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Regulation of C/EBPδ-Dependent Transactivation by Histone Deacetylases in Intestinal Epithelial Cells

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Abstract The C/EBPδ transcription factor is involved in the positive regulation of the intestinal epithelial cell acute phase response. C/EBPδ regulation by histone deacetylases (HDACs) during the course of inflammation remains to be determined. Our aim was to examine the effect of HDACs on C/EBPδ-dependent regulation of haptoglobin, an acute phase protein induced in intestinal epithelial cells in response to pro-inflammatory cytokines. HDAC1, HDAC3, and HDAC4 were expressed in intestinal epithelial cells, as determined by Western blot. GST pull-down assays showed specific HDAC1 interactions with the transcriptional activation and the b-ZIP C/EBPδ domains, while the co-repressor mSin3A interacts with the C-terminal domain. Immunoprecipitation assays confirmed the interaction between HDAC1 and the N-terminal C/EBPδ amino acid 36–164 domain. HDAC1 overexpression decreased C/EBPδ transcriptional activity of the haptoglobin promoter, as assessed by transient transfection and luciferase assays. Chromatin immunoprecipitation analysis showed a displacement of HDAC1 from the haptoglobin promoter in response to inflammatory stimuli and an increased acetylation of histone H3 and H4. HDAC1 silencing by shRNA expression increased both basal and IL-1β-induced haptoglobin mRNA levels in epithelial intestinal cells. Our results suggest that interactions between C/EBPs and HDAC1 negatively regulate C/EBPδ-dependent haptoglobin expression in intestinal epithelial cells. J. Cell. Biochem. 103: 1573–1583, 2008.

Key words: C/EBPδ; histone deacetylase; haptoglobin; inflammation; intestinal epithelial cells

The intestinal epithelial cell inflammatory reaction leads to the rapid and selective activation of several acute phase protein genes in response to multiple cytokines, including IL-1β [Chang, 2000]. Intestinal epithelial cells participate in an acute phase response (APR) by activating among others CCAAT/enhancer binding protein (C/EBP) transcription factors [Ramji and Foka, 2002]. The intestinal APR is modulated by C/EBPα and most importantly by the C/EBPβ and C/EBPδ isoforms [Gheorghiu et al., 2001]. We have shown that C/EBP isoforms regulate acute phase protein genes in intestinal epithelial cells in response to glucocorticoids [Boudreau et al., 1998], cAMP [Pelletier et al., 1998], TGFβ [Yu et al., 1999], and IL-1β [Desilets et al., 2000]. Furthermore, C/EBP isoform overexpression increases cAMP and IL-1β-dependent expression of the acute phase protein gene haptoglobin [Gheorghiu et al., 2001].

C/EBP isoforms interact through their C-terminal leucine zipper domains and bind DNA as dimers with the adjacent basic regions. In contrast to the C-terminal domain, the N-termini of C/EBP proteins diverge and contain positive and negative transcriptional regulatory regions [Ramji and Foka, 2002]. Co-activator complexes with histone acetyltransferase activities play a key role in C/EBP isoform-dependent transcriptional activation. Indeed, p300/CBP interaction with C/EBPα [Erickson et al., 2001], C/EBPβ

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[Mink et al., 1997; Guo et al., 2001], and C/EBPδ [Kovacs et al., 2003; Svotelis et al., 2005] is necessary for increased transactivation potential. In contrast, C/EBP isoforms may be involved in transcriptional repression through interaction with co-repressor complexes with histone deacetylase activities. Indeed, a transcriptional mSin3A-histone deacetylase 1 (HDAC1) co-repressor complex interacts with C/EBPβ to insure C/EBPα repression and inhibition of adipocyte differentiation [Wiper-Bergeron et al., 2003]. In addition, transcriptional repression of the PPARβ promoter by C/EBPα- or C/EBPβ-HDAC1-co-repressor complexes may be involved in the regulation of keratinocyte differentiation [Di-Poi et al., 2005]. These data indicate that C/EBP isoforms may be part of co-activating or co-repressing complexes, depending on cell status.

We have previously identified C/EBPδ domains involved in transcriptional activation of the acute phase protein gene haptoglobin [Svotelis et al., 2005]. However, little is known about the ability of C/EBPδ's to repress acute phase protein gene expression. In this study, we provide evidence that the C/EBPδ N- and C-terminal domains interact independently with HDAC-containing co-repressor complexes, leading to decreased haptoglobin expression. IL-1β treatment of intestinal epithelial cells leads to HDAC1 displacement from the haptoglobin promoter and increased histone H3 and H4 acetylation. Finally, HDAC1 silencing increases both basal and IL-1β-induced haptoglobin mRNA levels. Our results suggest that interactions between C/EBPs and HDAC1 negatively regulate C/EBPδ-dependent haptoglobin expression in intestinal epithelial cells.

MATERIALS AND METHODS

Cell Culture

The rat intestinal epithelial cell line IEC-6 (A. Quaroni, Cornell University, Ithaca, NY) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal bovine serum (FBS). IEC-6 cells at 80% confluence were stimulated with IL-1β (10 ng/ml) (R&D Systems, Minneapolis, MN) for 0, 4 or 24 h. The human colon carcinoma cell line Caco-2/15 [Beaulieu and Quaroni, 1991] was grown in DMEM supplemented with 10% FBS. Caco-2/15 cells were harvested at 30% confluence, at confluence or 5, 10, and 15 days after confluence. These cells differentiate into enterocytes after reaching confluence. The undifferentiated human intestinal epithelial cell line HIEC-6 [Perreault and Jean-Francois, 1996] was grown in DMEM supplemented with 10% FBS, 10 μg/ml insulin (Sigma–Aldrich Canada, Oakville, ON) and 5 ng/ml EGF (R&D Systems, Minneapolis, MN). The human transformed kidney cell line HEK 293T (provided by A. Nepveu, Department of Oncology, McGill University, Montreal, QC, Canada) was grown in DMEM with 10% FBS.

