Short Communication

The Effect of O-GlcNAc Glycosylation of Rat Liver Nucleoproteins on their Acute Phase-Dependent Binding Ability to the Hormone Responsive Element of the Haptoglobin Gene

I. Grigorov, I. Cvetković, T. Milosavljević, A. Uskoković, D. Bogojević, S. Ivanović-Matić, G. Poznanović and M. Petrović

Department of Molecular Biology, Institute of Biological Research, Belgrade, Serbia and Montenegro

Abstract. We examined whether the transcriptional activation of the rat haptoglobin (Hp) gene during the acute phase (AP) response reflects the O-linked N-acetylglucosamine (O-GlcNAc) status of liver nucleoproteins (NPs) and their binding for the hormone responsive element (HRE). After deglycosylation with N-acetylglucosaminidase of the O-GlcNAc glycoproteins obtained by WGA, affinity chromatography and South-Western analysis, it was observed that only increased HRE binding ability of p64/p70 in control and p51 obtained from turpentine-treated rats can be directly attributed to the presence of O-GlcNAc residues. Therefore, expression of the rat Hp gene could be controlled by this modification of certain trans-acting NPs.

Key words: O-GlcNAc glycosylation — *Trans*-acting proteins — DNA binding — Haptoglobin gene — Acute phase response

A unique form of nucleoplasmic and cytoplasmic protein glycosylation in which single, unmodified N-acetylglucosamine monosaccharides are O-glycosidically linked to serine or threonine residues (O-GlcNAc) has been found on many proteins that are essential to all aspects of cellular physiology. Among them are nuclear pore proteins, RNA polymerase II and its associated transcription factors, chromatin proteins, oncogenes, tumor supressors, regulatory proteins, small heat-shock proteins, enzymes and cytoskeletal proteins (Snow and Hart 1998; Comer and Hart 2000). Given the broad spectrum of proteins that contain this modification, there are likely to be many different functions for O-GlcNAc. Aside from an apparent role of O-GlcNAc-containing nucleoporins in nucleocytoplasmic transport (Hart 1997),

Correspondence to: Ilijana Grigorov, Department of Molecular Biology, Institute for Biological Research, Despot Stephen Blvd. 142, 11060 Belgrade, Serbia and Montenegro
E-mail: iligri@ibiss.bg.ac.yu

potential functions of glycosylation by O-GlcNAc include the conferral of protein stability, an involvement in nuclear localization signals, a role in the assembly of multimeric protein complexes and blocking of sites of protein phosphorylation (Snow and Hart 1998). Studies of transcription factor Sp1 suggest that O-GlcNAc protects this protein from proteasome degradation (Han and Kudlow 1997) and enhances its transcriptional activation function (Jackson and Tjian 1988). The possible involvement of the O-GlcNAc type of glycosylation in the enhacement of DNA-binding and transcriptional activities have been reported for HNF-1 (Lichtsteiner and Schibler 1989), p53 (Shaw et al. 1996) and the serum response factor (Reason et al. 1992). All three proteins appear to be glycosylated and deglycosylated in a dynamic manner in the sense that they possess varying amounts of O-GlcNAc residues in response to different physiological stimuli. This finding suggests that differential glycosylation is a reflection of important functional differences between transcription factors.

In this paper we investigated whether the transcriptional activation of the rat haptoglobin (Hp) gene during the acute phase (AP) response resulted from the O-GlcNAc glycosylation-related modulation of the binding of liver nucleoproteins (NPs) to the hormone responsive element (HRE). Hp belongs to the group of so-called AP proteins whose genes are markedly and transiently induced in response to acute inflammation, a variety of tissue injuries and traumas (Kushner and Mackiewicz 1993). The transcriptional activation of AP protein genes is incorporated in the complex interplay of cytokines, growth factors and glucocorticoid hormones, resulting in specific assemblies of *trans*-acting NPs on the promoters and enhancers of these genes. Accumulating evidence indicates that different combinations of a relatively small number of *trans*-acting proteins with similar DNA binding abilities, their capability to homo- and heterodimerise and their posttranslational modification play a critical role in achieving a high level of orchestration required for gene expression (Akira 1997). It was shown previously that the transcriptional activation of the rat Hp gene during the turpentine-induced AP response mainly coincided with an increase in the binding ability of several constitutively expressed NPs for the HRE located in the promoter region at position −170 to −56 (Ševaljević et al. 1995). Some of the proteins were identified as members of C/EBP (Grigorov et al. 1998; Milosavljević et al. 2003) and STAT (Grigorov et al. 2002) families of transcription factors and as HMG1 protein (Petrović et al. 1996; Grigorov et al. 2001).

