Activity of hepatocyte nuclear factor 1 α and hepatocyte nuclear factor 1 β isoforms is differently affected by the inhibition of protein phosphatases 1/2A

Véronique CARRIÈRE¹, Michel LACASA and Monique ROUSSET

INSERM U505, Université Pierre et Marie Curie, 15 rue de l'école de Médecine, 75006 Paris, France

Phosphorylation/dephosphorylation processes are known to control the activity of several transcription factors. The nutrition-dependent expression of sucrase–isomaltase and Na⁺/glucose co-transporter 1, two proteins implicated in the intestinal absorption of glucose, has been shown to be closely related to modifications of hepatocyte nuclear factor 1 (HNF1) activity. This study was conducted to determine whether phosphorylation/dephosphorylation processes could control HNF1 activity. We show that expression of the gene encoding sucrase–isomaltase is inhibited in the enterocytic Caco-2 clone TC7 by okadaic acid at a concentration that is known to inhibit protein phosphatases 1/2A and that does not affect cell viability. At the same concentration, phosphorylation of the HNF1 α and HNF1 β isoforms is greatly enhanced and their DNA-binding capacity is decreased. The phosphorylation state of HNF1 β isoforms

directly affects their DNA-binding capacity. In contrast, the decreased DNA-binding activity of the HNF1 α isoforms, which was observed after the inhibition of protein phosphatases 1/2A, is due to a net decrease in their total cellular and nuclear amounts. Such an effect results from a decrease in both the HNF1 α mRNA levels and the half-life of the protein. This is the first evidence for the implication of protein phosphatases 1/2A in the control of the activity of HNF1 isoforms. Moreover, these results emphasize a physiological role for the balance between phosphatases and kinases in the nutrition-dependent regulation of HNF1-controlled genes.

Key words: Caco-2 clone TC7, DNA binding, okadaic acid, protein phosphorylation, sucrase–isomaltase.

INTRODUCTION

The hepatocyte nuclear factors HNF1 α (also known as LF-B1) [1] and HNF1 β (also known as vHNF1 or LF-B3) [2] were initially found and studied in liver but they are also particularly abundant in stomach, kidney and intestine (reviewed in [3]). They bind to DNA as homodimers or as HNF1 α /HNF1 β heterodimers [4]. Among the six members of the family already characterized, HNF1 α -A, HNF1 α -B and HNF1 α -C as well as HNF1 β -A and HNF1 β -B are transactivators, whereas HNF1 β -C behaves as a transdominant repressor [5]. Invalidation of the *TCF1* gene (coding for the three HNF1 α polypeptides) in mice has indicated an important role of this gene in the control of glucose homeostasis [6–8]. Inactivation of the gene encoding HNF1 β has shown that it is essential to the differentiation of visceral endoderm and that it is part of a genetic network directing the expression of HNF4 [9,10]. It has been reported that mutations in the genes encoding HNF4 (which controls the expression of HNF1 α [11,12]), HNF1 α and HNF1 β induce hyperglycaemia and are responsible for type 1, type 3 and type 5 of maturity-onset diabetes of the young (MODY) respectively [13–16]. In fact, even MODY1 is a consequence of HNF4 mutations on HNF1 α expression [17].

Three functional HNF1-binding sites have been characterized within the promoter of sucrase–isomaltase (SI) [18,19]. In human adults this disaccharidase is exclusively present in the brush

border of the small intestine (reviewed in [20,21]). Most studies *ex vivo* on the expression of the gene encoding SI have used Caco-2 cells, a colon carcinoma cell line that spontaneously differentiates into enterocytes after confluence [22]. In these cells, all culture conditions leading to a high glycolytic flux and to a lower intracellular glycogen content [22,23] result in a transcriptional repression of the gene encoding SI. This is also true for clonal populations isolated from Caco-2 cells, which vary in their characteristics of glucose metabolism [24,25]. The glucose-dependent repression of the SI promoter requires the SIF3 (–176 to –156) and SIF4 (–222 to –209) HNF1-binding sites and results from a higher proportion of HNF1 β , and more particularly of inhibitory HNF1 β -C-containing dimers, bound to SIF3 and SIF4 [19]. However, under all conditions, the mRNA level of HNF1 β -C remained constant, suggesting that the DNA-binding capacity of HNF1 was controlled by mechanisms other than transcription. Modifications of the balance between HNF1 α and HNF1 β isoforms bound to their DNA sites were also reported *in vivo* for another HNF1-controlled gene. Indeed, a nutritional/circadian periodicity of the expression of the intestinal Na⁺/glucose co-transporter 1 (SGLT1), which is tightly controlled by HNF1 [26], was correlated with a circadian periodicity of the HNF1 β DNA-binding activity [27]. All these observed modifications of the HNF1 DNA-binding activity were related to variations in glucose and glycogen metabolism, which are known to be controlled by phosphorylation/

