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TRANSCRIPTION FACTOR p53 EXHIBITS INCREASED BINDING TO THE A2-MACROGLOBULIN GENE PROMOTER AND DECREASED GLYCOSYLATION IN FETAL AND ADULT RAT LIVER DURING THE ACUTE-PHASE RESPONSE

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Abstract — The binding affinity of p53 for the MG promoter was assessed by DNA-affinity chromatography with the extended α2-macroglobulin (MG) gene promoter (-852/+12) and immunoblot analysis. During the increased MG gene transcription observed in the fetus and the acute-phase (AP) response in both the fetus and the adult, p53 exhibited increased binding to the MG promoter. This increase was accompanied by decreased O-linked N-acetyl glucosamine glycosylation of p53. We suggest that the enzymatic removal of sugar moieties in vivo serves to activate the MG gene promoter binding potential of p53 and its participation in upregulated MG gene transcription.

Key words: α2-macroglobulin, p53, glycosylation, acute-phase response

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INTRODUCTION

Different types of trauma – bacterial or viral infections, as well as mechanical, thermal, and chemical injuries - lead to a disturbance in homeostasis. Consequently, in mammals a complex systemic reaction that is referred to as the AP response becomes activated in order to reestablish homeostatic balance (Kushner and Mackiewicz, 1987). During the AP response, pro-inflammatory cytokines and corticosteroids upregulate transcription factors that in turn induce the expression of AP protein genes in the liver (Baumann and Gauldie, 1994). A prominent AP protein in the rat is MG, whose serum concentration increases up to 100-fold during the AP response. MG is a glycoprotein that functions as a serum protease inhibitor and cytokine inactivator (Gonias, 1992). The generation of proteases is important in the development of different inflammatory conditions during the AP response, and their subsequent inhibition by MG plays an important role in the resolution of the inflammatory state (Schaefer et al., 2004). Significant MG plasma levels are also normally observed during embryogenesis, in childhood, and in pregnancy – periods of life characterized by intense tissue growth and remodeling (Glibetić et al., 1992, Bogojević et al., 1998).

Previously, we described the participation of a transcription factor, tumor supressor protein p53, in transcriptional regulation of the AP proteins haptoglobin and MG in the adult rat liver (Bogojević et al., 2002; Mihailović et al., 2005, 2007). While p53 seems to be dispensable for normal development, it plays an important role in regulating cell proliferation in response to DNA lesions (Lacroix et al., 2006). Thus, p53 is viewed as a node for stress signals transduced mainly through the ability of p53 to act as a transcription factor. The ability of p53 to regulate transcription is linked to its affinity for a specific DNA sequence in the promoter element. The p53 consensus DNA-binding sequence is 5′-puPuPuC(A/T)(A/T)GpyPyPy-3′ (El-Deiry et al., 1992). Computer search of the extended MG promoter element (-852/+12) revealed the presence of two potential p53 binding sites at positions -289/-298 and -131/-140 (Alggen Promo computer gene