Experiments were performed on two-month-old male Wistar rats. The AP response was induced by a single subcutaneous injection of turpentine oil (1 μl/g *per* body weight). The rats were sacrificed after 12 h when maximal transcription of the rat Hp gene is established (Ševaljević et al. 1989). Liver nuclear extracts (NEs) were isolated from control and turpentine-treated animals following the procedure of Gorski et al. (1986). *Trans*-acting NPs involved in the transcriptional regulation of the Hp gene were isolated from the NEs by DNA affinity chromatography. The DNA affinity column was prepared with the Hp gene HRE (−170/− 56 bp) which was linked to a CNBr-activated Sepharose 4B column (Pharmacia) according to

Kadonaga and Tjian (1986). NEs from the livers of control and turpentine-treated rats were applied to the HRE affinity column and the HRE-binding NPs were eluted with a buffer containing 1 mol/l KCl. Equal amounts of HRE-binding NPs from both livers were subjected to SDS-PAGE (Laemmli 1970) and stained with silver. As can be seen in Figure 1A, the DNA binding ability of several NPs changed in the course of the AP response. NPs with molecular masses from 20 to 70 kD (designated as p20 and p70, respectively) displayed an increased binding ability towards the HRE (Fig. 1A, lane 2) relative to the control sample (lane 1), while p32 and p51 displayed a DNA-binding only under AP conditions. This result was confirmed by South-Western analysis (Bowen et al. 1980). The NPs were separated by SDS-PAGE, blotted and probed with α (^{32}P)dCTP-labelled HRE (Fig. 1A, lanes 3 and 4).

The possibility that a differential O-GlcNAc glycosylation of NPs plays a role in the regulation of Hp gene transcription was assessed by examining the capability of wheat germ agglutinin (WGA) lectin that recognizes GlcNAc/sialic acid-bearing glycoproteins, to interact with putative carbohydrate residues of NPs. After eluting from the HRE affinity column equal amounts of HRE-binding NPs prepared from the livers of control and turpentine-treated rats were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-C, Amersham). The membrane was firstly incubated in TBST buffer (100 mmol/l Tris-HCl, pH 7.5; 15 mmol/l NaCl; 0.1% Tween 20) with 3 mg/ml BSA, and then in TBST with bi-

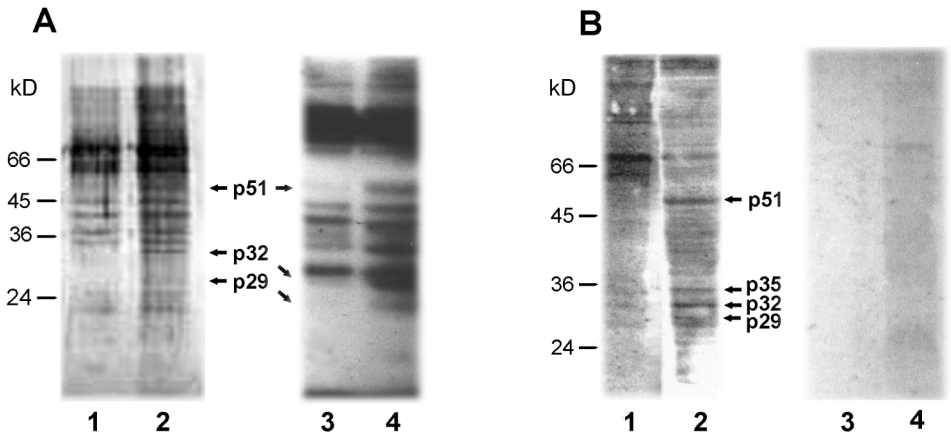

Figure 1. Detection of nuclear O-GlcNAc glycoproteins that bind the HRE of the rat Hp gene. **A.** HRE-binding NPs from the livers of control (lanes 1 and 3) and turpentine-treated rats (lanes 2 and 4) characterized by DNA affinity chromatography (lanes 1 and 2) and South-Western analysis (lanes 3 and 4). **B.** WGA-Western blot assay of HRE affinity-purified nucleoproteins prepared from control (lanes 1 and 3) and turpentine-treated rats (lanes 2 and 4) in the absence (lanes 1 and 2) or presence of 0.5 mol/l D-GlcNAc as a specific competitor (lanes 3 and 4). Molecular mass standards are indicated in kD.

otinylated WGA (10 mg/ml) in the presence/absence of N-acetyl-D-glucosamine (D-GlcNAc) (Sigma) as a specific competitor. The unbound lectin was removed by rinsing the membranes with TBST. After incubation of the membrane with an avidine-biotinylated alkaline phosphatase complex, the binding reaction was visualized with BCIP/NBT (INC kit SP-2001, Vector Laboratories). The obtained results revealed that the AP response was accompanied by increased O-GlcNAc glycosylation of p29, p32, p35 and p51, and deglycosylation of p64 and p70 (Fig. 1B, lane 2) with respect to the same proteins obtained from control rats (Fig. 1B, lane 1). The specificity of rat liver *trans*-acting NPs-WGA interactions was confirmed by performing a WGA-Western blot assay in the presence of 0.5 mol/l D-GlcNAc as a specific competitor (Fig. 1B, lanes 3 and 4).