Abbreviations used: DPP IV, dipeptidylpeptidase IV; EMSA, electrophoretic mobility-shift assay; HNF1, hepatocyte nuclear factor 1; OA, okadaic acid; RT-PCR, reverse-transcriptase-mediated PCR; SI, sucrase–isomaltase.

¹ To whom correspondence should be addressed (e-mail veronique.carriere-u505@bhdjussieu.fr).

dephosphorylation processes. Such processes also control the activity of several transcription factors, among them HNF4. Indeed, it has been shown that, under various nutritional conditions, the activity of HNF4 depends on its phosphorylation state [28]. As regards HNF1 by itself, which controls the expression of many genes, including those involved in the regulation of glucose metabolism [29], nothing had been known until now about the potential role of its phosphorylation state on the regulation of its activity.

The purpose of this study was therefore to determine whether protein phosphatases 1/2A, which are known to regulate glucose and glycogen metabolism, are implicated in the control of expression of the gene encoding SI and affect the DNA-binding capacity of HNF1 α and HNF1 β . We show here that HNF1 α and HNF1 β are phosphorylated and that the inhibition of protein phosphatases 1/2A by okadaic acid (OA) [30] decreases the mRNA level of SI and the DNA-binding capacity of both HNF1 α and HNF1 β isoforms. The hyperphosphorylation of HNF1 β directly affects its DNA-binding capacity. More surprisingly, the lower DNA-binding activity of HNF1 α isoforms is due to a net decrease in the nuclear amount of HNF1 α isoforms after the inhibition of protein phosphatases 1/2A. This results from an overall lowering in the amount of HNF1 α , which is due to a decrease in both the mRNA level and the half-life of the proteins. This is the first evidence for the implication of protein phosphatases 1/2A in the regulation of the activities of HNF1 α and HNF1 β isoforms.

MATERIALS AND METHODS

Chemical

Okadaic acid (OA) was obtained from Sigma (Saint-Quentin-Fallavier, France).

Cell culture

Caco-2 cell clone TC7 was isolated as reported previously [24]. TC7 cells were seeded at 2×10^5 cells per 25 cm² T flask (Corning Glassworks, Corning, NY, U.S.A.) and cultured in Dulbecco's modified Eagle's medium (25 mM glucose) supplemented with 1% (w/v) non-essential amino acids (Gibco, Paisley, Renfrewshire, U.K.) and 20% (v/v) heat-inactivated (30 min, 56 °C) fetal bovine serum (Boehringer Mannheim, Mannheim, Germany). The medium was changed 48 h after seeding and daily thereafter [24,25].

Assays of glucose consumption, lactic acid production, intracellular glycogen and protein

Glucose consumption was determined by measuring the amount of remaining glucose in the medium after the medium changes, with a Beckman Glucose Analyser 2. Lactic acid production was measured with an L-lactic acid assay kit (Boehringer-Mannheim, Meylan, France). Glycogen content was measured with anthrone as reported previously [23]. Protein content was measured with the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, U.S.A.).

RNA extraction and Northern blot analysis

Total RNA was extracted by the guanidinium isothiocyanate method [31]. After denaturation with glyoxal, 20 μ g was subjected to electrophoresis on 1% (w/v) agarose and transferred to nylon membranes (Appligene, Illkirsh, France) with $20 \times$ SSC (SSC

being 0.15 M NaCl/0.015 M sodium citrate). Prehybridization and hybridization were performed as described previously [25]. SI was detected with the cDNA probe SI2 [32] and dipeptidyl-peptidase IV (DPP IV) with DPI-101 [33]. GLUT1 was detected with pGEM4Z-HepG2GT GLUT1 obtained from G. I. Bell (Howard Hughes Medical Institute, University of Chicago, Chicago, IL, U.S.A.). Quantification was achieved with a densitometric scanner (Mark III CS; Joyce-Loebl, Gateshead, U.K.).