-852 CT
GCAGCAACCAGAGGATGGATGCATGATTTAATGAAATTGCCTCCAGGGCCTGGAGACTAGAGAGGGGAG
TGTTGGGCAAAAGTGTTTGTGTAGTGAATGGCAAAGTCAGCTGGGGTGTTGGTTAGGCACAGTAAAAAG
GGGTTATTTGACACTTGTTACTTTTCACCTTCTTAAAACAGAAATGAGTATATGTGAAGGTTAAGGTCA
ATGTTATGTTATACTGGAATTTAGAAATTCACTGTGAAGGTTACACTTACTGACCAGCAAGCCCAAGAC
CCTCCTGTCTCCTGTCCTCCAGAACCCAGATTCTAGCTAAGCATTAGGAAACCTAATCATTTCTCCTGG
CCCCGACACTTCATTTTCAGTCAATCTAATGTATAATAATACCTCTAAAGTCAATACCTACTTCCTAGT
TTGGTTCAAAATGCTTTCCATAGAAGGGTGCTGATCATGGGCCTCACGTTAAAGACAGGCCTTATGTGG
CCCAGAGCTTGCTGGCACACTCCGTTGGAGTGAACTATTCAAGCCTCCCAGGGCTGCTTAAGCCACAGC
C***TCCTTGCCAA***CTATCCAGACAGAAGCTCAGAGCATCCCTAAGAGGCTGTGGGGGAGGAGAAGCCGATT
ATCAAGTTTCATGTACAAAAGAGAAAAAGTGAGCAGTAACTGGAAAGTCCTTAATCCTTCTGGGAATTG
TGGCTAACGGGTCAGGAATTA***ACCTTGGCGG***TAATTAGGCCATCAGTGACTCTTTCAGAGAATGTTCAA
AAACCTCAGCTTTGTTTGGAGAACTCGTGTGGGTGGGAGCAGCTGTTTGCACAAAGAACAGCATAAAGT
CTAGCTGCTCCTCACCACGTCCAGGACCAGATCTC +12

p53 binding sequence: ACCTTGGCGG [-131/-140]
p53 binding sequence: TCCTTGCCAA [-289/-298]

Fig. 1. p53 consensus DNA-binding sequence ------ 5′-puPuPuC(A/T)(A/T)GpyPyPy-3′

analysis, available at www.alggen.lsi.upc.es/cgi/bin/promo) (Fig. 1).

In the present work, we examined the potential role of p53 in transcriptional regulation of the MG gene in the fetal and adult liver under basal conditions and during the AP response. Since the activation of p53 occurs primarily as a result of post-translational events rather than due to increased transcription of its gene (O r e n , 1999), in considering its involvement in MG gene regulation we correlated the affinity of p53 for the MG gene promoter with its post-translational (de)glycosylation.

MATERIALS AND METHODS

Animals

Male and female albino rats of the Wistar strain were used. Livers were isolated from 19-day-old fetuses removed from 10-week-old dams and 10-week-old male adults. To obtain sufficient material for one experiment, livers were pooled from up to five litters of 19-day-old fetuses (i.e., fetuses from up to five dams, depending on the number of fetuses in each litter), representing either the control or turpentine-treated groups. Livers from three adult (10-week-old) male rats were pooled for each group (control and turpentine-treated) per one experiment. From three to five separate experiments were performed for each group. The rats were kept at constant temperature, humidity, and dark/light intervals.

Induction of AP response

The AP response was induced by a subcutaneous injection of turpentine oil (1 μl/g of body weight) in the lumbar region of dams, neonatals, and male adults (B a u m a n n et al., 1984). The animals were killed 12 h after turpentine injection.

Isolation of rat liver nuclear extract

Nuclear extracts were prepared from livers of control and turpentine-treated rats following the procedure described by G o r s k i *et al.* (1986). Tissues were excised and homogenized in 10 mM Hepes (pH 7.6), 25 mM KCl, 1 mM spermidine, 1 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 M sucrose, and 10% glycerol. Liver nuclei were collected by centrifugation through a cushion of the same solution at 72,000 x g in a SW 28 rotor (Beckman L7-55) for 30 min at 4°C. The nuclei were resuspended in lysis

buffer [10 mM Hepes (pH 7.6), 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 10% glycerol]. The chromatin was pelleted by centrifugation of the lysate (82,000 x g, Ti 50 rotor, 60 min, 4° C). Solid $(NH_4)_2 SO_4$ was added to the supernatant. The precipitated proteins were sedimented at 82,000 x g in a Ti 50 rotor for 30 min at 4°C. Nuclear extracts were dialyzed against 25 mM Hepes (pH 7.6), 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol, resuspended in dialysis buffer, and kept at -80°C.