Western Blot Analysis

Nuclear extracts were prepared as described previously [Stein et al., 1989], and diluted in Laemmli buffer (62.5 mM Tris-HCl, pH 6.9, 2% SDS, 1% β-mercaptoethanol, 10% glycerol, and 0.04% bromophenol blue). Equal amounts of proteins were loaded on a 10% SDS–polyacrylamide gel. Proteins electroblotted on a PVDF membrane (Roche Molecular Biochemicals, Laval, QC, Canada) were incubated with rabbit polyclonal antibodies against HDAC1, HDAC3, HDAC4 (New England BioLabs, Mississauga, ON) and laminB (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, as described previously [Desilets et al., 2000]. Immune complexes were detected with the Super Signal West Pico Substrate system (Pierce, Rockford, IL) according to the manufacturer's instructions. Results are representative of three independent experiments.

DNA Constructs

All GST fusion constructs used have been described previously [Svotelis et al., 2005], including those containing the N-terminal region of C/EBPδ (amino acids 1–164), the N-terminal mutants δ1–70 (amino acids 1–70), δ1–108 (amino acids 1–108), Δ75–85 (deletion of amino acids 75–85) and the C-terminal region of C/EBPδ (amino acids 165–268), and the murine sarcoma virus (MSV) vector [Cao et al., 1991] expressing C/EBPδ and deletion mutants Δ36–164 or Δ70–164 have been described previously [Svotelis et al., 2005]. C/EBPδ PCR-amplified fragments encompassing amino acids 1–80 (240 bp), 1–97 (291 bp), 165–228 (189 bp), 165–242 (231 pb), 191–268 (231 bp), and 210–268 (174 bp) were cloned in-frame, downstream of the glutathione S-transferase (GST) sequence in the IPTG-inducible (isopropyl-β-thiogalactopyranoside) vector pGEX4T-2 (Amersham Biosciences, Baie d'Urfé, QC, Canada). The PCR-amplified C/EBPδ N-terminal

sequence encompassing amino acids 1–102 (306 bp) was cloned in-frame downstream of the GAL4 DNA-binding domain of the mammalian expression vector pM2 [Sadowski et al., 1992]. All PCR reactions were performed in the PCR Express Thermal Cycler (Hybaid, Franklin, MA) in 32 cycles: 1 cycle at 94°C, 1 min; 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s; and 1 cycle at 72°C for 5 min. The presence of the mutations was verified by restriction enzyme and sequence analysis (Sheldon Biotechnology Centre, McGill University, Montreal, QC, Canada; University Core DNA & Protein Services, University of Calgary, Calgary, AB, Canada).

GST Fusion Protein Purification and GST Pull-Down Assays

C/EBPδ GST fusion proteins were purified according to the GST Gene Fusion System protocol (Amersham Biosciences), as described previously [Houde et al., 2001]. HEK 293T cells were transfected by lipofection (Lipofectamine 2000, Invitrogen, Burlington, ON, Canada) with 3 μg of pcDNA3-HA-HDAC1, pcDNA3-HDAC3, or pcDNA3-HA-HDAC4 expression vectors (provided by Yang XJ, University McGill, Montréal, QC, Canada) and were lysed as described previously [Svotelis et al., 2005]. Total cell extracts (750 μg) were incubated with 10 μg of GST-fusion proteins coupled to glutathione-sepharose (Amersham Biosciences) for 2 h at 4°C. The beads were washed three times and protein complexes were eluted with Laemmli buffer [Svotelis et al., 2005]. Proteins resolved by SDS–PAGE (10% acrylamide) were electroblotted on a PVDF membrane (Roche Diagnostics, Laval, QC, Canada) for Western blot. Incubation with a rabbit or a mouse polyclonal antibody against HDAC3 (New England BioLabs) or HA-tag (Santa Cruz Biotechnology) was performed overnight at 4°C. Immunocomplexes were visualized with the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) according to the manufacturer's instructions. Protein concentration was determined with the Bio-Rad Protein Assay kit (Bio-Rad, Mississauga, ON, Canada). Equal loading of the GST fusion proteins was assessed by Ponceau Red staining. Where indicated, HA-HDAC1 staining was quantified by densitometry with the ImageQuant software (Amersham Biosciences) and normalized to GST-C/EBPδ fusion constructs Ponceau staining. Experiments have been performed independently three times.

Co-Immunoprecipitation Assays

HEK 293T cells were transfected with MSV, MSV-C/EBPδ, MSV-C/EBPδ (Δ36–164) or MSV-C/EBPδ (Δ70–164), and either pcDNA3 or pcDNA3-HA-HDAC1 expression vectors for 48 h, and lysed as described before [Laprise et al., 2002]. Cell lysates cleared by centrifugation were incubated with either a rabbit polyclonal antibody against C/EBPδ or a mouse monoclonal antibody against HA-tagged proteins (Santa Cruz Biotechnology) overnight at 4°C. Sixty microliters of pre-equilibrated protein A-agarose (Amersham Biosciences) were added for 60 min at 4°C. Immunocomplexes were then harvested by centrifugation and washed four times with ice-cold lysis buffer. Proteins were solubilized with Laemmli buffer and analyzed by SDS–PAGE and immunoblotting using anti-HA-tagged or anti-C/EBPδ antibodies. Results are representative of three independent experiments.

