Short Communication

Acute-Phase-Dependent Binding Affinity of C/EBPβ from the Nuclear Extract and Nuclear Matrix towards the Hormone Response Element of the α_2-Macroglobulin Gene in Rat Hepatocytes

D. Bogojević , M. Mihailović, M. Petrović, S. Dinić, S. Ivanović-Matić and G. Poznanović

Molecular Biology Laboratory, Institute for Biological Research, 29 Novembra 142, 11000 Belgrade, Serbia and Montenegro

Abstract. Interactions of nuclear extract and nuclear matrix proteins from rat hepatocytes with the hormone response element of the α_2-macroglobulin gene were studied. By Western and South-Western blot analysis we have shown the presence of C/EBPβ in the examined nuclear fractions as well as its increased binding affinity to the examined gene sequence during the acute-phase response. The results suggest that both nuclear protein fractions could participate in the transcriptional regulation of the α_2-macroglobulin gene in the rat hepatocytes.

Key words: C/EBPβ — α_2-macroglobulin gene — Nuclear extract — Nuclear matrix — Hepatocytes

The acute-phase response represents a cascade of reactions that are initiated to prevent tissue damage and activate repair processes in order to reestablish homeostasis. Various types of stress, such as tissue injury or infections, induce dramatic changes of concentrations of several plasma proteins known as the acute-phase proteins. α_2-macroglobulin (α_2MG) is a prominent acute-phase protein in the rat. It is a tetrameric molecule that contains two non-covalently linked and identical dimers where the 180-kDa monomers are covalently linked by disulfide bridges (Borth 1992). α_2MG is a broad-spectrum protease-binding protein with an immune-suppressive activity and its plasma concentration increases during the inflammatory process. It is primarily synthesized in the liver, although, fibroblasts, macrophages and decidua express α_2MG as well. The increase of α_2MG concentration in the serum is the result of the transcriptional activation of its gene, and the corresponding increase of the mRNA rates and protein synthesis. Hepatic α_2MG gene expression is primarily mediated by cytokine interleukin 6

Correspondence to: Dr. Desanka Bogojević, Molecular Biology laboratory, Institute for Biological Research, 29 Novembra 142, 11060 Belgrade, Serbia and Montenegro
E-mail: dekana@ibiss.bg.ac.yu

(IL-6) and glucocorticoids (Kunz et al. 1989). The regulatory elements involved in the IL-6-dependent transcription induction of the rat α_2MG gene are located between −852 and −777 and between −404 and −165 relative to the cap site. A glucocorticoid response element lying upstream from the transcription start site between −38 and −33 is required for the induction of the rat α_2MG gene by glucocorticoids (Kunz et al. 1989). Transcriptional regulation of the α_2MG gene depends on interactions between the above *cis*-acting sequences and *trans*-acting proteins. Namely, it was shown that hepatocyte DNA-binding proteins participate in the regulation of expression of liver-specific genes. Among the regulatory *trans*-acting proteins that participate in the transcriptional regulation of the α_2MG gene, the IL-6-activated signal transducers and activators of transcription (STAT) (Alonzi et al. 2001) and CCAAT-enhancer binding protein (C/EBP) family of transcription factors found in nuclear extracts of rat hepatocytes (Baumann and Gauldie 1994; Petrović et al. 1995) assume a prominent role. C/EBP proteins (α, β, δ) belong to a major group of liver-enriched transcription factors that regulate the expression of numerous liver-specific genes (Xanthopoulos and Mirkovitch 1993). In rat liver, C/EBPβ is an important regulator of liver functions such as nutrient metabolism, liver regeneration and the acute phase response (Ruminy et al. 2001). C/EBPβ is upregulated during the acute-phase response (Takiguchi 1998) and is involved in the transcriptional regulation of acute phase reactants (Baumann and Gauldie 1994). Initial data revealed the presence of *trans*-acting proteins in the hepatic soluble nuclear protein extract, which has often been used for transcription factor identification and characterization (Alam et al. 1992). More recent findings indicate that the insoluble nuclear protein fraction – the nuclear matrix, also has an important role in transcription. Interactions between the nuclear matrix and consensus binding sequences for a number of transcriptional activators have been shown (van Wijnen et al. 1993; Nardozza et al. 1996), suggesting the association of transcription factors with the nuclear matrix. Assuming that both nuclear fractions, the nuclear extract and the nuclear matrix are involved in the transcriptional regulation of the α_2MG gene, we examined the distribution of C/EBPβ between them, and its binding affinity to the regulative element of the α_2MG gene in different physiological states.