DNA affinity chromatography

In order to investigate whether p53 exhibits binding affinity for the MG gene promoter, soluble nuclear proteins and internal nuclear matrix proteins were purified with a DNA affinity column and then examined by Western analysis. Affinity chromatography of sequence-specific DNA binding proteins from rat liver was performed by the method of Kadonaga and Tjian (1986). A fragment of the MG gene (-825/+12) was annealed and ligated to obtain oligomers and then covalently coupled to Sepharose CL-2B with cyanogen bromide to yield the affinity resin. The DNA affinity resin was equilibrated in a Bio-Rad Econo-Column with dialysis buffer [25 mM Hepes (pH 7.6), 0.1 mM EDTA, 1 mM DTT, and 10% glycerol] containing 0.1 M KCl. Equal quantities of fetal and adult liver nuclear extracts prepared from control and experimental animals were combined with competitor DNA (salmon sperm), allowed to stand for 30 min, and then passed through the DNA-Sepharose resin by gravity flow ("flow-through" fraction). The resin was washed with dialysis buffer containing 0.1 M KCl ("rinse" fraction). The passage of buffer through the column was stopped, and dialysis buffer containing 1 M KCl was added to the column. The resin was mixed with the buffer using a glass rod and allowed to stand for 10 min. After passage of buffer through the column, the eluate was collected. This step was repeated with dialysis buffer containing 1 M KCl.

Wheat-germ agglutinin affinity chromatography

Wheat-germ agglutinin (WGA) affinity chromatography was performed to examine the level of the N-acetylglucosamine (GlcNAc)-modified p53. Equal amounts of nuclear proteins prepared from fetal and adult liver in the basal state and 12 h after induction of the AP response were passed through the WGA column (Jackson and Tjian, 1989). The glycosylated proteins were purified with 0.4 M N-acetylglucosamine and the presence of p53 was assessed by immunoblot analysis.

SDS-polyacrylamide gel electrophoresis and Western immunoblot analysis

Protein concentrations were determined according to the method of Lowry et al. (1951). For SDS-polyacrylamide gel electrophoresis (SDS-PAGE), proteins (20 µg) were loaded onto 4% stacking/12% separating slab gels as described by Laemmli (1970). Nucleoproteins separated by SDS-PAGE were transferred to PVDF membranes (Hybond-P, Amersham Pharmacia Biotech), and Western immunoblot analysis was performed by the procedure of Towbin et al. (1979) with polyclonal p53 antibody (FL-393, Santa Cruz Biotechnology, USA). After incubation with blocking solution [0.05% Tween 20, 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 3% non-fat condensed milk], the membranes were incubated with specific antibodies for 2 h at room temperature. After rinsing, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin as a secondary antibody for 1 h. Immunoreactive bands were identified by an enhanced chemiluminescence (ECL) detection system (Santa Cruz Biotechnology) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Elevated MG gene transcription was shown to lead to a 100-fold increase in circulating MG during the AP response (Okubo et al., 1981). Expression of the MG gene is regulated at the transcriptional level, relying on the binding affinity of hepatocyte regulatory DNA-binding proteins for the promoter region of the MG gene (De Simone and Cortese, 1988). To assess the involvement of p53 in MG gene expression, nuclear protein extracts were prepared from control and AP fetal and adult livers 12 h after turpentine administration, when

maximal transcriptional activity of the MG gene was observed (Glibetić et al., 1992). Immunoblot analysis showed that p53 was present in the control nuclear extracts (Fig. 2, lanes 1 and 3) and that after induction of the AP response the relative amounts of p53 underwent a similar degree of increase in the nuclear extracts prepared from both fetal (lane 2) and adult (lane 4) rats.