Analysis by reverse-transcriptase-mediated PCR (RT-PCR)

The reverse-transcription experiments were performed as described previously [25], with 7 μ g of total RNA in a total volume of 100 μ l. The PCR conditions used were essentially as described by Bach and Yaniv [5], including the oligonucleotides used for the specific amplification of human HNF1 α and HNF1 β isoforms. Each PCR was performed on 5 μ l of the reverse-transcription cDNA mixture in a total volume of 100 μ l. An amplification of a segment of the 18 S ribosomal cDNA, with the use of 5 μ l of a 1:1000 dilution of each reverse transcription and primer 18-A and primer 18-S, was used as internal control. Aliquots of 30 μ l of the PCR mixture were taken at cycle 14, 16 or 18 (each cycle consisted of 30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C) and were subjected to electrophoresis on a 2% (w/v) agarose gel and blotted to a nylon membrane. The probe for the different HNF1 α isoforms was a fragment obtained by PCR on plasmid pRSV-HNF1-A [5] with primers PHABC 1336 and PHABC 1689 [5], encompassing an internal sequence common to HNF1-A, HNF1-B and HNF1-C. The probe for HNF1 β isoforms was a fragment obtained by PCR on plasmid pRSV-vHNF1-C [5] with primers deltaVHC1 and deltaVHC2 [5] encompassing an internal sequence common to HNF1 β -A/HNF1-B and HNF1-C. The probes were ³²P-labelled with a megaprime DNA-labelling kit (Amersham, Les Ulis, France). Quantification was achieved with a PhosphorImager.

The sequences of the sense and anti-sense oligonucleotide primers for 18 S ribosomal RNA were respectively 5'-CGAA-GACGATCAGATACCG-3' (sense) and 5'-CCAGACAAAT-CGCTCCACC-3' (anti-sense).

Preparation and electrophoretic mobility-shift assay (EMSA) of nuclear extracts

Nuclear extracts were prepared from TC7 cells as described by Hoppe-Seyler et al. [34], in the presence of protease inhibitors (0.5 μ M PMSF, 50 μ g/ml leupeptin and 25 μ g/ml aprotinin) and with or without protein phosphatases inhibitors (2 mM Na₃VO₄, 50 mM NaF, 40 mM β -glycerophosphate and 25 mM PP_i). EMSAs were performed as described previously [19]. To test the action of endogenous nuclear protein phosphatases, nuclear extracts prepared with or without protein phosphatases inhibitors were incubated at 30 °C during 30 min. The HNF1 DNA-binding was detected with the strong SIF3 HNF1-binding site from the SI promoter [18]. For supershift experiments, nuclear extracts were preincubated with anti-HNF1 α IgG (Santa Cruz Biotechnology, Tebu, Le Perray-en-Yvelines, France) at 20 °C for 30 min before the addition of the radioactive SIF3 probe. DNA binding by HNF4 was detected with CIIIB probe [35]. DNA binding by the transcription factors AP1 and AP3 was detected with PEA1-PEA3 probe [36].

Metabolic labelling of HNF1 with [³²P]P_i

Confluent TC7 cells were labelled for 24 h with 4 mCi/ml [³²P]P_i (ICN, Irvine, CA, U.S.A.) in phosphate-free Dulbecco's modified

Eagle's minimum (25 mM glucose) supplemented with 1% (w/v) non-essential amino acids and 20% (v/v) heat-inactivated (30 min, 56 °C) fetal bovine serum with or without 100 nM OA. The cells were lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer [20 mM Tris/HCl (pH 8)/1% (v/v) Nonidet P40/0.5% deoxycholate/0.1% SDS/1 mM PMSF/1% (w/v) aprotinin/25 mM benzamidine/2 mM Na_3VO_4 /50 mM NaF/40 mM β -glycerophosphate/25 mM pyrophosphate with complete protease inhibitor cocktail (Boehringer Mannheim)] containing 0.42 M NaCl. Cells were disrupted by three freezing–thawing cycles. Cell debris was pelleted and the supernatant was diluted with 2 vol. of RIPA buffer without NaCl, adsorbed on Protein A–Sepharose beads (Tebu) and subjected to immunoprecipitation overnight at 4 °C with a saturating amount of polyclonal anti-HNF1 antibody rHCT-284 [37]. The beads were washed four times with RIPA buffer containing 150 mM NaCl; the immunocomplexes were eluted at 100 °C in Laemmli buffer [125 mM Tris/HCl (pH 6.8)/4% (w/v) SDS/20% (v/v) glycerol/0.004% Bromophenol Blue/10% (v/v) 2-mercaptoethanol], resolved by SDS/PAGE [10% (w/v) gel (37.5 acrylamide:1 bisacrylamide)] and autoradiographed.

