Anisoosmotic regulation of the Nopp140 mRNA in H4IIE rat hepatoma cells and primary hepatocytes

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Abstract Using the differential display polymerase chain reaction osmosensitive regulation of mRNA levels of the nucleolar phosphoprotein of 140 kDa (Nopp140) was found in H4IIE rat hepatoma cells. These levels were downregulated after hypoosmotic exposure in H4IIE cells and primary rat hepatocytes. Hyperosmotic incubation increased Nopp140 mRNA levels in H4IIE cells but not in hepatocytes. Inhibition of $p38^{MAPK}$ or MAP kinase kinase upstream of Erk-1 and Erk-2 decreased Nopp140 mRNA levels but did not prevent their osmosensitivity. Because Nopp140 is involved in the regulation of transcriptional activity it could play a role in the osmosignalling pathway towards gene expression in H4IIE cells and hepatocytes.

Key words: Cell volume; Differential display polymerase chain reaction; Gene expression; Liver cell; Transcription factor

1. Introduction

Anisoosmotic changes in cellular hydration are important modulators of metabolic cell function and gene expression (for review see [1]). This involves not only the regulation of osmoregulatory genes, such as the expression of aldose reductase [2] or specific sodium-coupled transporters for *myo*-inositol [3,4], betaine [4,5] and taurine [4,6], but also the expression of genes encoding proteins that are not linked to osmoregulation. Examples of the latter are the rapid increases in mRNA levels for β-actin [7], inducible nitric oxide synthetase [8], cyclooxygenase-2 [9], c-jun [10] and phosphoenolpyruvate carboxykinase [11].

In mammals, little is known about the signal transduction pathways leading to the transcriptional regulation of genes in response to osmotic stress; however, mitogen-activated protein (MAP) kinase cascades appear to play an important role [12,13]. Furthermore, an osmosensitive transcriptional regulation of h-sgk, a serum- and glucocorticoid-regulated serine/threonine protein kinase, was identified [14]. Recently, osmosensitive mRNA expression of Mi-2 autoantigen, a member of the SNF/RAD 54 helicase family, was shown in H4IIE hepatoma cells and rat primary hepatocytes [15].

In the present study, the differential mRNA display polymerase chain reaction (DDRT-PCR) method [16,17] was applied in order to identify osmoregulated genes, which might participate in the osmosignalling pathway in mammalian cells.

2. Materials and methods

2.1. Cell culture

H4IIE rat hepatoma cells (American Type Culture Collection [ATCC] CRL 1600, Rockville, MD, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM)-Ham's F12/5% CO_2/5 mM glucose/ 2.5 mM L-glutamine at 37°C, pH 7.55, supplemented with 10% fetal bovine serum. When cells had reached almost confluence, they were washed twice with Dulbecco's PBS and the culture was continued in serum-free medium for an additional 16–24 h. Thereafter, cells were maintained under various test conditions. In some experiments, cells were pretreated with the pyridinyl imidazole SB 203580 (10 μM), a specific inhibitor of p38 MAP kinase [20], with PD 098059 (10 μM), a MAP kinase kinase inhibitor blocking the signal transduction to the MAP kinases Erk-1 and Erk-2 [21] or with H7 (10 μM), a potent inhibitor of cyclic nucleotide-dependent protein kinases [22] for 1 h in different experiments and then exposed to test media, also containing the inhibitors for 6 h.

For isolation of liver parenchymal cells (PC), male Wistar rats (300–400 g body weight) were raised in the local institute for laboratory animals and held, fed ad libitum on stock diet, according to the local ethical guidelines. PC were prepared by collagenase perfusion as described previously [23]. Cells were plated on collagen-coated dishes at a density of about 1×10^6 cells/ml and were then cultured at 37°C in DMEM/ 5% CO_2, pH 7.4/glucose (5 mM), supplemented with 100 nM insulin, 100 nM dexamethasone, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 5% fetal bovine serum for 24 h. Thereafter, cells were maintained under various test conditions.

The osmolarity changes were performed by appropriate changes of the NaCl concentration.