Since there are two potentional p53-binding sites in the promoter region of the MG gene (Fig. 1), the participation of p53 in transcriptional regulation of the MG gene was examined by DNA-affinity chromatography using the extended MG gene promoter (Kadonaga and Tjian, 1986). Immunoblot analysis of different protein fractions obtained after chromatography (Fig. 3) revealed that p53 in the fetal liver possessed binding affinity for the MG promoter (lane 1). At 12 h of the AP response, p53 displayed increased promoter binding (lane 2). Although p53 did not bind to the MG promoter (lane 3) in the control adult liver, at 12 h of the AP response it exhibited promoter binding (lane 4). Thus, increased MG gene transcriptional activity was always accompanied by increased binding affinity of p53 for the MG promoter. This finding suggests that p53 participated in transcriptional regulation of the MG gene.

In normal cells, p53 is latent. However, a variety of conditions and agents that cause genotoxic stress rapidly induce the expression of p53, which regulates genes mediating cell cycle arrest and apoptosis. AP protein genes are induced during intense cellular stress characterized by macrophage activation and a cytokine-elicited oxidative burst (Koj, 1998). Both local and global inflammation frequently occur simultaneously with tissue hypoxia. Evidence for a direct link between low oxygen supply and induction of the AP response is provided by the observation that genes for interleukins (IL) 1 and 6, the mediators of the AP response, are induced in endothelial cells *in vitro* by hypoxia and that AP gene expression is stimulated following exposure of human hepatoma cells to moderate hypoxia (Yan et al., 1995). A similar increased binding of p53 to an AP protein gene regulatory sequence during the AP response was observed at the hormone response element of the haptoglobin gene (Bogojević et al., 2002; Mihailović et al., 2005). Since hypoxia is a physiological inducer of wild-type p53 (Won et al., 1998), the findings presented here suggest that hypoxia underlies the induction of p53 and its participation in the inducible expression of MG, haptoglobin, and probably other AP protein genes. Several viral and cellular promoters that do not contain known p53-binding sites are repressed by p53, whereas promoters containing p53-binding sites are transactivated (Ginsberg et al., 1991). The presence of predicted p53-binding sites on the MG promoter, together with the observed increased binding affinity of p53 for the MG promoter during the AP response, lend additional support for a role of p53 in

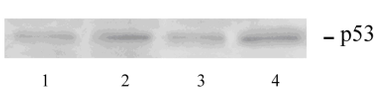

Fig. 2. Western immunoblot analysis of rat liver nuclear proteins with p53 antibody. Proteins (20 μg) were separated by 12% SDS-PAGE, electrotransferred onto PVDF membranes and incubated with polyclonal rabbit antibody raised against rat p53. The antigen-antibody complex was visualized by the ECL detection system. Lanes 1 and 2 – 19 day-old fetal liver; lanes 3 and 4 – 2.5-month-old adults. Lanes 1 and 3 – nuclear proteins isolated from control livers; lanes 2 and 4 – nuclear proteins prepared 12 h after induction of the AP response.

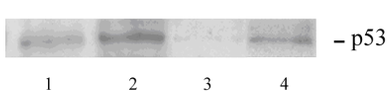

Fig. 3. Western immunoblot analysis of nuclear proteins with p53 antibody after DNA-affinity chromatography.

Equal quantities of liver nuclear proteins were eluted from the DNA affinity column with 1 M KCl, and Western immunoblot analysis was performed with p53 antibody. Lanes 1 and 2 – 19-day-old fetal liver; lanes 3 and 4 – 2.5-month-old adult. Lanes 1 and 3 –nuclear proteins isolated from control livers; lanes 2 and 4 – nuclear proteins obtained 12 h after induction of the AP response.

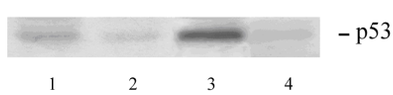

Fig. 4. Western immunoblot analysis of nuclear proteins with p53 antibody after WGA column.