To examine whether the obtained AP-related increase of O-GlcNAc glycosylation of p29, p32, p35 and p51 affects their ability to bind the Hp gene HRE, South-Western analysis with deglycosylated rat liver NPs was performed. 25 μg of glycoproteins containing O-GlcNAc residues, that were purified from NEs of control and treated rats by WGA affinity chromatography (Jackson and Tjian 1989), were separated by SDS-PAGE and transferred to PVDF membranes (Fig. 2A). The glycoproteins on the PVDF membranes were deglycosylated by incubation with β-N-acetylglucosaminidase (4 units U/ml in 50 mmol/l sodium-citrate buffer, pH 5.0) (Sigma) for 4 h at 37°C. The membranes were then incubated with the

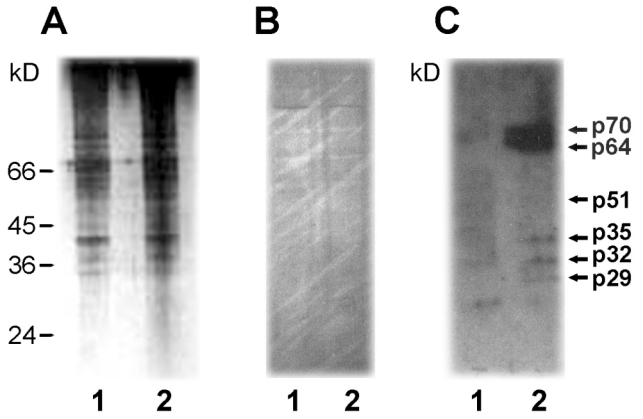

Figure 2. DNA binding properties of nuclear O-GlcNAc glycoproteins that bind the HRE of the rat Hp gene. **A.** Silver staining of WGA affinity purified O-GlcNAc glycoproteins from liver nuclear extracts of control (lane 1) and turpentine-treated (lane 2) rats. **B.** PVDF membranes bearing WGA affinity purified O-GlcNAc glycoproteins from liver nuclear extracts of control (lane 1) and turpentine-treated (lane 2) rats were deglycosylated with β-N-acetylglucosaminidase and probed with biotinylated WGA. **C.** South-Western analysis of deglycosylated glycoproteins prepared from the livers of control (lane 1) and turpentine-treated rats (lane 2) using α (^{32}P)dCTP-labelled HRE of the Hp gene. Molecular mass standards are indicated in kD.

labelled HRE. The WGA-Western blot assay of deglycosylated NPs confirmed that deglycosylation was highly efficient (Fig. 2B). South-Western profiles revealed that only control p64 and p70 (Fig. 2C, lane 1) and p51 obtained from treated rats (lane 2) lost their abilities to bind the HRE after deglycosylation. This finding suggests that O-GlcNAc glycosylation is a major structural modification that regulates the interaction of these proteins with DNA under different physiological conditions.

In several documented instances posttranslational modifications of proteins by O-glycosylation and O-phosphorylation were shown to be reciprocal and to occur on the same or adjacent serine or threonine hydroxyl groups (Kelly et al. 1993; Chou et al. 1995a; Comer and Hart 2000). Furthermore, all O-GlcNAc-modified proteins identified up-to-date also occur as phosphorylated proteins. Since several recent reports have provided direct support for a relationship between O-phosphorylation and O-glycosylation (Haltiwanger et al. 1997; Griffith and Schmitz 1999; Lefebvre et al. 1999), we investigated this aspect of modulation of protein DNA-binding of rat liver NPs during the AP response. The NEs isolated from control livers were *in vitro* phosphorylated in the presence of endogenous kinases and unlabelled ATP (Piccoletti et al. 1993), separated by SDS-PAGE and subjected to South-Western analysis with (α^{32}P)dCTP-labelled HRE. The autoradiogram showed that *in vitro* phosphorylation (Fig. 3, lane 2) increased the binding ability of control p32, p35, p38, p64 and p70 to levels similar to those observed during the AP response (lane 3). However, *in vitro* phosphorylation had no effect on the DNA binding ability of p51. Thus, we assumed that p51 required O-GlcNAc glycosylation for its enhanced DNA binding ability, whereas p64 and p70 required the removal of O-GlcNAc residues in order to be phosphorylated during the AP response. Besides, the AP-related variations in the p32 glycosylation status did not appear to be important for the