2.2. Northern blot analysis

Total RNA from near-confluent H4IIE hepatoma cell cultures or rat liver parenchymal cells in cluster 6 dishes (Costar, Cambridge, MA, USA) was isolated using the RNeasy Total RNA Kit (Qiagen, Hilden, Germany). Liver, small intestine, spleen, testis and kidney were isolated from Wistar rat and about 1 g of tissue was immediately homogenized in 10 ml of guanidine isothiocyanate solution on ice with an Ultra-Turrax instrument at high speed and total RNA was isolated as described in [24]. RNA samples were electrophoresed in 0.8% agarose/3% formaldehyde and then blotted onto Duralon-UV membranes (Stratagene, Mannheim, Germany) with 20×SSC (3 M NaCl, 0.3 M trisodium citrate). Following the blotting procedure the membranes were UV-crosslinked (Hoefer UV-crosslinker 500, Hoefer, San Francisco, CA, USA) and observed under UV illumination to determine RNA integrity and location of the 28S and 18S rRNA bands. Hybridization was carried out with approximately 10^6 cpm/ml [α-^{32}P]dCTP-labelled Nopp140, phosphoenolpyruvate carboxykinase (PEPCK) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probes as described in [4]. The plasmid containing the cDNA probe for PEPCK was kindly provided by Dr. R. Hanson (Case Western Reserve University, Cleveland, OH, USA) [25]. The 1.0 kb cDNA fragment of GAPDH used for standardization was

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Abbreviations: DDRT-PCR, differential display polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; Nopp140, nucleolar phosphoprotein of 140 kDa; PC, liver parenchymal cells; PEPCK, phosphoenolpyruvate carboxykinase

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from Clontech (Palo Alto, CA, USA). The probes were labelled by random priming under the conditions recommended by the supplier (Pharmacia, Freiburg, Germany). After hybridization and washing steps, blots were exposed to Kodak X-Omat AR or Kodak BIOMAX film at −70°C with intensifying screens. Densitometric evaluation was performed with the E.A.S.Y. RH system (Herolab, Wiesloch, Germany).

2.3. Differential mRNA display PCR

Almost confluent H4IIE cells were exposed for 8 h to hypoosmotic (205 mosmol/l), normoosmotic (305 mosmol/l) and hyperosmotic (405 mosmol/l) medium. Total RNA was extracted using the RNeasy Total RNA Kit (Qiagen, Hilden, Germany). Differential mRNA display [16,17] was performed using the primer kit for mRNA display amplification (GeneExScreen Primer Kit, Biometra, Göttingen, Germany) [26], AMV reverse transcriptase (Promega, Heidelberg, Germany) and Taq DNA polymerase (Eurogentec, Seraing, Belgium) according to the manufacturers' descriptions. Briefly, 0.2 μg of each sample was reverse transcribed and a 1:10 dilution of these probes was amplified by PCR. PCR products were resolved on prepared, rehydrated 10% polyacrylamide gels spread on a plastic foil (CleanGel, Pharmacia, Freiburg, Germany). The gels were analyzed by silver staining under the conditions recommended by the supplier (Pharmacia). cDNA bands that appeared to be markedly different under anisoosmolar conditions compared with normoosmolar controls were cut from the gel. DNA was extracted and reamplified by PCR with use of the same primer pairs (GeneExScreen Primer Kit) and cloned in pGEM-T vector (Promega) before sequencing. The complete sequence of both cDNA strands was determined by cycle sequencing with the DNA sequencing kit, ABI Prism Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Weiterstadt, Germany).

3. Results and discussion

The DDRT-PCR method was used for the identification of osmoregulated genes. After culture of H4IIE cells in normoosmolar medium (305 mosmol/l), the cells were exposed to hypoosmolar (205 mosmol/l) and hyperosmolar (405 mosmol/l) medium for 8 h. Following the isolation of total RNA, DDRT-PCRs were performed with different sets of primers from a primer kit established by Bauer and co-workers [26]. Among several DDRT-PCR products obtained, a 360 bp cDNA product amplified with the upstream primer 7 (U7; 5′-TCGATACAGG-3′) and the downstream primer 2 (D2; 5′-TGGATTGGTC-3′) was of particular interest (Fig. 1).