Experiments were performed on male Wistar rats. The acute-phase response was induced by a subcutaneous injection of turpentine oil (1 μl/g *per* body weight) 12 h before sacrifice. The nuclear extract (Gorski et al. 1986) and the nuclear matrix (Belgrader et al. 1991) were isolated from the livers of controls and rats 12 h after the induction of the acute-phase response when the transcription rate of the α_2MG gene reaches its maximal level (Ševaljević et al. 1989). Equal quantities of soluble nuclear proteins and nuclear matrices were separated by 12% SDS-PAGE (Laemmli 1970). The relatively complex protein composition of the nuclear protein fractions was revealed by Coomassie staining (Figure 1). Considerable differences between the hepatic nuclear extract and the nuclear matrix protein profiles were observed while the acute-phase response was not followed by discernible changes of

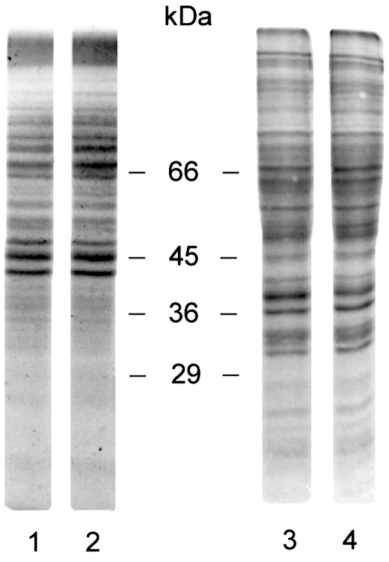

Figure 1. Electrophoretic patterns of nuclear extract and nuclear matrix proteins from control and acute-phase rat livers. Twenty μg of proteins were subjected to 12% SDS-PAGE and the gels were stained with Coomassie Brilliant Blue R-250. Lanes 1 and 2 – nuclear extracts from control and 12 h turpentine-treated rat livers; lanes 3 and 4 – nuclear matrices from control and acute phase livers, respectively. Molecular mass standards are indicated.

the protein profiles in either protein fraction (Figure 1, lanes 2 and 4) with respect to the appropriate control (Figure 1, lanes 1 and 3).

In order to investigate the DNA binding affinities of hepatic nuclear proteins to the hormone response element (HRE) of the α_2MG gene, South-Western blot analysis was performed according to Bowen et al. (1980). Soluble nucleoproteins and the insoluble nuclear matrix structure isolated from the livers of control and turpentine-treated rats were separated by SDS-PAGE, blotted and probed with (α^{32}P)dCTP-labeled restriction fragment (−852/12) of the α_2MG gene (Figure 2, A and C). The sequence specificity of the above interaction was demonstrated in experiments performed in the presence of an excess of unlabeled DNA (data not shown). The pattern of the control soluble nuclear protein fraction compared to the insoluble nuclear matrix demonstrated a higher binding affinity for the examined gene sequence, resulting in the formation of prominent bands at 29, 35, 45 and 70 kDa, as well as a number of fainter ones (Figure 2, lane 1). At the same time, 32, 35 and 42 kDa nuclear matrix proteins bound the DNA (Figure 2, lane 5). During the acute phase response the binding affinities for α_2MG HRE increased in both nuclear fractions. Compaired with the control sample, bands of higher intensity in the 29, 35, 45, 68 and 70 kDa region were observed in the soluble protein fraction under turpentine-induced condition (Figure 2, lane 2). The acute-phase matrix pattern was characterized by the considerably higher binding affinity of the 32–35 kDa proteins in regard to control sample and the appearance of a new band at 45 kDa (Figure 2, lane 6). However, both soluble and insoluble nuclear proteins were characterized by radioactive bands at 35 kDa. Based on literature data we have presumed that the 35 kD protein is an isoform of C/EBP proteins (Yiangou et al. 1998). The