Pulse–chase study

To analyse the stability of HNF1 α , confluent TC7 cells were pulse-labelled for 2 h with 600 μCi of [^{35}S]cysteine/methionine (Tran ^{35}S -label; ICN, Orsay, France) and chased for various durations in the presence of excess non-radioactive cysteine and methionine (1 and 10 mM respectively) added to the culture medium. The treatment of TC7 cells with OA (100 nM) was started 20 h before pulse-labelling and was maintained during the chase. Cells were lysed and subjected to immunoprecipitation with anti-HNF1 α IgG as described above. The immunocomplexes were eluted in Laemmli buffer and resolved by SDS/PAGE [10% (w/v) gel]. The gel was fixed, incubated in Amplify (Amersham, Little Chalfont, Bucks., U.K.), dried and detected by fluorography. Quantification was achieved with a Mark III CS densitometric scanner.

Western blot analysis

The proteins were prepared from 10^7 TC7 cells. Nuclear extracts were separated by SDS/PAGE [10% (w/v) gel] and transferred to nitrocellulose. For membrane saturation, 4% (v/v) low-fat milk in PTMN (0.05% Tween 20/0.4% low fat milk/150 mM NaCl in PBS) was used. PTMN was used for washing and diluting the antibodies. The membrane was incubated with both anti-HNF1 α and anti-HNF1 β IgG (both at 1 $\mu\text{g}/\text{ml}$). After incubation with anti-goat antibodies coupled with horseradish peroxidase (Biosys, Compiègne, France), detection was performed by enhanced chemoluminescence with an ECL $^{\text{®}}$ kit (Amersham).

RESULTS

Inhibition of protein phosphatases 1/2A blocks the expression of the gene encoding SI

The potential role of phosphorylation/dephosphorylation processes on the regulation of expression of HNF1-dependent genes was analysed by using the target gene encoding SI. TC7 cells were treated at confluence (day 7) and for 24 h with increasing concentrations of the strong protein inhibitor of phosphatases 1/2A, OA [38] (Figure 1A). The level of SI mRNA was analysed by Northern blotting, as well as the levels of the hexose

Figure 1 Analysis of SI, GLUT1 and DPP IV mRNA levels and of glucose utilization parameters in control and OA-treated TC7 cells

(A) Northern blot analysis of the expression of SI, GLUT1 and DPP IV mRNA species in confluent TC7 cells, treated or not for 24 h with increasing concentrations of OA. The same amount of total RNA (20 μg) was loaded in each lane. The membrane was hybridized simultaneously with the SI and GLUT1 probes. After dehybridization, the same membrane was hybridized with the DPP IV probe. Similar results were obtained with two independent cultures. (B) Glucose utilization parameters and SI mRNA levels. Confluent TC7 cells, treated or not with 100 nM OA, were analysed at 5, 10 and 24 h after the change of medium. Results are expressed as percentages of the corresponding control for each time. Similar results were obtained with three independent cultures. The S.D. (not shown) was less than 10%.

transporter GLUT1 and of the brush-border hydrolase DPP IV, used here as controls. As reported previously [25], in control cells, during the 24 h after confluence, SI mRNA species increased and GLUT1 mRNA species decreased, whereas the DPP IV mRNA species remained constant (Figure 1A, left two lanes). Treatment with 10 nM OA inhibited the decrease in the GLUT1 mRNA species and reproducibly induced a slight increase in the level of DPP IV mRNA species. Under these conditions there was no effect on the SI mRNA level. It was only when the cells were treated with 100 nM OA that a blockade in the classical increase in the SI mRNA level was observed. The viability and the morphology of the cells were not altered even after 48 h of treatment with 100 nM with OA (results not shown). When used at 1 μM , OA had a deleterious effect on the cells within less than 24 h.