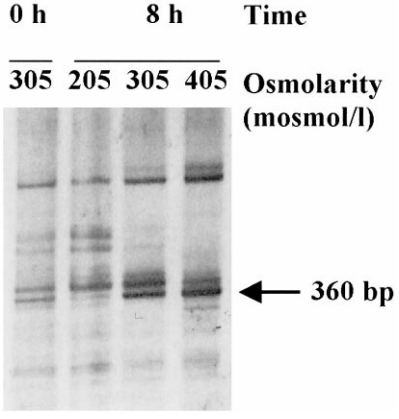

Fig. 1. Separation of DDRT-PCR products on a silver-stained 10% polyacrylamide gel. H4IIE cells were grown in normoosmolar medium (305 mosmol/l) and they were switched at time point 0 (0 h) for 8 h to the same or hypoosmolar (205 mosmol/l) or hyperosmolar (405 mosmol/l) medium. Total RNA was extracted, reverse transcribed and amplified by PCR with primer pair D2/U7 from the primer kit GeneExScreen (Biometra).

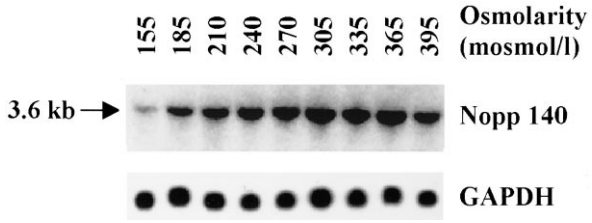

Fig. 2. Effect of anisoosmotic exposure on Nopp140 and GAPDH mRNA levels in H4IIE rat hepatoma cells. H4IIE cells were incubated for 6 h in media with different osmolarities. The osmolarity changes were performed by appropriate changes of the NaCl concentration. Thereafter, the cells were harvested for RNA isolation and subjected to Northern blot analysis for Nopp140 and GAPDH (10 μg total RNA per lane). This experiment is representative of three separate experiments.

The sequence of the 360 bp cDNA product was determined after subcloning into pGEM-T vector. A search with the BLAST Search Result service of the National Center for Biotechnology Information (NCBI) yielded a 100% identity to positions 3250–3609 of the sequence of the rat nucleolar phosphoprotein of 140 kDa (Nopp140) (clone pTM17 in [19]).

This PCR product was used for testing the anisoosmolar regulation of the Nopp140 in H4IIE and rat primary liver PC. After switching H4IIE cells or PC from normoosmolar medium to hypoosmolar and hyperosmolar medium, respectively, the mRNA abundance was tested by Northern blotting. Fig. 2 shows that in H4IIE cells the Nopp140 mRNA levels under the influence of a 6 h anisoosmotic exposure were dependent on the osmolarity of the medium. When compared with the normoosmotic conditions, changes in Nopp140 mRNA levels were detectable after 6 h of hypoosmotic and hyperosmotic exposure in H4IIE cells (Fig. 3). Detectable was one transcript with the size of 3.6 kb whereas Meier and Blobel identified two Nopp140 transcripts of 3.6 and 3.2 kb in N1S1 rat hepatoma cells [19].

In PC, hypoosmotic exposure also led to a time-dependent decrease in Nopp140 mRNA levels but in contrast to the expression in H4IIE cells Nopp140 mRNA levels were not affected by hyperosmotic exposure of 1–24 h (Fig. 4). One might speculate that in H4IIE cells the sensitivity of the

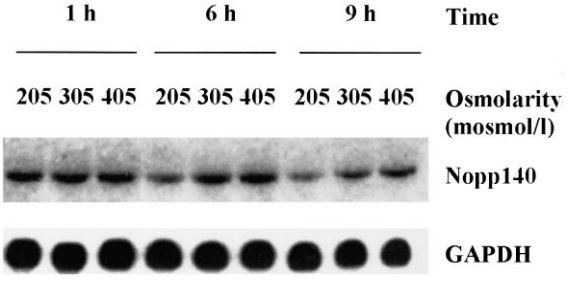

Fig. 3. Time course of the anisoosmolarity effects on relative Nopp140 mRNA levels in rat H4IIE hepatoma cells. H4IIE cells were incubated for the time periods indicated in normoosmotic (305 mosmol/l), hypoosmotic (205 mosmol/l) or hyperosmotic (405 mosmol/l) medium. The osmolarity changes were performed by increasing or lowering the NaCl concentration by 50 mM. At the time points indicated, cells were harvested for RNA isolation and subjected to Northern blot analysis for Nopp140 and GAPDH (10 μg total RNA per lane). This experiment is representative of three separate experiments.

