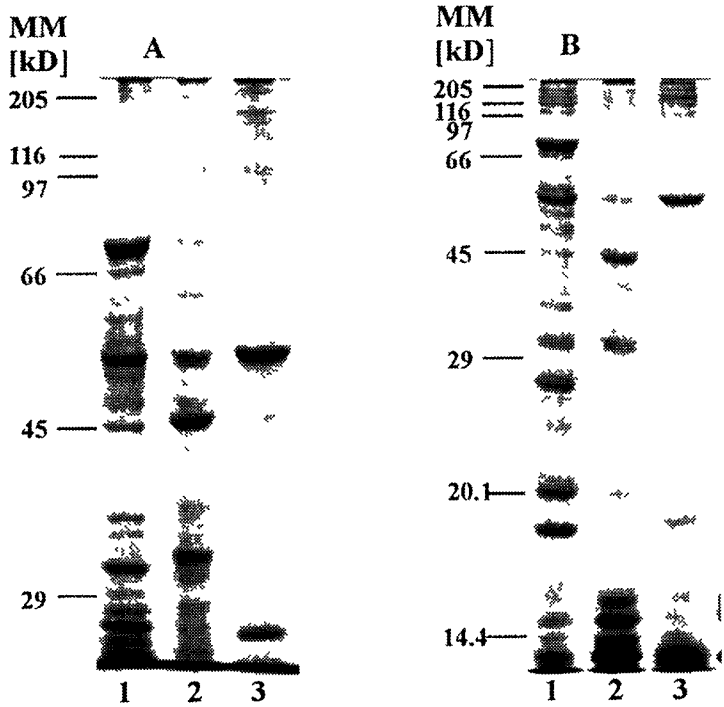

Figure 1. SDS-PAGE of 0 35 mol/l NaCl soluble chromatin proteins loosely bound to DNA from liver of hamster (lane 1), chicken (lane 2) and frog (lane 3) on 8% (A) and 11 2% (B) acrylamide slab gels Bars indicate the position of standard proteins Gels were stained with Coomassie Brilliant Blue R-250 and 50 μg proteins were applied per lane

Chromatin proteins obtained from liver nuclei of the examined vertebrates were separated by one-dimensional electrophoresis performed in slab polyacrylamide gels containing 0.1% SDS and 8% or 11 2% acrylamide (pH 8.8) with 3% stacking gel (pH 6.8) according to Laemmli (1970). Typical polypeptide patterns of liver chromatin proteins loosely bound to DNA from hamster, chicken and frog obtained after SDS-PAGE and after staining with Coomasie Brilliant Blue R-250 are shown in Fig. 1. In electrophoretic conditions performed in this investigation the chromatin proteins were distributed from about 14 to above 200 kDa. Significant quantitative and some qualitative differences in the electrophoretic patterns of the liver chromatin proteins were observed. Chromatin proteins separated by one-dimensional SDS-PAGE (Fig. 1) were transferred onto Immobilon-P transfer membranes and tested for ConA and GNA binding (see Materials and Methods). The results of these experiments are demonstrated in Figs. 2 and 3. Now, it is known that glycoproteins stain weakly in polyacrylamide gels by commonly used protein stains such as Coomassie Brilliant Blue R-250 or silver, probably because of steric interference

