Activation of haptoglobin gene expression by cAMP involves CCAAT/enhancer-binding protein isoforms in intestinal epithelial cells

Nadine Pelletier, François Boudreau, Shun-Jiang Yu, Sonia Zannoni, Véronique Boulanger, Claude Asselin*

Groupe de recherche en biologie du développement, Département d'anatomie et biologie cellulaire, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, Que. J1H 5N4, Canada

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Abstract CCAAT/enhancer-binding protein (C/EBP) isoforms are expressed in rodent intestine and in the rat intestinal epithelial cell line IEC-6 but their role remains to be determined. Treatment of IEC-6 cells with the adenylate cyclase activator forskolin led to coordinate induction of C/EBP isoforms α, β and δ at the mRNA and protein levels. Transient transfection assays showed that their expression is controlled at the transcriptional level. Forskolin treatment induced haptoglobin mRNA levels. Electrophoretic mobility shift and supershift assays demonstrated an increase in DNA-binding activities of the three C/EBP isoforms to the haptoA and haptoC C/EBP DNA-binding sites of the proximal haptoglobin promoter. Site-specific mutations of both sites led to a decrease in transcriptional induction by forskolin, suggesting that C/EBP isoforms are involved in the cAMP-dependent regulation of the acute-phase protein gene haptoglobin in intestinal epithelial cells.

Key words: CCAAT/enhancer-binding protein; Haptoglobin; cAMP; Intestinal epithelial cell

1. Introduction

In response to inflammation or tissue injury, several plasma proteins known as acute-phase plasma proteins (APPs) are induced [1]. In rat liver, haptoglobin is an APP whose synthesis is increased during the acute-phase response [2]. Multiple roles have been ascribed to haptoglobin including binding and clearance of hemoglobin, inhibition of superoxide production and stimulation of angiogenesis [1]. In hepatocytes, transcription of haptoglobin is regulated by cytokines such as interleukin-1 (IL-1) and interleukin-6 (IL-6) and by glucocorticoids [3,4]. Analysis of acute-phase response gene promoters in hepatocytes, including the haptoglobin promoter, has led to the identification of regulatory elements interacting with members of the CCAAT/enhancer-binding protein family of transcription factors (C/EBPs) [1,2]. C/EBPs are characterized by a leucine zipper dimerization motif enabling the formation of homo- or heterodimers and a basic DNA-binding domain [5]. C/EBP family members, including C/EBPα, C/EBPβ and C/EBPδ, play major roles in the control of proliferation and differentiation of many cell types [6,7]. C/EBPβ (NF-IL6) and C/EBPδ are involved in the cytokine-dependent transcriptional activation of acute-phase response genes in hepatocytes [2].

Human intestinal epithelial cells secrete and respond to multiple cytokines, and are part of an acute-phase response both in vivo and in vitro [8]. In fact, several APPs are expressed and regulated by multiple cytokines in human colon carcinoma cell lines [9]. We have shown that the APP genes and C/EBP isoforms are expressed in rat intestinal epithelial cells in vivo and in vitro [10–13]. However, the regulatory pathways and the transcription factors involved in the control of APP gene expression in intestinal epithelial cells remain to be determined.

In order to understand the regulatory pathways involved in C/EBP isoform and APP gene expression in the intestinal epithelial cell context, we have investigated the regulation of C/EBP isoforms in response to cAMP levels and verified their role in the regulation of the APP gene haptoglobin in the IEC-6 cell line. This rat intestinal epithelial cell line is considered a useful in vitro model for the study of the regulation of intestinal epithelial cell proliferation and the acute-phase response [14–16]. We found that increases in cAMP levels lead to coordinate induction of C/EBP isoforms, and that C/EBP isoforms are directly involved in the cAMP-dependent regulation of the APP gene haptoglobin in intestinal epithelial cells.

2. Materials and methods

2.1. Cell culture

The rat intestinal epithelial cell line IEC-6 [17] was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Twenty-four hours before stimulation, complete medium was replaced with DMEM without serum. IEC-6 cells at 80% confluence were stimulated with forskolin (1 μM) and IBMX (3-isobutyl-1-methylxanthine) (0.1 mM) (Sigma, St. Louis, MO).