Transient Transfections and Luciferase Assays

HEK 293T were seeded in 24-well plates and transfected by lipofection (Lipofectamine 2000, Invitrogen) [Svotelis et al., 2005]. pFRluc (Clontech, Palo Alto, CA) was cotransfected with the GAL4 constructs without or with the N-terminal C/EBPδ amino acid 1–102 domain. Haptoluc, containing the 395 bp portion of the rat haptoglobin promoter (−396 to −2) inserted into the pGL3 basic luciferase reporter plasmid (Promega, Madison, WI) [Pelletier et al., 1998], was co-transfected with the MSV constructs without or with the C/EBPδ coding sequence, and the pcDNA3 construct without or with HA-HDAC1, and cells were lysed after 48 h [Desilets et al., 2000]. Luciferase activity was measured for 20 s as described before [Pelletier et al., 1998]. The pRL-SV40 renilla luciferase vector (Promega) was used as a control for transfection efficiency. Results shown are representative of experiments repeated four times, each in triplicate. Results were analyzed by the student's *t*-test and were considered significantly different at $P \leq 0.05$.

Chromatin Immunoprecipitation Assays

IEC-6 cells were treated with IL-1β for 0 and 4 h. Chromatin immunoprecipitation (CHIP) assays were performed as previously described

[Blais et al., 2005] using a CHIP assay kit (Upstate Biotechnology, Lake Placid, NY). Cells were crosslinked with 1% formaldehyde for 10 min at 37°C. Chromatin was immunoprecipitated with rabbit polyclonal antibodies against acetylated histone H3, acetylated histone H4 (Upstate Biotechnology), C/EBPβ and C/EBPδ (Santa Cruz Biotechnology), and HDAC1 (New England BioLabs). One percent of the lysate was used to verify the amount of DNA for each immunoprecipitation. Immunoprecipitated DNA was purified and diluted 1:20 prior to PCR amplification using the following primers: haptoglobin-5′ (−131 to −111), 5′-GGTTTGCTTTTGTGGTTACTGG-3′; haptoglobin-3′ (+40 to +59), 5′-ACGTGTGGACTGTGGACTCA-3′ [Marinkovic and Baumann, 1990; Pelletier et al., 1998]. Amplified DNAs were separated and visualized on a 2% agarose gel. As a specificity control, an upstream −1,292 to −1,115 region of the haptoglobin promoter was not amplified after immunoprecipitation with HDAC1 and C/EBP antibodies, and weakly amplified by acetylated histone antibodies, as compared to the proximal promoter region (data not shown). Experiments were performed three times independently.

Retroviral shRNA Infection

A shRNAmir against HDAC1, cloned in the pSM2 retroviral vector, was selected (Open Biosystems, Huntsville, AL) and transfected by lipofection in HEK 293T cells, with helper amphotropic DNA (A. Nepveu, McGill University). Two days after transfection, supernatants were filtered and used to infect IEC-6 cells at 40% confluence in 60 mm plates, in the presence of 4 μg polybrene (Sigma-Aldrich Canada) for 6 h. Two days after transfection, cells were split and selected in medium containing 2 μg/ml puromycin (Sigma–Aldrich Canada). Cell clones were selected for a decrease in HDAC1 protein expression, as compared to empty vector infected cells. HDAC1 protein levels were assessed by Western blot, quantified by densitometry with the ImageQuant software (Amersham Biosciences) and normalized to LaminB. HDAC1-specific shRNA expression did not activate interferon-responsive genes.

Northern Blot Analysis

Total cellular RNAs prepared with the TRIZOL reagent (Invitrogen) were electrophoresed in an agarose gel containing formaldehyde, and transferred to nylon membranes (Biodyne B, Pall Corp., Pensacola, FL), as described previously [Souleimani and Asselin, 1993]. Hybridizations were performed with random-primed ^{32}P-labeled probes (Rediprime™ II random prime labeling system, GE Healthcare, Buckinghamshire, UK) from a 1 kb rat haptoglobin DNA fragment [Pelletier et al., 1998] and a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) fragment as a RNA loading control. Quantification of band intensity was performed with the ImageQuant software (Amersham Biosciences). Results are representative of three independent experiments.

RESULTS

HDAC1, HDAC3, and HDAC4 are Expressed in Intestinal Epithelial Cells

Levels of HDAC expression were assessed by Western blot of nuclear extracts from subconfluent, confluent and post-confluent human colon carcinoma Caco-2/15 cells, human transformed kidney HEK 293T cells, and non-transformed intestinal epithelial crypt cells of human [Perreault and Jean-Francois, 1996] or rat origin [Quaroni and May, 1980]. HDAC1 and HDAC3 were ubiquitously expressed in human carcinoma Caco-2/15 and in crypt cells, as observed before [Wilson et al., 2006]. As opposed to its expression in Caco-2/15 cells, HDAC4 was not expressed in the nucleus of non-transformed crypt-derived HIEC and IEC-6 cells (Fig. 1). In contrast to HDAC1, protein levels of both HDAC3 and HDAC4 were reduced in differentiating Caco-2 cells. Thus, HDAC1, HDAC3, and HDAC4 are expressed in intestinal epithelial cells.