The effect of OA (100 nM) on the level of SI mRNA and on glucose consumption parameters was further analysed during the 24 h after the medium change at confluence (Figure 1B). During the first 10 h there was no difference between control and OA-treated cells with regard to the level of SI mRNA. However,

Figure 2 Phosphorylation of HNF1

(A) Confluent TC7 cells were metabolically labelled with [32 P] P_i in the presence or absence of OA (100 nM) for 24 h before being harvested. Total cellular extracts were immunoprecipitated with anti-HNF1 antibody (rHCT-284). The immunoprecipitated proteins were separated by SDS/PAGE and visualized by autoradiography. The bottom image shows autoradiography resulting from a longer exposure than in the top image. (B) Western blotting was performed with the same total cellular extracts as in A. The membrane was incubated with both anti-HNF1 α and anti-HNF1 β IgG (Santa Cruz Biotechnology). The positions of molecular mass markers are indicated (in kDa) between the panels.

between 10 and 24 h we observed an inhibition of the increase in SI mRNA. During the 24 h treatment, the same slight increase in the DPP IV mRNA level, reported above, was observed (results not shown). The inhibition of protein phosphatases 1/2A by OA was proven by its known effect on glucose metabolism: there was an inhibition of glycogen accumulation and an increase in the glycolytic flux (glucose consumption and lactic acid production) in comparison with the control cells (Figure 1B).

Inhibition of protein phosphatases 1/2A induces the hyperphosphorylation of HNF1 α and HNF1 β

To determine whether the phosphorylation status of HNF1 α and HNF1 β was affected by the concentration of OA that induced the modifications of expression of the gene encoding SI, confluent TC7 cells were labelled with [32 P] P_i in the presence or absence of 100 nM OA (Figure 2). Total cellular extracts were immunoprecipitated with anti-HNF1 antibodies; the immunocomplexes were fractionated by SDS/PAGE and detected by autoradiography (Figure 2A). In control TC7 cells, HNF1 α and HNF1 β were already phosphorylated; on treatment with OA there was a marked increase in the level of phosphorylation of HNF1 α and HNF1 β . The presence of multiple 32 P-labelled proteins corresponding to HNF1 α and HNF1 β in control TC7 cells suggested that most, if not all, of the HNF1 α and HNF1 β isoforms were phosphorylated. The inhibition of protein phosphatases 1/2A by OA treatment resulted in a hyperphosphorylation of all the discernible HNF1 α and HNF1 β isoforms. However, among the detectable isoforms of HNF1 α and HNF1 β , the amplitudes of

Figure 3 Western blot analysis of HNF1 α and HNF1 β isoforms in control and OA-treated TC7 cells

(A) Nuclear extracts (50 μ g) from confluent control TC7 cells and from cells treated for 24 h with increasing concentrations of OA were subjected to electrophoresis and transferred to nitrocellulose membrane. The membrane was incubated with both anti-HNF1 α and anti-HNF1 β IgG (Santa Cruz Biotechnology). (B) Nuclear extracts (50 μ g) from confluent TC7 cells treated or not with 100 nM OA for 24 h. The membrane was incubated with both anti-HNF1 α and anti-HNF1 β IgG. Western blotting was performed with a longer gel-migration time than in (A), to permit the better separation of the isoforms of HNF1 α and HNF1 β . Similar results were obtained from two independent cultures. The positions of molecular mass markers are indicated (in kDa) at the right.

hyperphosphorylation of the fastest migrating isoforms within each family were reproducibly greater. For HNF1 β , this fastest migrating isoform most probably corresponded to HNF1 β -C. An immunoblot analysis of the same total cellular extracts (Figure 2B) showed that, whereas the amount of HNF1 β remained constant, the level of HNF1 α was considerably decreased in OA-treated cells.

Protein phosphatases 1/2A control the nuclear amount of HNF1 α isoforms

To test whether the low level of HNF1 α proteins observed in total cellular extracts from OA-treated cells (Figure 2B) resulted in a lower nuclear content of HNF1 α , nuclear extracts purified from control or OA-treated TC7 cells were analysed by Western blotting (Figure 3). It clearly seemed that the nuclear extracts of cells treated or not with increasing concentrations of OA for 24 h contained similar amounts of the HNF1 β isoforms. By contrast, the nuclear amounts of the HNF1 α isoforms were greatly decreased when the cells were treated with 100 nM OA (Figure 3). This last effect of OA, as those reported previously (Figure 1), occurred between 10 and 24 h of treatment (results not shown).