2.2. Northern analysis

Total cellular RNAs prepared by the guanidinium isothiocyanate-phenol method [18] were subjected to agarose gel electrophoresis with formaldehyde and transferred to nylon membranes (Nytran, Schleicher and Schuell) [19]. Equal RNA loading (20 μg) was confirmed by ethidium bromide staining and by hybridization either to a rat glyceraldehyde 3-phosphate dehydrogenase gene probe [20] or α-tubulin probe [21]. Northern analysis was performed as described [22]. Hybridizations were performed with the following random primed ^{32}P-labeled probes (Multiprime kit, Amersham): 1.8 kb rat C/EBPα fragment [23]; 1.5 kb murine C/EBPβ fragment [24]; 1.0 kb murine C/EBPδ fragment [24]. The 1040 bp haptoglobin cDNA probe was obtained by RT-PCR from IEC-6 total RNAs with the following oligonucleotide primers: HP+1774 (5′-GAGCTGTCGTCACTCTCCTG-3′) and HP+4338 (5′-CAGCCCTGAACTAGTTCTTG-3′). The nature of the amplified DNA was confirmed by DNA sequencing.

*Corresponding author. Fax: (1) (819) 564-5320.
E-mail: casselin@courrier.usherb.ca

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Autoradiograms exposed in a linear range were quantified by densitometric analysis with a Pharmacia LKB XL Ultroscan and signals were normalized to those of the control. Results are representative of three different experiments.

2.3. Western blot analysis

Nuclei were isolated as described by Stein et al. [25]. Nuclear extracts and Western blot analysis were prepared as described previously [11]. Proteins (20–40 μg) resolved by SDS-PAGE were electroblotted on nitrocellulose membranes according to Towbin et al. [26]. Incubations with affinity-purified rabbit polyclonal antisera (anti-C/EBPα, 0.4 μg/ml; anti-C/EBPβ, 0.1 μg/ml; anti-C/EBPδ, 0.4 μg/ml) (Santa Cruz Biotechnology, Santa Cruz, CA) were performed overnight at room temperature. The immune complexes were detected with the Super Signal Substrate system (Pierce, Rockford, IL) according to the manufacturer's instructions. The intensities of the signals were measured by densitometric analysis with a Pharmacia LKB UltroScan and normalized to a Coomassie blue staining control for protein loading. Results are representative of three different experiments.

2.4. Electrophoretic mobility shift assays

Nuclear extracts were prepared according to Stein et al. [25]. Gel retardation assays were done as described previously [11]. Samples were electrophoresed in a 4% polyacrylamide gel containing 0.5% Tris-borate buffer and 2% glycerol. The following C/EBP-binding sites from the rat haptoglobin promoter [3] were used for electrophoretic mobility shift assays: haptoA, −166 to −145, 5′-CCAAGTATGAAGCAAGAGCTCA-3′; haptoC, −88 to −67, 5′-GCCGACATTGTGCAAACACAGA-3′. Supershift assays with concentrated C/EBP isoform rabbit polyclonal antisera (Santa Cruz Biotechnology, Santa Cruz, CA) were performed as described previously [11].

2.5. Site-specific mutagenesis of the rat haptoglobin promoter

A 396 bp portion of the rat haptoglobin promoter (−396 to −2) [3] was inserted in the pGL3 basic luciferase reporter plasmid (Promega, Madison, WI). Specific mutations were introduced into hapto-luc at the haptoA and haptoC nuclear factor-binding sites indicated in Fig. 5 by using oligonucleotide-directed mutagenesis [27]. Complementary oligonucleotides containing a restriction enzyme 6 base sequence replacing the C/EBP-binding site core were generated. The haptoA sequence 5′-GCCAAGTATGAAGCAAGAAGAGCTCAGCTCT-3′ (−167 to −140) was modified in the haptoA mutant 5′-GCCAAGTATGACAGCTGAGCTCAGCTCT-3′ and the haptoC sequence 5′-AAGGCCGACATTGTGCAAACACAGAAAT-3′ (−91 to −64) in the haptoC mutant 5′-AAGGCCGACATCAGCTGAACACAGAAAT-3′. These changes were based on the consensus DNA-binding sites (underlined) for C/EBPs [28]. Double haptoA and haptoC mutants were constructed by replacing the C/EBP haptoC site in the haptoA mutant by a *Nde*I site. A mutation was also introduced in the haptoB STAT3 DNA-binding sequence [29] 5′-CTTTGTGGTTACTGGAACAGTCACTGAC-3′ (−125 to −97) by introducing a *Eco*RV site in the haptoB mutant 5′-CTTTGTGGTTAGATATCCAGTCACTGAC-3. Mutations were confirmed first by restriction enzyme digestion, then by DNA sequencing.