C/EBPδ Interacts With HDACs

Our previous work has shown that the p38 MAPK and the co-activator p300 are involved in the positive regulation of C/EBPδ transcriptional activity [Svotelis et al., 2005]. To determine whether HDACs played a role in C/EBPδ regulation, we first verified the interaction of C/EBPδ with HDACs. GST fusion proteins with the C/EBPδ N-terminal or C-terminal domain (Fig. 2A) were used in pull-down assays of total protein extracts from HEK 293T cells transfected with respectively, HA-tagged HDAC1, HDAC3, or HA-tagged HDAC4 expression vectors. HDACs were detected by Western blot with anti-HA or anti-HDAC3 antibodies

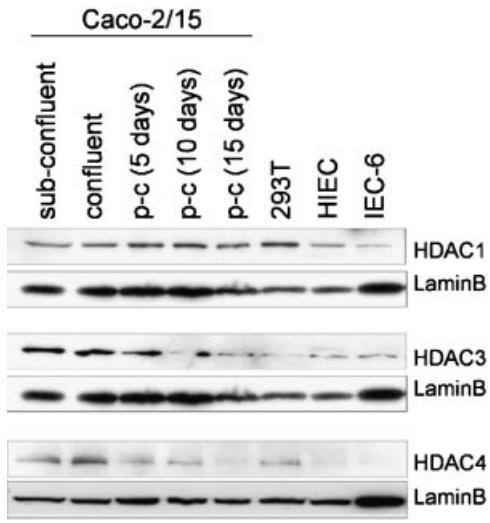

Fig. 1. HDAC1, HDAC3, and HDAC4 are expressed in intestinal epithelial cells. Nuclear extracts from Caco-2/15 cells prepared after various periods post-confluence (p-c), HEK 293T, HIEC, and IEC-6 cells were separated on a 10% SDS–PAGE gel, transferred onto a PVDF membrane and analyzed by Western blot for expression of HDAC1, HDAC3, HDAC4, and laminB as a control of nuclear protein loading. The results are representative of three independent experiments.

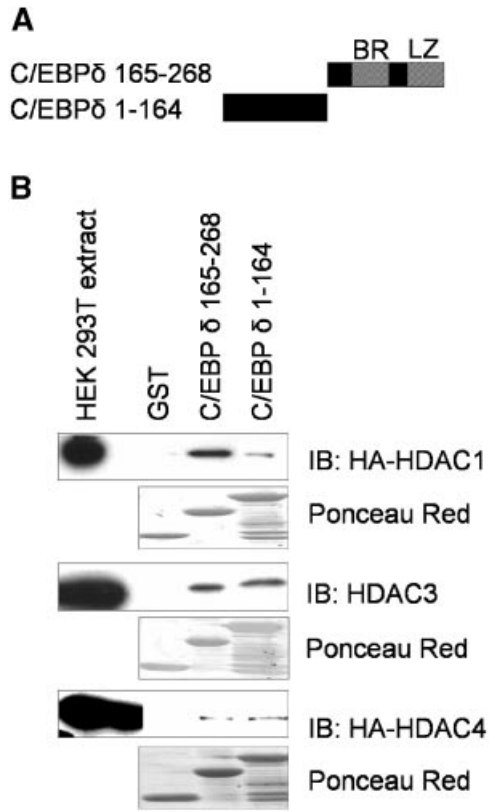

Fig. 2. HDAC1, HDAC3, and HDAC4 interact with both C/EBPδ N-terminal and C-terminal domains. **A**: Schematic representation of the C/EBPδ N-terminal (1–164) and C-terminal (165–268) domains, with the location of the basic DNA-binding region (BR) and the leucine zipper (LZ). **B**: GST fusion proteins were used in pull-down assays with 750 μg of total cell extracts from HEK 293T cells transfected with HA-HDAC1, HDAC3, and HA-HDAC4 expression vectors. Protein complexes were separated on a 10% SDS–PAGE gel and transferred onto a PVDF membrane for detection by Western blot (IB) with anti-HA-tag or anti-HDAC3 antibodies. The amount of protein loaded is shown by Ponceau Red staining. The results are representative of three different experiments.

(Fig. 2B). HDAC1, HDAC3, and HDAC4 interacted in vitro not only with the C/EBPδ N-terminal transactivation domain, but with the C-terminal DNA-binding domain as well.

HDAC1 Interacts With C/EBPδ Between Amino Acids 36 and 164 In Vivo

We focused on HDAC1 to determine the C/EBPδ domains involved in HDAC interactions. To demonstrate the in vivo interaction between HDAC1 and C/EBPδ, HEK 293T cells were co-transfected with C/EBPδ and HA-HDAC1 expression vectors. Cellular extracts were immunoprecipitated either with anti-C/EBPδ or anti-HA, and analyzed by anti-HA or C/EBPδ immunoblotting. The results show that HDAC1 was co-immunoprecipitated with C/EBPδ, and C/EBPδ was co-immunoprecipitated with HDAC1 (Fig. 3A). To identify the C/EBPδ domain responsible for HDAC1 interaction, we co-transfected two C/EBPδ N-terminal deletion mutants, namely Δ36–164 and Δ70–164 [Svotelis et al., 2005] (Fig. 3B). Immunoprecipitation assays showed that, in comparison to the interaction between wild-type C/EBPδ and HDAC1, deletion of amino acids 70–164, and to a larger extent deletion of amino acids 36 to 164, led to a decrease in HDAC1 interaction with C/EBPδ (Fig. 3C). This suggests that the C/EBPδ N-terminal region encompassing amino acids 36 and 164 interacts with HDAC1, and that other regions, including the leucine zipper-DNA-binding domain, may be involved.