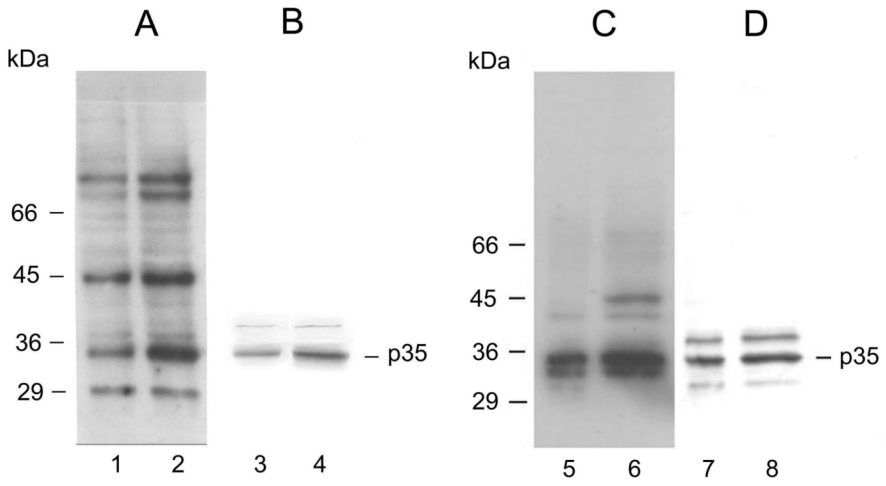

Figure 2. South-Western blot analysis of hepatic nuclear extract and nuclear matrix proteins with hormone response element of the α_2MG gene (A, C). Immuno-Western blot analysis of the same samples with C/EBPβ antibody (B, D). Twenty μg of nuclear proteins were separated by SDS-PAGE, blotted and probed with (α^{32}P) dCTP-labeled restriction fragment (−852/12) (A, C). The same nitrocellulose filters were incubated with C/EBPβ polyclonal rabbit antibody and stained with BCIP/NBT (B, D). Lanes 1 and 3 – nuclear extract from control rat liver; lanes 2 and 4 – nuclear extract from acute-phase liver; lanes 5 and 7 – nuclear matrix from control liver; 6 and 8 – nuclear matrix from acute-phase liver. South-Western blot analysis was repeated with 3–5 separately isolated nuclear protein fractions for each time point.

presence of the C/EBPβ consensus binding site T/(T/G)NNG(A/C/T)AA(T/G) (Baumann et al. 1992) in the promoter region of the α_2MG gene suggested that the detected 35 kDa DNA-binding protein belongs to the C/EBP family.

By Western blotting analysis we identified the presence and relative changes of C/EBPβ levels in the soluble and matrix nuclear fractions. Western immunoblot analysis was performed following the procedure of Towbin et al. (1979) using antibodies to rat C/EBPβ (C19, Santa Cruz Biotechnology, CA, U.S.A.) and staining with BCIP/NBT. Western blotting analysis revealed the presence of C/EBPβ isoforms (30, 35, 38 kDa) in all of the nuclear samples examined (Figure 3). It is assumed that the described isoforms are the products of differential translational initiation at multiple AUG sites within the single C/EBP mRNA (Descombes and Schibler 1991). The major isoform was a 35 kDa protein described in the literature as a transcriptional regulator (Ossipow et al. 1993). Higher concentrations of all isoforms, in particular of the 35 kDa C/EBPβ isoform, were detected in the control nuclear matrix relative to the nuclear extract protein pattern (Figure 3, lanes 3 and 1). The acute-phase response was accompanied by an increase in the 35 kDa isoform in both nuclear fractions (Figure 3, lanes 2 and 4). However, the elevation of