2.6. Transient transfections and luciferase assays

Transient transfections were performed with the cationic lipid Lipofectin (Gibco-BRL, New York). Murine C/EBPα promoter sequences from −355 to +7 [30], rat PCR-amplified C/EBPβ promoter sequences from −396 to +31 [31], and murine PCR-amplified C/EBPδ promoter sequences from −265 to +32 were subcloned in pGL3basic (Promega). These constructs, the hapto-luc with the single and double haptoA and haptoC mutants, and the NF-IL6-pGL3 promoter containing two copies of the NF-IL6 DNA-binding site (5′-TGATTGTGCAATTGTAGATTGTGCAATGT-3′ [32]), were transfected for 7 h in IEC-6 cells. The pRL-SV40 renilla luciferase vector (Promega) was used as a transfection efficiency control. After 24 h of induction, cells were lysed in 300 μl of extraction buffer containing 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 1% Triton X-100 and 1 mM dithiothreitol. Luciferase activity was then measured for 20 s in a Lumat LB 9507 luminometer (EG&G Berthold, USA). The activity of renilla luciferase was used to normalize for variations in transfection efficiency. Promoter activity of each construct was expressed as firefly/renilla luciferase activity ratio. Each experiment was done three times in triplicate.

3. Results

3.1. Expression of C/EBP isoforms in response to forskolin

C/EBP family members are differentially expressed in intestinal epithelial cells both in vivo and in vitro [10,11]. To understand the regulation of these transcription factors in intestinal epithelial cells, we analyzed the effect of protein kinase A (forskolin and IBMX) activators on the expression of C/EBP isoforms in the rat intestinal epithelial cell line IEC-6. Forskolin treatment resulted in induction of C/EBP isoform mRNAs with different kinetics, as determined by Northern analysis and comparison to a GAPDH mRNA loading control (Fig. 1). C/EBPα mRNA levels increased rapidly 2.5-fold ($P < 0.05$) after 1 h and remained elevated. C/EBPβ mRNAs increased rapidly and transiently with a 4.9-fold ($P < 0.005$) induction after 1 h. In contrast, C/EBPδ mRNA levels increased later with maximal induction at 24 h (3.7-fold, $P < 0.005$). Short-term induction of C/EBPα and C/EBPβ mRNAs by forskolin was still observed in the presence of the protein synthesis inhibitor cycloheximide, suggesting that induction of these genes did not need new protein synthesis (data not shown). Addition of the transcription inhibitor actinomycin D to stimulated cells did not show significant increased stabilities of C/EBP isoform mRNAs. The half-lives

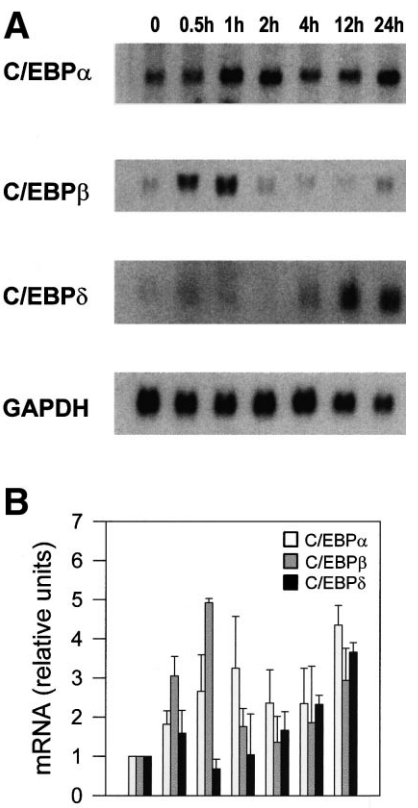

Fig. 1. Effect of forskolin on C/EBP isoform mRNA levels. Total RNAs were isolated from IEC-6 cells treated with forskolin/IBMX for 0, 0.5, 1, 2, 4, 12 and 24 h as indicated. Equal amounts of RNA (20 μg) were electrophoresed and analysed by Northern blot with sequential hybridization of ^{32}P-labeled C/EBPα, C/EBPβ, C/EBPδ and GAPDH DNA probes. C/EBP isoform mRNA levels were quantitated by densitometric analysis in comparison to a GAPDH control. Means and S.E.M. were calculated with values obtained from three independent experiments.