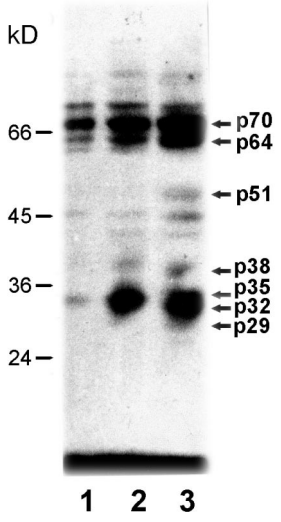

Figure 3. South-Western analysis of rat liver nucleoprotein HRE-binding after *in vitro* phosphorylation. Lanes 1 and 3 – liver NPs prepared from control and turpentine-treated rats; lane 2 – control liver NPs pretreated with unlabelled ATP (50 μmol). Molecular mass standards are indicated in kD.

regulation of its ability to bind the HRE. In our previous papers we identified p29 and p32 as HMG1 (Grigorov et al. 2001) and an isoform of C/EBPβ (Milosavljević et al. 2003), respectively. Our preliminary data suggest that p51 belongs to the NF-kB transcription factor family (unpublished data).

The data presented here are in agreement with the results from several authors who provided evidence that O-GlcNAc glycosylation influences the DNA binding ability of transcription factors (Jackson and Tjian 1988; Reason et al. 1992; Shaw et al. 1996). The exact mechanism by which this type of glycosylation achieves this is still unknown. In those cases when glycosylation increases the ability of transcription factors to bind DNA, the glycosylation sites are removed from the DNA-binding domain. Thus, it appears that glycosylation alters protein conformation so that its DNA-binding ability is changed (Shaw et al. 1996). Glycosylation also appears either within the transactivation domain (Chou et al. 1995b) or within PEST sequences (Rechsteiner and Rogers 1996; Jiang and Hart 1997). Thereby it could lead to changes of the transactivating potential or stability of the transcription factor, which could be a case for p32.

Acknowledgements. This work was supported by the Ministry for Science and Technology of Serbia and Montenegro, grant No. 1722.

References

Akira S. (1997): IL-6 regulated transcription factors. Int. J. Biochem. Cell Biol. **29**, 1401—1418

Bowen B., Steinberg J., Laemmli U. K., Weintraub H. (1980): The detection of DNA-binding proteins by protein blotting. Nucleic Acids Res. **3**, 1—20

Chou T. Y., Hart G. W., Dang C. V. (1995a): c-Myc is glycosylated at threonine 58, a known phosphorylation site and a mutational hot spot in lymphomas. J. Biol. Chem. **270**, 18961—18965

Chou T. Y., Dang C. V., Hart G. W. (1995b): Glycosylation of the c-Myc transactivation domain. Proc. Natl. Acad. Sci. U.S.A. **92**, 4417—4421

Comer F. I., Hart G. W. (2000): O-glycosylation of nuclear and cytosolic proteins. Dynamic interplay between O-GlcNAc and O-phosphate. J. Biol. Chem. **275**, 29179—29182

Gorski K., Carneiro M., Schibler U. (1986): Tissue specific *in vitro* transcription from the mouse albumin promoter. Cell **47**, 767—776

Griffith L. S., Schmitz B. (1999): O-linked N-acetylglucosamine levels in cerebellar neurons respond reciprocally to pertubations of phosphorylation. Eur. J. Biochem. **262**, 824—831

Grigorov I., Milosavljević T., Cvetković I., Petrović M. (1998): Participation of two C/EBPβ transcription factor in the acute-phase regulation of the rat haptoglobin gene. Cell Biol. Int. **22**, 685—693

Grigorov I., Milosavljević T., Cvetković I., Petrović M. (2001): HMG-1 as regulatory transacting protein in the acute-phase-induced expression of the rat liver haptoglobin gene. Gen. Physiol. Biophys **20**, 401—412

Grigorov I., Lazić T., Cvetković I., Milosavljević T., Petrović M. (2002): Opposite nuclear level and binding activity of STAT5B and STAT3 proteins with rat haptoglobin gene under normal and turpentine induced acute phase conditions. Mol. Biol. Rep. **28**, 217—222