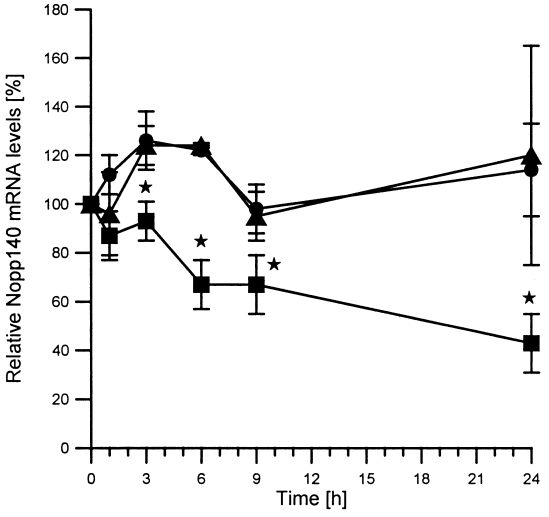

Fig. 4. Time course of the anisoosmolarity effects on relative Nopp140 mRNA levels in rat primary liver parenchymal cells. Cells were incubated for the time periods indicated in normoosmotic (305 mosmol/l), hypoosmotic (205 mosmol/l) or hyperosmotic (405 mosmol/l) medium. The osmolarity changes were performed by increasing or lowering the NaCl concentration by 50 mM. At the time points indicated, cells were harvested for RNA isolation and subjected to Northern blot analysis for Nopp140 and GAPDH. Nopp140 mRNA levels were normalized for GAPDH mRNA levels, and the relative Nopp140 mRNA levels found at time point zero were set at 100% and the relative Nopp140 mRNA levels found in hypo- (■), normo- (▲), or hyperosmotic (●) media were expressed as percentages thereof. Data are given as means ± S.E.M. and are from 3–4 different experiments for each condition.

Nopp140 mRNA levels against medium hyperosmolarity is related to the malignant phenotype of these tumor cells.

In addition, the Nopp140 mRNA band of 3.6 kb was also detected in rat small intestine, testis, spleen and kidney, and this expression was higher in these tissues than in rat liver (Fig. 5).

Nopp140 acts as a nuclear localization signal (NLS) binding protein and is one of the most phosphorylated proteins in the cell [19]. Casein kinase II phosphorylates Nopp140 to its unusually high degree. A similar phosphorylation occurs in yeast cells [27]. Antibody cross-reactivity reveals the yeast SRP40 gene product as immunologically related to rat

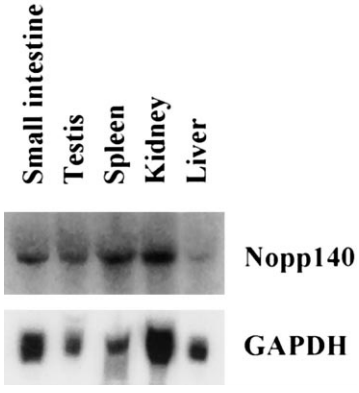

Fig. 5. Detection of Nopp140 and GAPDH mRNA levels in rat tissues. Liver, testis, small intestine, spleen and kidney from male Wistar rats were isolated for RNA preparation and total RNA was then subjected to Northern blot analysis for Nopp140 and GAPDH (15 μg total RNA per lane).