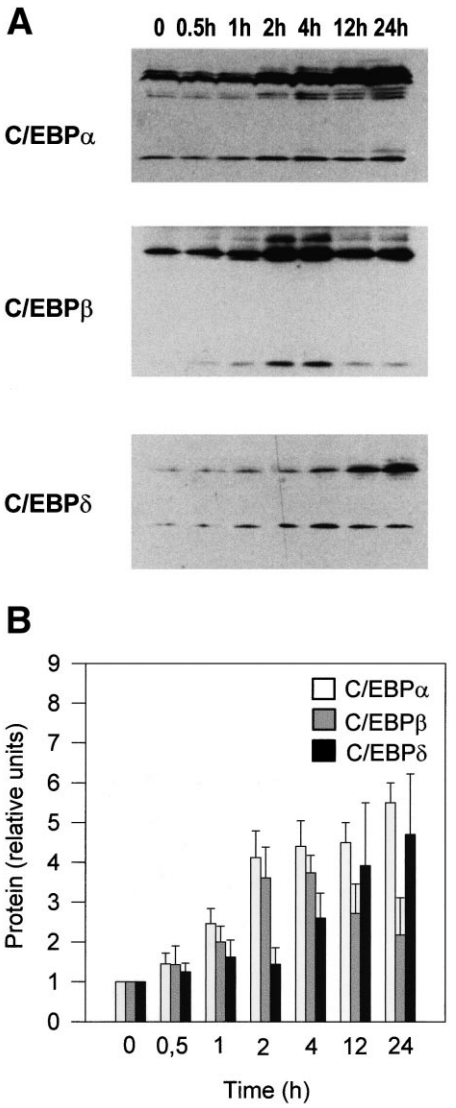

Fig. 2. Effect of forskolin on C/EBP isoform protein levels. Nuclear protein extracts were isolated from 0, 0.5, 1, 2, 4, 12 and 24 h forskolin/IBMX-treated IEC-6 cells. 50 μg of protein extracts was applied to each lane, separated by SDS-PAGE, and transferred to nitrocellulose membranes for Western blotting analysis. C/EBP isoform protein levels were quantitated by densitometric analysis in comparison to a Coomassie blue staining control for protein loading. Means and S.E.M. were calculated with values obtained from three independent experiments.

measured for C/EBPα, β and δ were respectively ∼60, ∼38 and ∼25 min (data not shown).

To verify whether changes in C/EBP isoform mRNA expression led to modifications in protein levels, we performed Western blot experiments on nuclear proteins isolated from IEC-6 cells stimulated with forskolin (Fig. 2). Forskolin treatment resulted in increasing amounts of the C/EBPα p42 and p30 proteins between 1 and 24 h (5.5-fold, $P < 0.05$). The C/EBPβ LAP and LIP proteins were induced rapidly and transiently with 3.6-fold increases after 2 h ($P < 0.005$) and 3.7-fold increases after 4 h ($P < 0.001$). In contrast, the C/EBPδ p33 protein was induced later between 4 and 24 h (4.7-fold, $P < 0.05$). These data indicate that C/EBP isoform protein levels vary with different kinetics in response to forskolin.

In order to determine whether C/EBP isoforms were transcriptionally activated by forskolin, we subcloned the C/EBP isoform proximal promoter regions in the pGL3basic vector. These constructs were transfected in IEC-6 cells and luciferase activities were measured after a 24 h induction period. Forskolin treatment led to significant inductions from the three promoters, respectively 3.9-fold for C/EBPα, 3.6-fold for C/EBPβ and 2.7-fold for C/EBPδ ($P < 0.005$). Thus, C/EBP isoforms are differently regulated by forskolin, mostly at a transcriptional level. The coordinate regulation of C/EBP isoforms by forskolin prompted us to verify the transcriptional activity of a NF-IL6-pGL3 luciferase reporter construct. Forskolin induced 3-fold the luciferase levels over the control values after 24 h. These data indicate that forskolin treatment leads to increased transactivation of a promoter containing C/EBP DNA-binding sites in intestinal epithelial cells.