HDAC1 Interacts With the C/EBPδ N-Terminal Domain Between Amino Acids 70 and 97

To determine the domains involved in HDAC1 interaction, we generated several GST fusion proteins with different portions of the C/EBPδ N-terminal region (Fig. 4A). GST pull-down assays of total protein extracts from HEK 293T cells transfected with HA-tagged HDAC1 were followed by anti-HA immunoblotting. Results demonstrate that deletion between

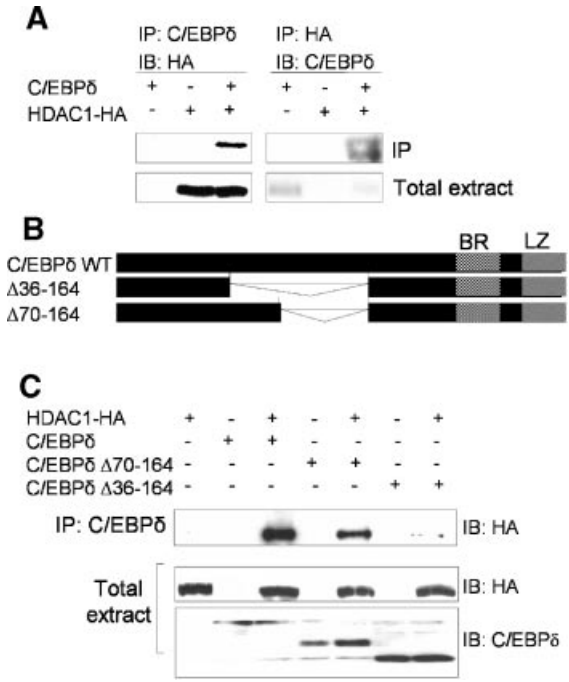

Fig. 3. HDAC1 interacts with C/EBPδ between amino acids 36 and 164 in vivo. **A**: HEK 293T cells were transfected with empty vectors or with C/EBPδ and HA-HDAC1 expression vectors. Lysates were immunoprecipitated (IP) with antibodies against either C/EBPδ or HA-tag. Total extracts or immunoprecipitates were separated on a 10% SDS–PAGE gel and transferred onto a PVDF membrane for detection by Western blot (IB) with anti-HA-tag or anti-C/EBPδ. **B**: Schematic representation of C/EBPδ N-terminal deletion mutants, with the location of the basic DNA-binding region (BR) and the leucine zipper (LZ). **C**: HEK 293T cells were transfected with empty vectors or with C/EBPδ, C/EBPδ Δ70–164 and C/EBPδ Δ36–164, along with HA-HDAC1 expression vectors. Lysates were immunoprecipitated (IP) with antibodies against C/EBPδ. Total extracts or immunoprecipitates were separated on a 10% SDS–PAGE gel and transferred onto a PVDF membrane for detection by Western blot (IB) with anti-HA or anti-C/EBPδ. The results shown are representative of three different experiments.

amino acids 70 and 97, or between amino acids 75–85 reduced C/EBPδ interaction with HDAC1 (Fig. 4B). This suggests that amino acids 70–97 are important for the interaction of the C/EBPδ N-terminal region with HDAC1.

HDAC1 Interacts With the Leucine Zipper and the Basic Region of the C/EBPδ C-Terminal Domain

The same analysis was performed to identify the C/EBPδ C-terminal region interacting with HDAC1. We generated four GST C-terminal deletion constructs (Fig. 4C). Results show that deletion of either the C/EBPδ basic region (BR, 210–268) or the leucine zipper (LZ, 165–228 and 165–242) abolished the interaction with HDAC1 (Fig. 4D). In contrast, the co-repressor mSin3A [Silverstein and Ekwall, 2005] interacted exclusively with the C-terminal domain (Fig. 4E). Thus, both the C/EBPδ basic and leucine zipper regions are necessary for HDAC1 and C/EBPδ interaction.

HDAC1 Inhibits C/EBPδ-Dependent Transactivation

We have previously shown that C/EBPs are the major regulators of the acute phase protein gene haptoglobin in intestinal epithelial cells [Gheorghiu et al., 2001]. To determine the effect of HDAC1 on C/EBPδ transcriptional activity, we co-transfected C/EBPδ and HDAC1 expression vectors, with a Hapto-luc reporter construct in HEK 293T cells. While HDAC1 expression did not affect basal transcriptional activity of the Hapto-luc reporter construct (Fig. 5A), C/EBPδ-dependent transactivation was significantly decreased with HDAC1 expression (Fig. 5B, $P < 0.05$). We then verified the effect of HDAC1 on the transcriptional activation of the GAL4 DNA-binding site-luciferase vector pFRluc, in response to a GAL4-fusion construct containing C/EBPδ N-terminal amino acids 1–102. HDAC1 expression decreased significantly pFRluc transcriptional activation by the C/EBPδ N-terminal domain (Fig. 5C, $P < 0.05$). Therefore, these results suggest that HDAC1 inhibits C/EBPδ transactivation activity, and that the C/EBPδ N-terminal domain may be involved.

HDAC1 is Involved in the Negative Regulation of the Haptoglobin Gene in Intestinal Epithelial Cells

To determine whether HDAC1 was indeed a negative regulator of haptoglobin expression, we first performed chromatin immunoprecipitation assays on IEC-6 rat intestinal epithelial cells treated with or without the pro-inflammatory cytokine IL-1β. In unstimulated cells, HDAC1 and low levels of C/EBPδ, along with some histone H3 and H4 acetylation, were detected (Fig. 6). IL-1β treatment increased C/EBPβ and C/EBPδ recruitment, and decreased HDAC1 on the haptoglobin promoter. In addition, IL-1β treatment led to increased acetylation of histones H3 and H4. This reduction in HDAC1 recruitment was not associated with a decrease in HDAC1 protein levels after IL-1β stimulation, as observed in other cell types [Vashisht Gopal et al., 2006] (data not shown). Taken together, these results suggest that IL-1β may promote the transcriptional activation of the haptoglobin