Haltiwanger R. S., Busby S., Grove K., Li S., Mason D., Medina L., Moloney D., Philipsberg G., Scartozzi R.(1997): O-glycosylation of nuclear and cytoplasmic proteins: regulation analogous to phosphorylation? Biochem. Biophys. Res. Commun. **231**, 237—242

Han I., Kudlow J. E. (1997): Reduced O glycosylation of Sp1 is associated with increased proteasome susceptibility. Mol. Cell. Biol. **17**, 2550—2558

Hart G. W. (1997): Dynamic O-linked glycosylation of nuclear and cytoskeletal proteins. Annu. Rev. Biochem. **66**, 315—335

Jackson S. P., Tjian R. (1988): O-glycosylation of eukaryotic transcription factors: Implications for mechanisms of transcriptional regulation. Cell **55**, 125—133

Jackson S. P., Tjian R. (1989): Purification and analysis of RNA polimerase II transcription factors by using wheat germ agglutinin affinity chromatography. Proc. Natl. Acad. Sci. U.S.A. **86**, 1781—1785

Jiang M. S., Hart G. W. (1997): A subpopulation of estrogen receptors are modified by O-linked N-acetylglucosamine. J. Biol. Chem. **272**, 2421—2428

Kadonaga J. T., Tjian R. (1986): Affinity purification of sequence-specific DNA binding proteins. Proc. Natl. Acad. Sci. U.S.A. **83**, 5889—5893

Kelly W. G., Dahmus M. E., Hart G. W. (1993): RNA polimerase II is a glycoprotein-modification of the COOH-terminal domain by O-GlcNAc. J. Biol. Chem. **268**, 10416—10424

Kushner I., Mackiewicz A. (1993): Molecular biology, biochemistry, and clinical applications. In: Acute Phase Proteins (Eds. A. Mackiewicz , I. Kushner and H. Baumann.), pp. 4—14, CRC Press, Inc., Boca Raton, Fl

Laemmli U. K. (1970): Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature **227**, 680—685

Lefebvre T., Alonso C., Mahboub S., Dupire M. J, Zanetta J. P., Caillet-Boudin M. L., Michalski J. C. (1999): Effect of okadaic acid on O-linked N-acetylglucosamine levels in a neuroblastoma cell line. Biochim. Biophys. Acta **1472**, 71—81

Lichtsteiner S., Schibler U. (1989): A glycosylated liver-specific transcription factor stimulates transcription of the albumin gene. Cell **57**, 1179—1187

Milosavljević T., Lazić T., Uskoković A., Petrović M., Grigorov I. (2003): Expression of the rat liver haptoglobin gene is mediated by isoforms of C/EBPα, $-\beta$ and $-\delta$ proteins. Gen. Physiol. Biophys. **22**, 181—190

Petrović M., Grigorov I., Milosavljević T., Bogojević D., Šekularac S., Ševaljević Lj. (1996): Structural and functional homology between the 29kDa rat liver nucleoprotein and the high mobility group 1 protein. Mol. Biol. Rep. **23**, 79—85

Piccoletti R., Bendinelli P., Arienti D., Maroni P., Bernelli-Zazzera A. (1993): Phosphorylation pattern of liver proteins during the early stages of the acute-phase response. Cell Biol. Int. 17, 425—432

Reason A. J., Morris H. R., Panico M., Marais R., Treisman R. H., Haltiwanger R. S., Hart G. W., Kelly W. G., Dell A. (1992): Localization of O-GlcNAc modification on the serum response transcription factor. J. Biol. Chem. **267**, 16911—16921

Rechsteiner M., Rogers S. W. (1996): PEST sequences and regulation by proteolisis. Trends Biochem. Sci. **21**, 267—271

Shaw P., Freeman J., Bovey R., Iggo R. (1996): Regulation of specific DNA binding by p53: evidence for a role for O-glycosylation and charged residues at the carboxy-terminus. Oncogene **12**, 921—930

Snow D. M., Hart G. W. (1998): Nuclear and cytoplasmic glycosylation. Int. Rev. Cytol. **181**, 43—74

Ševaljević Lj., Marinković-Pajović S., Grigorov I., Bogojević D., Ivanović-Matić S., Petrović M. (1995): Acute phase-dependent changes in the binding of rat liver nu-

cleoproteins to the cytokine response element of the rat haptoglobin gene. Int. J. Biochem. Cell. Biol. **27**, 185—194

Ševaljević Lj., Petrović M., Bogojević D., Savić J., Pantelić D. (1989): Acute-phase response to scalding: changes in serum properties and acute-phase potein concentrations. Circ. Shock **28**, 293—307

Final version accepted: April 26, 2004