3.2. *C/EBP-dependent induction of the haptoglobin gene by forskolin*

C/EBP isoforms have been implicated in vivo and in vitro in the regulation of APP genes during the inflammatory response in hepatocytes [1,2]. We have suggested that C/EBPs may play a role in the regulation of the acute-phase response in intestinal epithelial cells. Indeed, APP genes are induced during inflammation in rat intestinal epithelial cells in vitro and in vivo [12,13]. We thus verified whether targets for C/EBPs, such as APP genes, were induced in IEC-6 cells by forskolin. Northern analysis showed that haptoglobin mRNAs were induced by forskolin with increasing levels until 12 h (Fig. 3).

Two major C/EBP-binding sites (haptoA and haptoC) have been described in the haptoglobin promoter in hepatocytes [3]. In order to determine whether forskolin treatment modulated the binding of nuclear proteins to these sites, electrophoretic mobility shift assays were performed with nuclear extracts isolated from IEC-6 cells stimulated with forskolin. Forskolin treatment led to a steady increase in complex formation to both haptoA and haptoC DNA-binding sites (Fig. 4, data not shown). In contrast, binding to the haptoB STAT3 DNA-binding sequence was not affected (data not shown). Binding

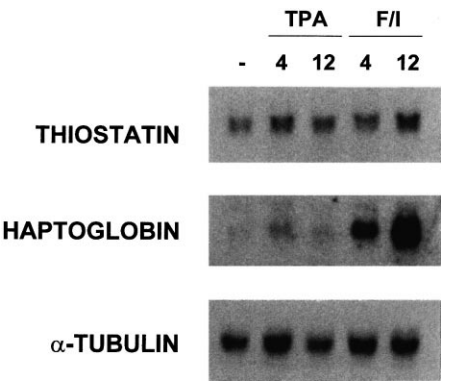

Fig. 3. Induction of haptoglobin by forskolin. Total RNAs were isolated from IEC-6 cells treated with TPA or forskolin/IBMX for 4 and 12 h. Equal amounts of RNA (20 μg) were electrophoresed and analyzed by Northern blot with sequential hybridization of ^{32}P-labeled thiostatin, haptoglobin and α-tubulin DNA probes.

to the haptoA and haptoC sites was specific since competition with a 50-fold molar excess of unlabeled haptoA, haptoC and NF-IL6 oligonucleotides resulted in a reduction in DNA-binding activity (data not shown). We then determined the nature of C/EBP isoforms in these complexes by supershift assays. In non-stimulated cells, the C/EBP complex binding both sites contained low amounts of the C/EBPα and δ isoforms in contrast to larger amounts of C/EBPβ, as assessed by supershift and by a decrease in the intensity of the major retarded complex (Fig. 4). Forskolin induced C/EBPα, C/EBPδ and to a lower extent C/EBPβ in both complexes after 12 h. These data show that both haptoA and haptoC sites bind C/EBP isoforms and that forskolin treatment increases C/EBP-binding activity through changes in the levels of specific isoforms interacting with both sites.

In order to determine the importance of the haptoA and haptoC sites and the implication of C/EBP isoforms in the regulation of the haptoglobin gene, we introduced site-specific mutations in the rat haptoglobin −396 to +31 promoter to inactivate the individual DNA-binding sites (Fig. 5A). The absence of competition by cold mutated oligonucleotides in electrophoretic mobility shift assays confirmed the inactivation of the haptoA and haptoC C/EBP DNA-binding sites (data not shown). We then verified the response of the mutants to forskolin in transient transfection assays. Forskolin treatment led to 4.2-fold increases in luciferase activity of the hapto-luc construct (Fig. 5B). Mutation of haptoA and haptoC respectively decreased basal activity by 30% and 50% while inactivation of both sites decreased luciferase activity by 70%. Individual mutations reduced the response to forskolin by about 40%. Mutation of both the haptoA and haptoC sites abolished the inducibility of the haptoglobin promoter by forskolin (Fig. 5B). In contrast, mutation of the STAT3 haptoB DNA-binding sequence did not affect significantly the response of the haptoglobin promoter to forskolin. These data show that forskolin act independently through the haptoA and haptoC sites. Both sites are necessary for a full transcriptional response of the haptoglobin promoter to forskolin.