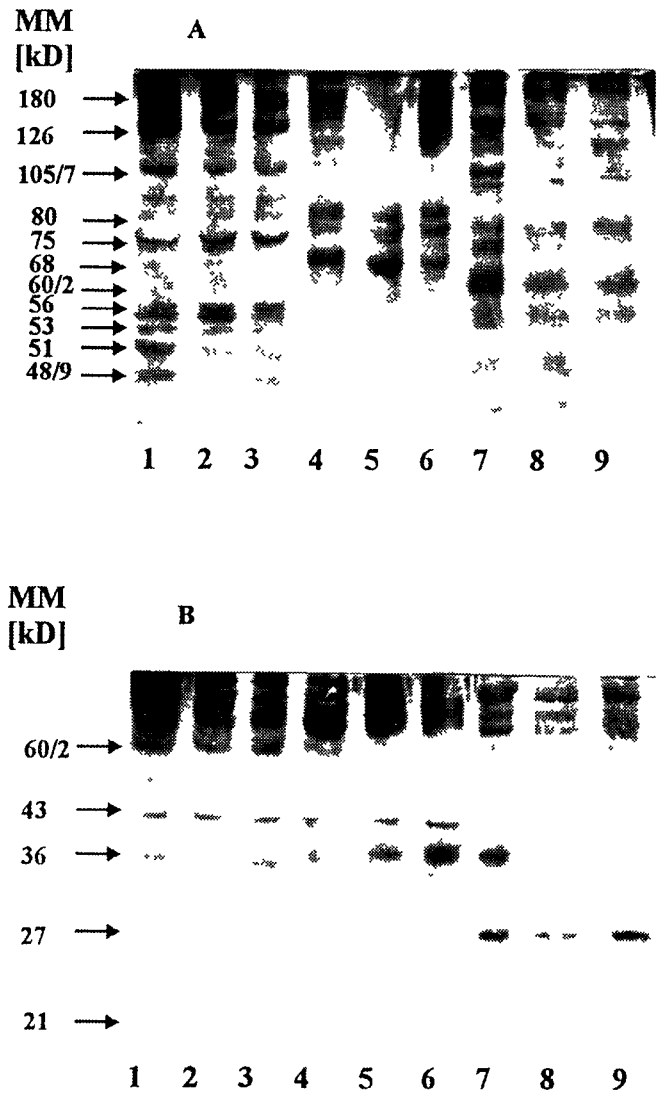

Figure 2. Liver chromatin proteins loosely bound to DNA (three separate preparations, 50 μg) from hamster (lanes 1–3), chicken (lanes 4–6) and frog (lanes 7–9) electrophoresed on 8% (A) and 11 2% (B) acrylamide slab gels were transferred onto Immobilon-P membranes and tested for ConA binding

(Møller and Poulsen 1995). So, some glycoprotein bands which were undetected by protein staining with Coomassie Brilliant Blue R-250 were revealed by lectins. The patterns shown in Fig. 2 allow to compare glycoproteins which carry mannose as

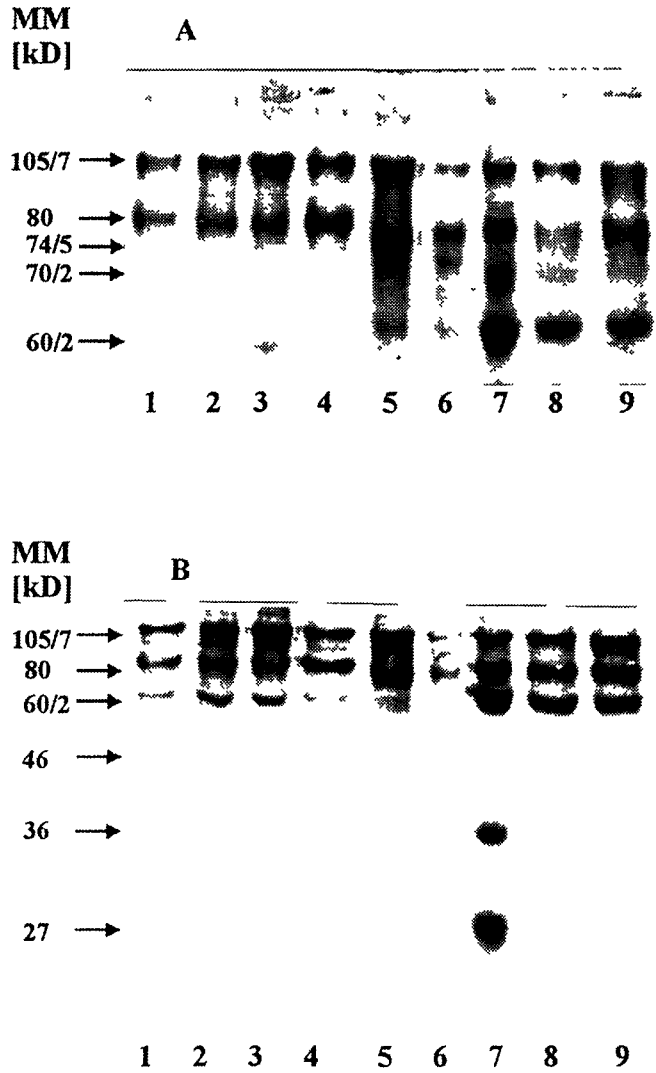

Figure 3. Liver chromatin proteins loosely bound to DNA (three separate preparations, 50 μg) from hamster (lanes 1–3), chicken (lanes 4–6) and frog (lanes 7–9) electrophoresed on 8% (A) and 11 2% (B) acrylamide slab gels were transferred onto Immobilon-P membranes and tested for GNA binding

a terminal sugar of the oligosaccharide. It can be seen that numerous chromatin proteins bind ConA Our detailed comparative analysis of liver chromatin glycoproteins indicates that the majority of glycoproteins visualized by ConA binding were

common at least in two of three animal species The differences in intensity of the bands corresponding to analogous glycoproteins of these examined species could be attributed to a different level of their glycosylation or to a different amount of the same protein However, the limitation, which was given by the used system of electrophoresis, makes a comparison of glycoproteins recognized by ConA existing as minor components impossible The major liver chromatin proteins loosely bound to DNA recognized by ConA are characterized by molecular masses in the range 21–43, 48/49 56, 75 105/7 and 126–180 kDa in the case of hamster, 21–43 and 68–80 – chicken and 27–36, 60/2–80 – frog