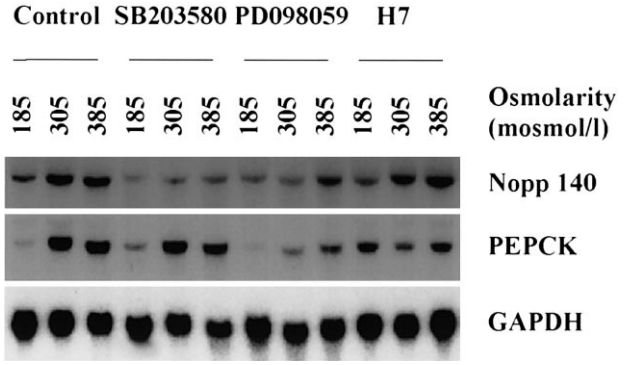

Fig. 6. Influence of SB 203580, PD 098059 and H7 on the anisotonicity effects on Nopp140 and PEPCK levels in H4IIE rat hepatoma cells. H4IIE cells were pretreated with the p38 MAP kinase inhibitor pyridinyl imidazole SB 203580 (10 μM), with MEK inhibitor PD 098059 (10 μM), or with the protein kinase inhibitor H7 for 1 h and then exposed for 6 h in normoosmotic (305 mosmol/l), hypotonic (185 mosmol/l) or hypertonic (385 mosmol/l) medium in the presence of the inhibitors. In control experiments H4IIE cells were exposed without inhibitor. The osmolarity changes were performed by appropriate changes of the NaCl concentration. Thereafter, cells were harvested for RNA isolation and subjected to Northern blot analysis for Nopp140, PEPCK and GAPDH (10 μg total RNA per lane). This experiment is representative of three separate experiments.

Nopp140, and sequence comparison shows the two proteins to be structurally similar [27]. The SRP40 protein plays a role in preribosome assembly or transport and depends on its carboxy-terminal domain for proper localization to the yeast nucleoskeleton [28]. In mammals, Nopp140 appeared to shuttle between the nucleolus and the small nuclear RNAs containing coiled bodies, and to chaperone the transport of other molecules [29]. Furthermore, Nopp140 functions as a transcriptional activator of the gene for α1 acid glycoprotein (AGP), which is a liver-derived plasma glycoprotein, whose level increases during the acute-phase response [18]. In addition, Nopp140 not only functions as a transcriptional activator per se but also mediates the interaction between a specific motif binding transcription factor, AGP/enhancer binding protein (AGP/EBP), and a general transcription factor, TFIIB, that results in the synergistic activation of the gene for AGP [18].

The mechanisms by which cell volume changes affect gene expression are largely unclear, although hypoosmotic exposure of H4IIE cells was shown to induce an activation of MAP kinases, which could account for a swelling-induced induction of the c-jun gene due to c-jun phosphorylation [12]. On the other side, in these cells MAP kinase seems not to be involved in the osmoregulation of PEPCK mRNA levels which is sensitive to inhibition of cyclic nucleotide-dependent protein kinases by H7 (Fig. 6 and [11]). Furthermore, $p38^{MAPK}$ is involved in hyperosmolarity-induced upregulation of MAP kinase phosphatase MKP-1 mRNA expression [30]. In the present study, addition of the p38 MAP kinase inhibitor SB 203580 or MAP kinase kinase inhibitor PD 098059 blocking the signal transduction to the MAP kinases Erk-1 and Erk-2 decreased Nopp140 mRNA levels to 56 ± 16% ($n = 4$) and 79 ± 20% ($n = 4$), respectively, but did not prevent their osmosensitivity (Fig. 6). Further, H7 did not significantly influence either osmosensitivity or the basal level (139 ± 31%, $n = 4$) of Nopp mRNA expression (Fig. 6). The data suggest

that the recently identified osmosensitive signal transduction pathways which involves an activation of Erk or p38 MAP kinases are apparently not involved in the osmoregulation of Nopp140 mRNA levels in H4IIE cells.

Recently, an osmosensitive mRNA expression of a further nuclear protein called Mi-2 autoantigen was shown in H4IIE hepatoma cells and rat primary hepatocytes [15]. The Mi-2 autoantigen is a member of the SNF/RAD 54 helicase family and may be involved in the regulation of transcriptional activity. Therefore it is interesting to speculate that both Mi-2 and Nopp140 could be part of the signal transduction pathway which triggers the cellular response towards osmotic stress. Perhaps Nopp140 is a downstream part of the osmosignalling pathway by regulating transcriptional activation in the nucleus and transport of molecules. Further investigations are necessary to elucidate the function of Nopp140 protein and the regulation of Nopp140 mRNA after exposure to stress factors.

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