Inhibition of protein phosphatases 1/2A decreases the DNA-binding activity of HNF1 α and HNF1 β

To determine the impact of the inhibition of protein phosphatases 1/2A on the DNA-binding activity of HNF1 α and HNF1 β , EMSAs were performed with the SIF3 probe, which corre-

Figure 4 DNA-binding activity of HNF1 in nuclear extracts from control and OA-treated TC7 cells

(A) EMSA was performed with the SIF3 (HNF1) probe and nuclear extracts (5 μ g) from confluent control TC7 cells and from cells treated for 24 h with increasing concentrations of OA. (B) Supershift experiments were performed after preincubation of the same nuclear extracts at room temperature with anti-HNF1 α IgG (Santa Cruz Biotechnology) for 30 min. Similar results were obtained in three independent cultures. (C) Nuclear extracts from control TC7 cells were prepared in the presence or absence of protein phosphatases inhibitors in lysis and extraction buffers and were then preincubated at 30 °C for 30 min. EMSAs were performed with SIF3 probe. Similar results were obtained in two independent experiments. (D) EMSA was performed with the same nuclear extracts as in (B), with the PEA1-PEA3 (AP1-AP3) probe. In (B), (C) and (D), EMSAs were performed with a much longer gel migration time than in (A) to obtain a better separation of the three HNF1/DNA complexes; the free probe therefore cannot be seen in (B), (C) and (D). Abbreviation: NS, non-specific binding.

sponded to the strongest HNF1-binding site of the SI promoter. TC7 cells were therefore treated at confluence and for 24 h with increasing concentrations of OA (Figure 4A). Treatment with 1 or 10 nM OA did not modify the DNA binding by HNF1 in comparison with control cells. It was only when the cells were treated with 100 nM OA that an overall decrease in SIF3-HNF1 complexes was observed, which resulted from a net decrease in the binding of the HNF1 α -containing and HNF1 β -containing dimers. The SIF3-HNF1 β homodimer complexes were revealed better in supershift experiments with anti-HNF1 α antibodies (Figure 4B, compare lanes 3 and 4). The decrease in HNF1 α DNA-binding activity observed in OA-treated cells could be explained by the lowering of the nuclear amount of HNF1 α (Figure 3). However, another mechanism must account for the decrease in the content of complexes implicating HNF1 β . To test the hypothesis of a direct effect of protein phosphatases, EMSAs were performed with nuclear extracts of control cells prepared in the presence or absence of protein phosphatase inhibitors in nuclear extraction buffers and incubated at 30 °C for 30 min. When the endogenous nuclear protein phosphatases were not inhibited, a significant increase in DNA binding by both HNF1 α and HNF1 β was observed (Figure 4C); treatment of these nuclear extracts *in vitro* with lambda phosphatase did not provoke any additional effect (results not shown).

The decrease in DNA-binding activity in OA-treated cells was specific for HNF1. Indeed, as reported previously in many studies [39-41], an increase in AP1 DNA-binding activity was observed after treatment with OA (Figure 4D). Under the same conditions the DNA-binding activity of the transcriptional factor AP3 was unaffected (Figure 4D).

Protein phosphatases 1/2A inhibition decreases the mRNA level of HNF1 α isoforms

RT-PCR analyses were performed to determine whether the lower total and nuclear amounts of HNF1 α isoforms in OA-treated cells resulted from a decrease in HNF1 α mRNA level. As reported previously for the parental Caco-2 cell line [5], the HNF1 α -A and HNF1 β -A/B mRNA species were also predominant in TC7 cells. As shown in Figure 5(A), a small decrease in the mRNA level of the HNF1 α isoforms (50 % of that of untreated cells) was observed when the cells were treated with 100 nM OA. By contrast, the mRNA level of the HNF1 β isoforms was unchanged under all conditions. Because transcription of the gene encoding HNF1 α [11,12], and not of that encoding HNF1 β [42], is closely dependent on the activity of HNF4, the effect of inhibition of protein phosphatases 1/2A on the DNA-binding activity of HNF4 was analysed. Here again, it was only when the cells were treated with 100 nM OA that a lower HNF4 DNA-binding, compatible with the decrease in the HNF1 α mRNA level, was observed (Figure 5B).