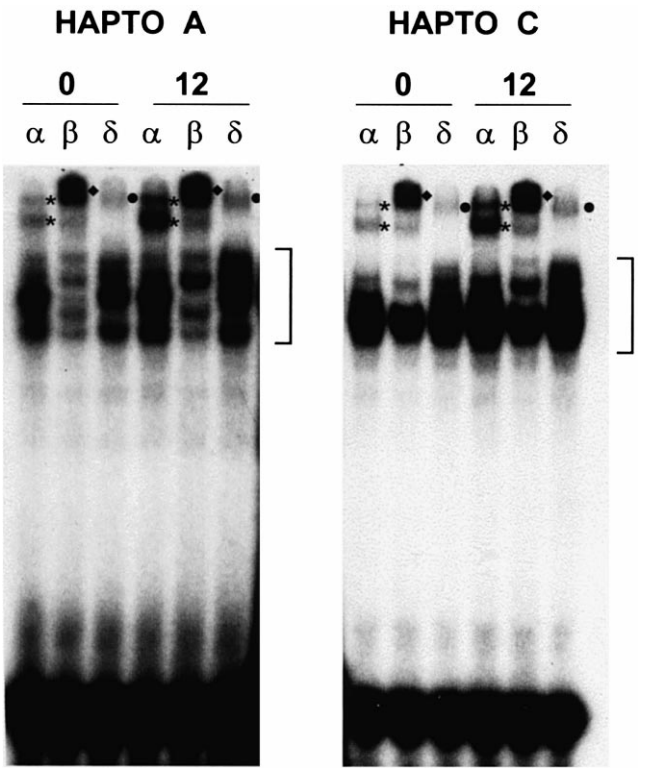

Fig. 4. Forskolin induces the DNA-binding capacity of C/EBP isoforms. Nuclear extracts from 0 and 12 h forskolin-treated cells were preincubated with specific antibodies against C/EBPα, C/EBPβ and C/EBPδ for 30 min prior to the addition of the specific haptoA and haptoC DNA-binding site labeled oligonucleotides. The position of the retarded complexes (C) is indicated by a bracket. The position of the specific retarded complexes is indicated (C/EBPα, *; C/EBPβ, ♦; C/EBPδ, ●).

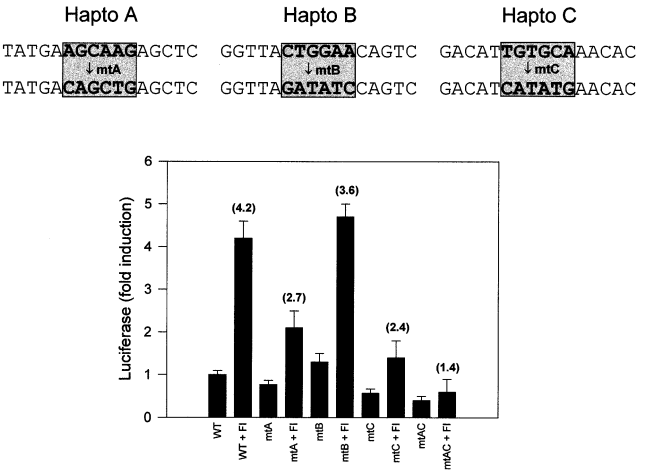

Fig. 5. The haptoA and haptoC sites are both necessary for forskolin-mediated induction of haptoglobin. A: Mutations in the haptoA, haptoB and haptoC, or both haptoA and haptoC sites were introduced by replacement of the respective interacting sequence with the restriction enzyme sequence shown in bold. B: To determine whether these sites were involved in forskolin-dependent haptoglobin induction, IEC-6 cells were transiently transfected with the respective mutants and the pSV40-renilla luciferase transfection control DNA. Cells were then treated with forskolin during 24 h. Luciferase activities were normalized to renilla luciferase activity and are expressed as fold induction in comparison to non-treated cells. Error bars represent the standard deviations between triplicate transfections in three independent experiments. The numbers in parentheses above bars represent the induction of luciferase activity in comparison to control cells. $P \leq 0.005$.

4. Discussion

Over the past few years, intestinal epithelial cells have been shown to play a central role in the inflammatory response of the intestine [8]. However, the regulation of APP genes and the transcription factors involved have not been clearly defined. We show here that C/EBP isoforms and the APP gene haptoglobin are regulated by forskolin, an adenylate cyclase activator. Furthermore, we demonstrate that C/EBPs are directly implicated in the cAMP-dependent regulation of haptoglobin.