Equal quantities of liver nuclear proteins were eluted from the WGA column with 0.4 M N-acetylglucosamine, and Western immunoblot analysis was performed with p53 antibody. Lanes 1 and 2 – 19 day-fetal liver; 3 and 4–2.5-month-old adult. Lanes 1 and 3 represent nuclear proteins isolated from the control livers; lanes 2 and 4 are nuclear proteins obtained 12 h after induction of the AP response.

the upregulation of MG expression.

Rapid post-translational activation of signaling proteins, including p53, is achieved through covalent modifications such as phosphorylation, acetylation, and glycosylation (O r e n , 1999). The most frequent type of intracellular glycosylation is through O-linkage of N-acetylglucosamine (GlcNAc). To examine the level of GlcNAc-modified p53, equal amounts of nuclear proteins prepared from fetal and adult livers during the basal state and 12 h after induction of the AP response were passed through a WGA column (J a c k s o n and T j i a n , 1989). The glycosylated proteins were purified with 0.4 M N-acetylglucosamine, and the presence of p53 was assessed by immunoblot analysis (Fig. 4). As can be seen on lane 1, p53 in the control fetal liver was glycosylated, whereas the AP response was accompanied by its decreased glycosylation (lane 2). In the control adult liver, the level of p53 glycosylation was very high (lane 3), whereas the AP response was accompanied by a markedly decreased glycosylation of p53 (lane 4). Comparison of these findings with results obtained after DNA affinity chromatography and Western analysis revealed that increased binding affinity of p53 for the MG promoter during the AP response was accompanied by decreased glycosylation of p53.

Phosphorylation reactions positively regulate p53 DNA binding (A s h c r o f t et al., 1999). Previously, we showed that *in vitro* phosphorylation of control nuclei increased the binding affinity of p53 for the promoter region of the haptoglobin gene (B o g o j e v i ć et al., 2002). Phosphorylation is tightly and dynamically regulated by protein glycosylation, and sugar and phosphate molecules compete for a single amino acid residue. Hence, a decrease in the level of phosphorylation occurs after glycosylation and vice versa (H a l t i w a n g e r et al., 1997). Both O-glycosylation and phosphorylation affect the activation and stability of p53 (A s h c r o f t et al., 1999) and are sequentially implicated in the modulation of p53 functioning and transactivation of p53-dependent/regulated genes. F i o r d a l i s o et al. (1999) reported that hyperglycemia-induced enzymatic O-glycosylation of p53 in myocytes decreases over time progressively and is replaced by its phosphorylation and increased DNA binding. The demonstration that inhibition of O-glycosylation prevented p53 phosphorylation and p53 activity suggests that these post-translational modifications are functionally linked. Based on our results, we conclude that the enzymatic removal of sugar moieties activated the binding potential of p53 for the MG gene promoter.

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ТРАНСКРИПЦИОНИ ФАКТОР p53 ПОКАЗУЈЕ ПОВЕЋАН АФИНИТЕТ ВЕЗИВАЊА ЗА ПРОМОТОРСКИ РЕГИОН ГЕНА ЗА А2-МАКРОГЛОБУЛИН И СМАЊЕН СТЕПЕН ГЛИКОЗИЈАЦИЈЕ У ЈЕТРИ ФЕТУСА И АДУЛТА ПАЦОВА ТОКОМ АКУТНО-ФАЗНОГ ОДГОВОРА

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ДНК афинитетна хроматографија, са промоторским регионом гена за алфа2-макроглобулин (MG) (-852/+12), и имуноблот анализа су показале повећан афинитет везивања p53 за промоторски регион гена за MG у контролној јетри фетуса, као и улогу p53 у транскрипционој регулацији гена за MG и у феталној и адултној јетри током акутно-фазног одговора (АФО). Повећање везивања p53 за промоторски регион гена за MG је праћено смањењем степена гликозилације p53. Резултати сугеришу да у *in vivo* условима уклањање шећерних остатака са p53 омогућава његово везивање за промоторски регион MG гена, као и улогу у транскрипционој активацији испитиваног гена током АФО.