Protein phosphatases 1/2A control the stability of the HNF1 α proteins

Pulse-chase experiments were performed to test whether the low level of HNF1 α isoforms observed in OA-treated cells could also have resulted from an additional post-transcriptional mechanism, such as a decrease in protein stability. A preliminary experiment with a 1 h labelling pulse revealed a decreased HNF1 α half-life in

Figure 5 RT-PCR analysis of the different HNF1 α and HNF1 β mRNA species and HNF4 DNA-binding activity in control and OA-treated TC7 cells

(A) The RNA species were extracted from confluent TC7 cells treated or not with increasing concentrations of OA. The numbers of PCR cycles are indicated. To improve the detection of the HNF1 α -A-specific and HNF1 β -C-specific fragments, a separate PCR was performed on cDNA samples from control cells exclusively with the specific corresponding primers (lane C). Top panel: RT-PCR-amplified fragments of HNF1 α -A and HNF1 α -C mRNA with a combination of specific primers for the three HNF1 α isoforms as described in the Materials and methods section. The level of HNF1 α -B mRNA was very low and was not detected in these experiments. Middle panel: RT-PCR-amplified fragments of HNF1 β -A/B and HNF1 β -C mRNA species with a combination of specific primers for the HNF1 β isoforms. The primers did not distinguish between HNF1 β -A and HNF1 β -B isoforms. Bottom panel: RT-PCR-amplified fragments of 18 S rRNA used to normalize the reverse transcription step. (B) EMSA was performed with the CIIIB (HNF4) probe and nuclear extracts (5 μ g) from confluent control TC7 cells and from cells treated for 24 h with increasing concentrations of OA. Similar results were obtained from two independent cultures.

OA-treated cells. However, owing to the low level of 35 S incorporation in the HNF1 α isoforms, an accurate quantification of this experiment was impossible (results not shown). The labelling period was therefore extended to 2 h (Figure 6A, zero time). The decrease in HNF1 α synthesis in OA-treated cells (Figure 6A, zero time) was compatible with the lower mRNA levels (Figure 5A). In parallel, the inhibition of protein phosphatases 1/2A induced a decrease in the half-life of the proteins, which was 2 h in control cells and only approx. 1 h in OA-treated cells (Figure 6B).

Figure 6 Stability of HNF1 α protein in control and OA-treated TC7 cells

(A) TC7 cells were metabolically labelled with [35 S]methionine/cysteine for 2 h (zero time) and chased for the indicated durations in the presence of excess non-radioactive methionine and cysteine. Total cellular extracts were immunoprecipitated with anti-HNF1 α IgG (Santa Cruz Biotechnology), subjected to SDS/PAGE and detected by fluorography. (B) Quantification of detectable HNF1 α isoforms as deduced from a scan of (A). Results are expressed as percentages of the value obtained at time 0 for each condition. Similar results were obtained from two independent cultures. Symbols: ■, control; □, OA.

DISCUSSION

Many studies have reported the direct or indirect implication of phosphorylation/dephosphorylation processes in the activity of transcription factors. Although it has been observed previously that in HepG2 and Hep3B hepatoma cell lines HNF1 α was a phosphoprotein [43], the results reported here, for the enterocytic Caco-2 clone TC7, show for the first time the implication of endogenous protein phosphatases 1/2A in the phosphorylation state of all the HNF1 α and HNF1 β isoforms and in their DNA-binding capacity. On the basis of consensus sequences, HNF1 α and HNF1 β contain a number of potential phosphorylation sites for serine/threonine kinases such as casein kinase 2 and protein kinase C. However, the potential role of each phosphorylatable site remains to be determined.