Some of the protein bands stained by ConA were recognized also by *Galanthus nivalis* agglutinin (GNA), this kind of recognition by GNA – a lectin specific for terminal bound mannoses (α 1–2, 1–3, 1–6 to mannose) – strongly indicated the presence of a high mannose type of oligosaccharide (Fig 3) A comparison of glycoproteins present in the fraction of chromatin proteins loosely bound to DNA from liver of the examined animals reveals that the main glycoprotein components are the same It can be seen that GNA-binding glycoproteins present in 0 35 mol/l NaCl soluble fraction of chromatin proteins from hamster, chicken and frog liver cells have approximate molecular masses of 27, 36, 46, 60/2, 74/5, 80 and 105/7 kDa

Control immunodetections indicated that all the above-mentioned glycoproteins bind ConA and GNA in a specific manner There was no lectin binding if ConA or GNA was added together with 0 2 mol/l methyl-α-D-mannopyranoside, which blocked the lectin active site (data not shown)

Discussion

A considerable amount of information has been obtained on tissue-specificity of mammalian chromatin glycoproteins but much less is presently known about glycoproteins originating from different animal species especially those representing different classes of vertebrates

Eufemi et al (1991) have compared the glycoproteins from liver and heart chromatin fractions differing in affinity to DNA from two mammalian species (pig and rabbit) and a bird (chicken) Comparative analysis of ConA binding glycoproteins present in the fraction of chromatin proteins tightly bound to DNA of liver and heart from chicken, rabbit and pig revealed a lack of species-specificity However, some species-specific glycoproteins binding ConA were found to be present in the fraction of chromatin proteins loosely bound to DNA, i e extracted by 0 35 mol/l NaCl From an evolutionary point of view it seems interesting to note that much more similar were the glycoproteins of two mammals as compared to those of a mammal and a bird But the authors emphasize that tissue-specificity rather than species-specificity appears to be quite a general property of these proteins, suggesting for them a role in the mechanism of regulation of chromatin function

The results of this study indicate that numerous ConA and GNA binding glycoproteins are present in the fraction of chromatin proteins loosely bound to DNA

originating from liver cells of hamster, chicken and frog. The main glycoproteins recognized by ConA from hamster, chicken and frog liver were common at least in two of the three animal species. The differences in intensity of the bands corresponding to analogous glycoproteins of these animal species could be attributed to different levels of their glycosylation or to different amounts of the same protein.

Some glycoproteins were recognized by GNA, which indicates the presence of a high mannose type of oligosaccharide. The majority of glycoproteins binding GNA were the same in all cases, suggesting that there is no species specificity of glycoproteins present in this fraction. Our results support the suggestion of Eufemi et al. (1991) that species-specificity does not appear to be a general property of chromatin glycoproteins. The presence of similar chromatin glycoproteins loosely bound to DNA in animal species representing different classes of vertebrates may indicate that they play an important universal role in liver cells.

The fraction of chromatin proteins extracted by 0.35 mol/l NaCl contains a number of well-characterized proteins that play an important role in the structure and function of chromatin. Among them are the high mobility group proteins (HMG) and the low mobility group proteins (LMG) (Wiland et al. 1990). Considering the molecular masses of the main glycoproteins of this liver chromatin fraction from hamster, chicken and frog they could be included in LMG proteins. LMG proteins are heterogeneous as regards their molecular masses (from about 12 to 100 kDa) and amino acid composition. The function of these proteins has not been examined sufficiently. The lack of marked tissue specificity and the relative abundance suggest that some of the LMG proteins have a structural function (Wiland et al. 1990). So, the similarity of glycoproteins from the fraction of chromatin proteins loosely bound to DNA from hamster, chicken and frog liver may indicate that they have the same, probably structural function.

Acknowledgements. We wish to express our thanks to M. Radwan for photography. This work was supported by grant 505/672 from the University of Łódź.

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Final version accepted January 1, 2001