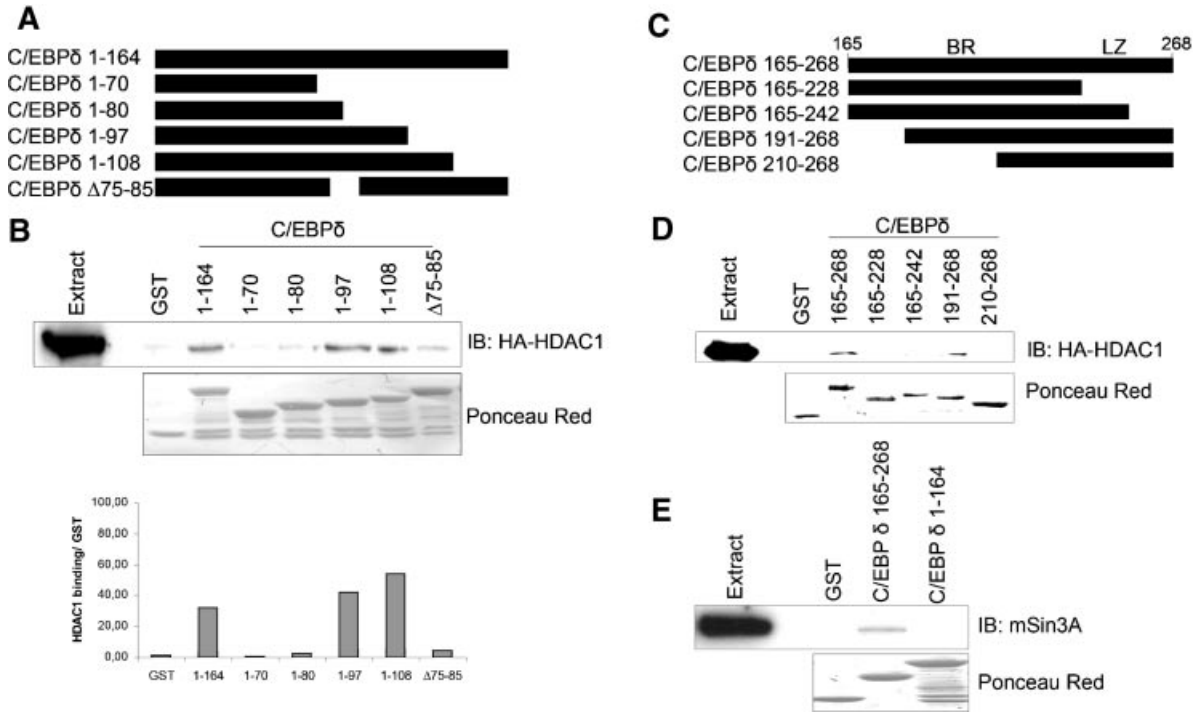

Fig. 4. HDAC1 interacts with both the C/EBPδ N-terminal and C-terminal domains. Schematic representation of the C/EBPδ N-terminal (**A**) or C-terminal (**C**) deletion mutants. The location of the basic DNA-binding region (BR) and the leucine zipper (LZ) is indicated. GST fusion proteins with C/EBPδ N-terminal (**B**) and C-terminal (**D**) domains were used in pull-down assays with 750 μg of total cell extracts from HEK 293T cells transfected with empty vector or HA-HDAC1 expression vector. Protein complexes were separated on a 10% SDS–PAGE gel and transferred onto a PVDF membrane for detection by Western blot (IB) with antibodies against the HA-tag (B,D) or mSin3A (**E**). The amount of protein loaded is shown by Ponceau Red staining. In (B), HA-HDAC1 staining of the Western blot was quantified by densitometry with the ImageQuant software and normalized to GST-C/EBPδ fusion constructs Ponceau staining. The results are representative of three different experiments.

promoter by recruiting C/EBPβ- and C/EBPδ-co-activator complexes, increasing acetylation of histones H3 and H4, and removing HDAC1-containing complexes from the promoter region.

To decrease HDAC1 expression, we generated IEC-6 cell clones expressing shRNAs against HDAC1 by retroviral infection. HDAC1 protein levels were indeed reduced by about 50% (clones #4 and clones #5) to 75% (clones #8 and clones #x) (Fig. 7A, $P < 0.001$). Haptoglobin expression was assessed by Northern blot of total RNAs isolated from unstimulated and IL-1β-stimulated cells. Results show that a decrease in HDAC1 protein levels correlated with an increase of both basal and IL-1β-induced haptoglobin mRNA levels (Fig. 7B, $P < 0.05$). These results confirm that HDAC1 may play an important role in the negative regulation of haptoglobin expression.

DISCUSSION

Our results support a role for C/EBPδ in basal transcriptional repression of the acute phase protein gene haptoglobin by recruiting HDAC-containing complexes. HDAC1, HDAC3 and HDAC4 were expressed in intestinal epithelial cells. C/EBPδ interacted with HDACs via the C-terminal domain. We have determined for the first time by deletion mutagenesis studies that both basic and leucine zipper domains were necessary for HDAC1 interaction, suggesting the importance of DNA-binding and dimerization for complex formation. While HDAC1 did not interact directly with C/EBPδ, we observed that the co-repressor mSin3A [Silverstein and Ekwall, 2005] interacts with the C-terminal domain. Thus, a co-repressor complex containing mSin3A and HDAC1 may bind the C/EBPδ C-terminal domain, as observed for C/EBPβ [Wiper-Bergeron et al., 2003].