In the present study we have shown that hyperphosphorylation of the HNF1 α isoforms, induced by the inhibition of protein phosphatases 1/2A, resulted in a decreased DNA-binding activity (Figure 4). This was the consequence of a decreased nuclear amount of HNF1 α (Figure 3). This lowering of the nuclear content of HNF1 α resulted from an overall decrease in the total cellular HNF1 α amount, owing to both a decreased mRNA level (Figure 5A) and a shortening of the half-life of the HNF1 α proteins (Figure 6). The specific decrease in the HNF1 α mRNA

level might be explained by the decrease of DNA binding by HNF4 (Figure 5B), which has already been reported to be sensitive to the action of protein phosphatase 1 *in vitro* [28]. Indeed, the transcription of HNF1 α [11,12], but not that of HNF1 β [42], is closely dependent on the activity of HNF4. Besides its effect on the level of HNF1 α mRNA, inhibition of protein phosphatases 1/2A decreased the half-life of HNF1 α protein (Figure 6). The HNF1 α half-life (2 h), estimated in our study from its endogenous expression in control TC7 cells, significantly differs from that reported previously (12 h [44]) with the use of transient expression experiments in C33 human epithelial cervical carcinoma cells, which lack endogenous HNF1 α expression and therefore probably the mechanisms that tightly control the expression of HNF1 α .

The effect, reported here, of protein phosphatases 1/2A on the HNF1 α half-life suggests that phosphorylation-dependent degradation is involved in the regulation of HNF1 α expression. Among the known mechanisms that control protein degradation, the ATP-dependent ubiquitin–proteasome pathway is involved in diverse regulatory cellular processes including the proteolytic control of short-lived transcription factors (reviewed in [45,46]). This protein degradation pathway is implicated in the activation of nuclear factor κ B which occurs after various cellular stimuli (reviewed in [47]). It has been shown that, in human Jurkat leukaemic T cells, the inhibition of protein phosphatases 1/2A by OA increases the phosphorylation of factor I κ B α , which is then subjected to proteasome-mediated degradation [48]. That a similar mechanism would also be involved in the regulation of HNF1 α isoforms merits further analysis.

Control of HNF1 α expression at a post-transcriptional level has previously been reported in diabetic animals. In streptozotocin-induced diabetic rats, the hepatic nuclear amount of HNF1 α was decreased, whereas the HNF1 α mRNA level remained unchanged [49]. Interestingly, the normalization of glycaemia of diabetic rats after treatment with phlorizin or vanadate restored both the level and the DNA-binding activity of HNF1 α [50].

Although resulting in the same final decrease in their DNA-binding activity, the mechanisms by which the inhibition of protein phosphatases 1/2A affects the HNF1 β isoforms was quite different from that which we described for HNF1 α . Indeed, we have shown that the OA-induced hyperphosphorylation of HNF1 β isoforms did not affect their total or nuclear amounts but directly inhibited their DNA-binding capacity (Figure 4). This phenomenon is in agreement with our previous results [19] in which glucose-metabolism-dependent variations in the DNA-binding capacity of HNF1 β -containing dimers were observed without any change in their mRNA levels. In the present study, an important increase in DNA binding by HNF1 β -containing dimers was observed when the endogenous protein phosphatases were not inhibited during the preparation of nuclear extracts in control cells. This concerned both HNF1 α and HNF1 β isoforms (Figure 4C). These results, obtained in conditions under which protein phosphatases but not kinases were functional, suggest that the balance between protein phosphatases 1/2A and protein kinases (particularly casein kinase 2 and protein kinase C) physiologically controls HNF1 activity. Moreover, the differences in the mechanisms involved in the regulation of HNF1 α and HNF1 β activities by this balance might result in different kinetics of effect on the HNF1 isoforms. Indeed, the impact on HNF1 β would be immediate, whereas that on HNF1 α implies a cascade of events resulting in a delayed effect. The equilibrium between protein phosphatases and protein kinases might thus tightly regulate the combination between the different isoforms, which are known to differ in their activation capacities

[5] and thus control the nutrition-dependent expression of the HNF1-targeted genes, such as those encoding SI [19] and SGLT1 [27]. We have also shown that the amplitude of hyperphosphorylation after the inhibition of protein phosphatases 1/2A differed greatly depending on the isoforms of HNF1 α and of HNF1 β . This focuses on the role of the phosphorylation status of each isoform of HNF1 α and HNF1 β in their dimerization and DNA-binding properties, more particularly for the transdominant-negative isoform HNF1 β -C. Phosphorylation/dephosphorylation processes, which also control the activity of HNF4 [28], affect HNF1 activity, indicating that such processes might have a central role in the modulation of the HNF network involved in the regulation of cellular differentiation and metabolism.

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