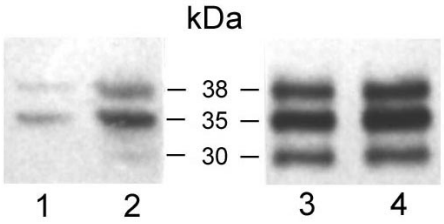

Figure 3. Western blot analysis of rat liver nuclear extract and nuclear matrix proteins with anti-C/EBPβ antibody. Western analysis was performed with C/EBPβ polyclonal rabbit antibody and stained with BCIP/NBT. Lanes 1 and 2 – nuclear extract proteins of control and 12 h turpentine-treated rat livers; lanes 3 and 4 – nuclear matrix proteins from control and animals 12 h after the induction of the acute-phase response. Western blotting analysis was repeated with 3–5 separately isolated nuclear protein fractions for each time point.

35 kDa-C/EBPβ isoform was much smaller in the nuclear matrix protein fraction (Figure 3, lane 4) in regard to the acute-phase extract sample. In order to assess whether the 35 kDa isoform of C/EBPβ is a protein that binds to the HRE of the α_2MG gene (Figure 2, A and C), we performed an additional experiment. Briefly, nitrocellulose filters with nuclear extract and matrix samples after South-Western blot analysis were incubated with the C/EBPβ antibody as required for Western blotting analysis (Figure 2, B and D). Bands at 30, 35 and 38 kDa in both the nuclear extract and nuclear matrix (Figure 2, lanes 3, 4, 7 and 8) samples were detected. By overlapping the films with radioactive signals with the stained nitrocellulose filters we were assured that protein with molecular mass of 35 kDa was an isoform of C/EBPβ that possessed an increased binding affinity towards the HRE of the α_2MG gene.

An increased concentration of the 35 kDa C/EBPβ isoform in both fractions during the acute-phase response (Figure 2, lanes 4 and 8) could not be the only explanation for the increased binding affinity towards the α_2MG HRE gene during inflammation (Figure 2, lanes 2 and 6). We propose that posttranslational modifications were also involved in this process (Ray and Ray 1994). Furthermore, significantly higher concentrations of C/EBPβ isoforms in the nuclear matrix fraction compared to the nuclear extracts (Figure 3, lanes 4 and 2) were accompanied by an inversion of the binding affinity towards the regulatory element of the α_2MG gene (Figure 2, lanes 6 and 2). This is reflected by an increased intensity of the binding affinity of hepatic soluble nuclear proteins, especially during the acute-phase response. Moreover, the relative increase in concentration of the 35 kDa C/EBPβ isoform in the acute-phase sample was more significant in the nuclear extract than in the nuclear matrix (Figure 3). We presume that disruption of homeostasis during the acute-phase response activated the release of *trans*-active proteins from a matrix-associated pool, thus rendering the factors more accessible for interactions with target sites on the DNA. However, according to the postulated role of the nuclear matrix in tissue-specific gene transcription (Getzenberg 1994) and the re-

sults presented herein, it can be assumed that the nuclear matrix does not only sequester regulatory proteins, but actively participates in α_2MG gene transcription by serving as a supporting structure that brings together specific DNA sequences with factors involved in the regulation of gene expression. The findings presented here show that both hepatic nuclear fractions are essential for the transcriptional regulation of the α_2MG gene and support the assertion that α_2MG gene expression is accomplished through an interplay between soluble and insoluble nuclear fractions.

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