Interestingly, the N-terminal region of C/EBPδ interacted with HDACs. Deletion of amino acids 36–164 decreased the in vivo interaction between C/EBPδ and HDAC1, as assessed by co-immunoprecipitation experiments. Removal of amino acids 70–97 reduced the in vitro binding capacity of C/EBPδ to

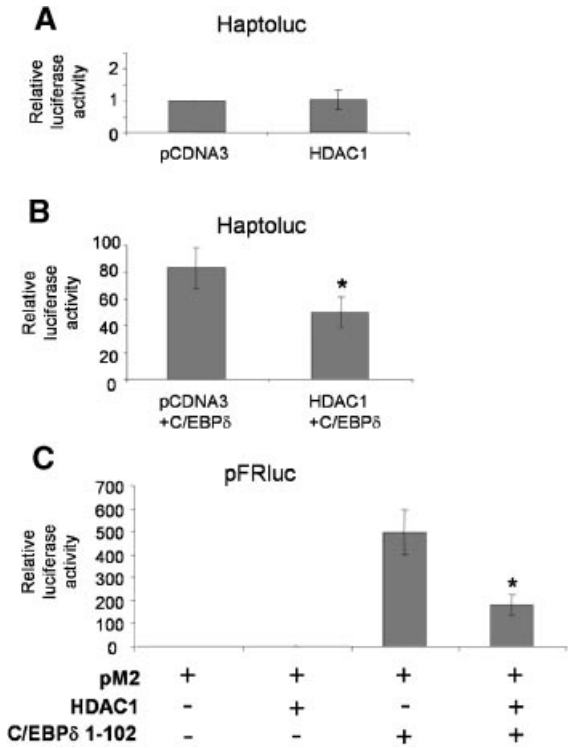

Fig. 5. HDAC1 inhibits C/EBPδ-dependent transactivation. HEK 293T cells were transiently transfected by lipofection with empty vector or HA-HDAC1 expression vector (**A**), or with HA-HDAC1 and C/EBPδ expression vectors (**B**), along with the Hapto-luc reporter construct. **C**: HEK 293T cells were transiently transfected by lipofection with the GAL4 empty vector or the GAL4 N-terminal C/EBPδ amino acid 1–102 domain expression vector, with or without empty vector or HA-HDAC1 expression vector, along with the pFR-luciferase reporter construct. Luciferase activity is expressed as fold-induction as compared to the level of induction measured in cells transfected with empty vectors. Error bars represent the standard deviations of a representative experiment of triplicate transfections, each repeated four times.

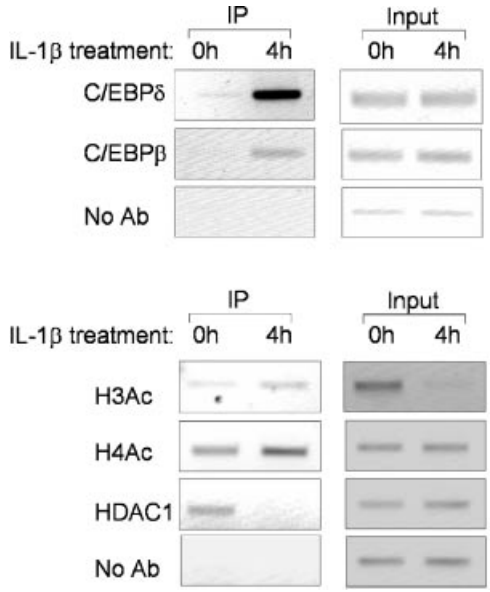

Fig. 6. Recruitment of HDAC1 to the haptoglobin promoter is inhibited by IL-1β treatment in IEC-6 cells. Chromatin immunoprecipitation assays were performed with chromatin extracts from IEC-6 cells treated with or without IL-1β (10 ng/ml) for 4 h. Chromatin was immunoprecipitated (IP) without antibody (No Ab), or with antibodies against C/EBPβ, C/EBPδ, acetylated histone H3, acetylated histone H4 and HDAC1. Samples were verified by PCR analysis with oligonucleotides amplifying the −131 to +59 region of the haptoglobin promoter. The amplified products were separated on 2% agarose gels. DNA levels were measured by PCR using 1% of input DNA prior to immunoprecipitation (Input). Experiments were performed three times independently.

HDAC1. While the co-repressor SMRT interacts with the N-terminal region of C/EBPβ [Ki et al., 2005], we were unable to detect interactions between the N-terminal domain of C/EBPδ and the co-repressors SMRT, N-Cor or mSin3A (data not shown). The nature of the co-repressor complex interacting with the N-terminal domain remains to be determined. C/EBPδ is phosphorylated by the p38 MAP kinase, and the N-terminal C/EBPδ domain interacts with the p38 MAP kinase [Svotelis et al., 2005] and the co-activator p300/CBP [Kovacs et al., 2003; Svotelis et al., 2005]. Thus, HDAC1 interaction may be modulated by post-transcriptional modifications of the N-terminal domain of C/EBPδ. For example, p38 MAP kinase phosphorylation could affect both HDAC1 and p300 recruitment.