In IEC-6 cells, the three C/EBP isoforms are induced by forskolin with different kinetics, as assessed by Northern and Western blot analysis and transfection assays. While the C/EBPα promoter sequences used in the transient transfection assays do not contain a cAMP responsive element, both C/EBPβ and C/EBPδ promoter sequences do [33–37]. The rapid and transient increases in C/EBPβ mRNA levels in response to forskolin suggest a direct effect of cAMP. In

hepatocytes, CREB controls C/EBPβ transcription through two CREB sites [34]. These binding sites are probably functional and involved in cAMP-dependent regulation of C/EBPβ in intestinal epithelial cells as well. The late appearance of C/EBPδ mRNAs suggest that C/EBPδ is indirectly regulated by cAMP and that the CRE-like sequences in the proximal promoter are not recognized by CREB-like proteins. As for C/EBPα, cAMP induces C/EBPα mRNA levels in preadipocytes [38]. The specific sequences in both C/EBPα and C/EBPδ promoters responsive to cAMP remain to be determined.

Forskolin treatment leads to sustained changes in C/EBP protein levels and increases the transcriptional activity of C/EBP DNA-binding sites containing promoters. In other cell types, cAMP differently regulates C/EBP isoform transactivation and DNA-binding activities. For example, protein kinase A phosphorylates C/EBPβ in vitro, resulting in an inhibition of DNA binding [39]. C/EBPβ is involved in the transactivation of the acetyl-CoA carboxylase in preadipocytes [38] without affecting DNA binding or translocation as in the rat pheochromocytoma cell line PC12 [40]. C/EBPα, while not phosphorylated by protein kinase A, acts as a cAMP-regulated nuclear regulator of the phosphoenolpyruvate carboxykinase (PEPCK) gene in hepatocytes [41,42]. Finally, C/EBPδ is involved in the cAMP-dependent regulation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene [43] and in the activation of IGF-1 transcription in osteoblasts [44]. Protein kinase A does not phosphorylate C/EBPδ protein [45]. It has been shown that C/EBP can heterodimerize with CREB/activating (ATF) transcription factors [46]. This heterodimerization leads the complex to asymmetric sequences binding both factors. CREB proteins and C/EBP isoforms bind asymmetric sequences in the promoter of the PEPCK and the CFTR gene [40,42]. However, cAMP-dependent transactivation of IGF-1 in osteoblasts and of the acetyl-CoA carboxylase in adipocytes requires a C/EBP specific DNA-binding site [38,44].

In intestinal epithelial cells, the haptoA and haptoC sites of the haptoglobin promoter bind C/EBP isoforms. Competition with CRE sequences in electrophoretic mobility shift assays did not affect the binding activity (data not shown). The sites also do not contain CRE-like sequences. Site-specific mutagenesis of both sites leads to an abolition of the forskolin-mediated induction of the haptoglobin promoter. Co-transfection of each isoform with the hapto-luc construct both in IEC-6 and CV-1 cells results in transactivation, suggesting that the three isoforms are involved in haptoglobin regulation (data not shown). The changes in the ratio of each isoform induced by cAMP should be important in the induction of the haptoglobin gene as well as the maintenance of its expression. cAMP could affect through phosphorylation the transactivation properties of the C/EBP isoforms or the interaction of C/EBPs with p300/CBP co-activators [47]. However, the kinetics of accumulation of C/EBP proteins do not indicate an effect of cAMP on translocation of C/EBP proteins to the nucleus.

In addition to C/EBPs, STAT3, a mediator of the IL-6 receptor signal, is involved in the regulation of haptoglobin [29]. Induction of STAT3 by increased synthesis of IL-6 by forskolin treatment could indirectly be responsible for haptoglobin induction through an autocrine pathway. However, our results point to a direct role of C/EBPs in forskolin-dependent induction of haptoglobin. First, binding to the haptoB STAT3 site is not increased after forskolin treatment. Second, mutation of the haptoB site does not affect significantly the response of the haptoglobin promoter to forskolin, while mutation of both haptoA and haptoC C/EBP interacting sites abolishes cAMP-dependent induction. Lastly, stimulation of IEC-6 cells by IL-6 does not induce the expression of haptoglobin (data not shown). In summary, we show for the first time that C/EBP isoforms are involved in the expression of the APP gene haptoglobin in intestinal epithelial cells and that C/EBP isoforms participate in the transduction of signals mediated by cAMP. Our results provide an outline of the intricate regulation of C/EBP isoforms involved in acute-phase protein expression.

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