Besides playing a role in the regulation of mammary cell proliferation [Sivko and DeWille, 2004; Sivko et al., 2004], apoptosis [Thangaraju et al., 2005], and adipocyte differentiation [Tanaka et al., 1997], C/EBPδ is involved in the regulation of acute phase protein gene expression during inflammation in various cell types [Ramji and Foka, 2002]. Indeed, we have previously shown that C/EBPs, including C/EBPδ, are the most important regulators of haptoglobin expression in intestinal epithelial cells [Pelletier et al., 1998; Yu et al., 1999; Desilets et al., 2000; Gheorghiu et al., 2001]. The interactions between HDAC1 and C/EBPδ may be functionally relevant for the regulation of acute phase protein gene expression. First, HDAC1 decreased C/EBPδ-dependent induction of the haptoglobin promoter, as assessed by transient transfection and luciferase assays. Second, HDAC1 decreased the transactivation mediated by the C/EBPδ N-terminal amino acid 1–102 domain. Third, chromatin immunoprecipitation experiments indicate that

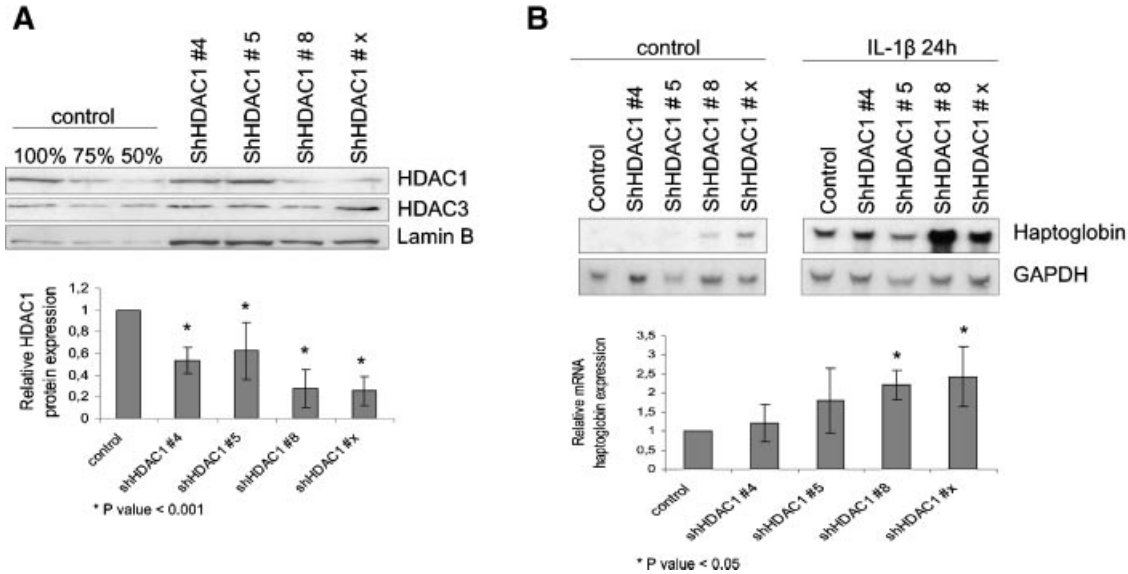

Fig. 7. HDAC1 silencing increases both basal and IL-1β-dependent induction of haptoglobin gene expression in IEC-6 cells. IEC-6 cells were infected with an empty vector or with the HDAC1 silencing expression vector (shHDAC1). **A**: IEC-6 cell control (total, 100%, or decreased amounts, 75% and 50%), or cell clones (#4, #5, #8, and #x) nuclear protein extracts were separated by SDS–PAGE and transferred to PVDF membranes for Western blot analysis of HDAC1, HDAC3, and laminB. HDAC1 protein levels were quantified by densitometry with the ImageQuant software and normalized to laminB ($P < 0.001$). **B**: Total RNAs were isolated from cell clones (#4, #5, #8, and #x) treated without (control) or with IL-1β (10 ng/ml) for 24 h. Equal amounts of RNAs were analyzed by Northern blot with ^{32}P-labelled haptoglobin and GAPDH probes. Haptoglobin mRNA levels from 24 h IL-1β treated cells were quantified by densitometry with the ImageQuant software and normalized to GAPDH ($P < 0.05$).

HDAC1 occupied the haptoglobin promoter C/EBP-containing region. IL-1β treatment resulted in a decrease in HDAC1 recruitment, an increase in both C/EBPβ and C/EBPδ DNA-binding, as well as histone acetylation as well. Finally, shRNA mediated inhibition of HDAC1 expression resulted in an increase of both basal and IL-1β-dependent induction of haptoglobin expression. The haptoglobin promoter may be occupied by C/EBP-containing co-repressor complexes, insuring transcriptional repression. These C/EBP proteins recruit co-repressor complexes from both the N-terminal and C-terminal domains. IL-1β treatment may enable the haptoglobin promoter to respond by acting on kinases, such as the p38 MAP kinase that alters the ability of C/EBPs to interact with co-repressor or co-activator complexes. It has been suggested that C/EBP isoforms may be involved at later stages of acute phase protein gene expression [Poli, 1998]. We hypothesize that a first and rapid step of cytokine induction leads to phosphorylation of the N-terminal domain of chromatin-associated C/EBPs, release of the co-repressor complex and recruitment of p300/CBP, without affecting the interaction of co-repressor complexes to the C-terminal domain. This would allow a low level of transcriptional activation. Subsequently, newly synthesized C/EBP isoforms, devoid of co-repressor complexes at both the N-terminal and the C-terminal region, would then be recruited to insure full transactivation potential.

Recent data show that C/EBPβ recruitment of HDAC1-containing repressor complexes inhibits the expression of PPARβ in murine keratinocytes [Di-Poi et al., 2005]. Furthermore, C/EBPβ, in association with HDAC1, is bound to the repressed C/EBPα gene promoter in mouse fibroblasts [Zuo et al., 2006]. Thus, we have shown for the first time that C/EBPs, including C/EBPδ, may actively repress acute phase protein expression by recruiting co-repressor complexes including HDAC1.

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