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Journal of Cellular Biochemistry 106:453–463 (2009)

Journal of Cellular Biochemistry

HDAC4 Contributes to IL-1-Induced mPGES-1 Expression in Human Synovial Fibroblasts Through Up-Regulation of Egr-1 Transcriptional Activity

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ABSTRACT

Microsomal prostaglandin E synthase-1 (mPGES-1) catalyzes the terminal step in the biosynthesis of PGE_2, which contributes to many physiopathological processes. We show here that inhibitors of histone deacetylase (HDAC) activity, trichostatin A (TSA), butyric acid (BA), and valproic acid (BA) prevented IL-1-induced mPGES-1 protein expression in human synovial fibroblasts. TSA also inhibited IL-1-induced mPGES-1 mRNA expression and promoter activation. Overexpression of HDAC4, but not of HDAC1, 2, 3, 5, or 6 enhanced, whereas HDAC4 silencing with small interfering RNA (siRNA) reduced, IL-1-induced mPGES-1 promoter activation, implying that HDAC4 contributes to mPGES-1 gene expression. Consistently, IL-1-induced mPGES-1 protein expression was prevented by siRNA for HDAC4. We also demonstrate that IL-1-induced HDAC4 recruitment to the mPGES-1 promoter. This recruitment was not accompanied by deacetylation of histones H3 and H4, suggesting that HDAC4 contributes to mPGES-1 induction independently of local deacetylation of histones H3 and H4. We then investigated whether HDAC4 regulates mPGES-1 expression by modulating the activity of Egr-1, a key transcription factor in IL-1-induced mPGES-1 expression. We found that HDAC4 overexpression enhances, whereas HDAC4 knockdown by siRNA reduces Egr-1-mediated activation of the mPGES-1 promoter. Together these data indicate that HDAC4 contributes to transcriptional induction of mPGES-1 by IL-1 through a mechanism involving up-regulation of Egr-1 transcriptional activity. J. Cell. Biochem. 106: 453–463, 2009. © 2008 Wiley-Liss, Inc.

KEY WORDS: MICROSOMAL PROSTAGLANDIN E SYNTHASE-1 (mPGES-1); INTERLEUKIN-1 (IL-1); EARLY GROWTH RESPONSE FACTOR-1 (EGR-1); HISTONE DEACETYLASE (HDAC)

The lipid mediator prostaglandin (PG) E_2 is produced by various cells and has been implicated in a wide range of physiological and pathological processes including cell growth, vascular homeostasis, inflammation, pain, arthritis and cancer [McCoy et al., 2002; Wang and Dubois, 2006]. The biosynthesis of PGE_2 from arachidonic acid requires two enzymatic activities. Cyclooxygenase (COX) enzymes convert arachidonic acid (AA) into PGH_2 which is in turn isomerized to PGE_2 by PGE synthase (PGES) enzymes [Murakami et al., 2000; Tanioka et al., 2000; Smith and Langenbach, 2001]. Two isoforms of the COX enzyme, COX-1 and COX-2, have been identified. COX-1 is constitutively expressed in most tissues, whereas COX-2 is inducible by various stimuli including pro-inflammatory signals [Smith and Langenbach, 2001]. Several PGES have been cloned and characterized including microsomal PGES-1 (mPGES-1), which was originally named MGST-L-1 (membrane-bound gluthatione *S*-transferase-1-like-1), mPGES-2, and cytosolic PGES (cPGES, or the heat shock protein-associated protein p23). cPGES is constitutively expressed and is functionally coupled with COX-1 to promote immediate PGE_2 production [Tanioka et al., 2000]. In contrast, mPGES-1 is markedly up-regulated by inflammatory or mitogenic stimuli and functionally coupled with COX-2 for delayed PGE_2 production [Murakami et al., 2000]. mPGES-2 is constitutively expressed in various cells and tissues and can be coupled with both COX-1 and COX-2 [Murakami et al., 2003].

The expression of mPGES-1 is up-regulated in several cell types after treatment with pro-inflammatory stimuli such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) and is down-regulated by anti-inflammatory glucocorticoids [Murakami et al., 2000; Stichtenoth et al., 2001]. The transcriptional induction of mPGES-1

Grant sponsor: Canadian Institutes of Health Research (CIHR); Grant number: MOP-84282; Grant sponsor: Fonds de la Recherche du Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CHUM).

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Received 26 September 2008; Accepted 20 November 2008 • DOI 10.1002/jcb.22027 • 2008 Wiley-Liss, Inc.

Published online 29 December 2008 in Wiley InterScience (www.interscience.wiley.com).

is primarily controlled by Egr-1 through two Egr-1 binding motifs located in the proximal region of the mPGES-1 promoter [Naraba et al., 2002; Cheng et al., 2004; Subbaramaiah et al., 2004]. The importance of mPGES-1 in induced PGE_2 production was evidenced in studies using mPGES-1 deficient mice. In these mice, induced PGE_2 production was almost completely abrogated [Uematsu et al., 2002; Engblom et al., 2003; Trebino et al., 2003; Boulet et al., 2004]. Importantly, mPGES-1 deficiency was protective in animal models of conditions in which PGE_2 has been implicated such as inflammation, pain, arthritis [Trebino et al., 2003; Kamei et al., 2004], pyresis [Engblom et al., 2003], bone loss [Inada et al., 2006], stroke [Ikeda-Matsuo et al., 2006], and atherogenesis [Wang et al., 2006], which implies that mPGES-1 is a potential target for therapeutic intervention.

The reversible acetylation of histone proteins plays a critical role in the control of gene transcription [Jenuwein and Allis, 2001; Urnov, 2003]. In general, acetylation which is catalyzed by histone acetyltransferases (HATs) is associated with transcription activation through relaxed chromatin structure. In contrast, histone deacetylation by deacetylases (HDACs) is associated with transcription repression via chromatin condensation [Jenuwein and Allis, 2001; Urnov, 2003]. However, emerging evidence indicates that gene regulation by acetylation/deacetylation is more dynamic and complex, and that HATs can act as repressors and HDACs as activators. For instance, HDAC inhibitors were reported to prevent the inducible expression of several genes including IL-1, IL-2, IL-8, IL-12, TNF-α, Id-1, and a number of IFNα and γ responsive genes [Huang et al., 1997; Koyama et al., 2000; Leoni et al., 2002; Nusinzon and Horvath, 2003; Xu et al., 2003; Klampfer et al., 2004; Chang and Pikaard, 2005; Leoni et al., 2005]. Moreover, global analysis of gene expression showed that inhibition of HDAC activity can result in the induction of gene expression [Mariadason et al., 2000; Chambers et al., 2003; Chang and Pikaard, 2005; Reid et al., 2005]. In addition, genome-wide genetic studies with yeast clearly demonstrated that HDACs also participate in transcriptional activation [Vidal and Gaber, 1991; Nawaz et al., 1994; Bernstein et al., 2000], and gene profiling experiments have identified many Drosophila genes that are activated by overexpression of individual HDACs [Cho et al., 2005]. HATs and HDACs were also reported to impact gene expression by modulating the activity of a variety of signaling proteins and transcription factors such as C/EBPβ [Xu et al., 2003], HIF-α [Kato et al., 2004; Qian et al., 2006], and GR [Qiu et al., 2006] STAT1 [Klampfer et al., 2004], STAT2 [Nusinzon and Horvath, 2003], and STAT5 [Xu et al., 2003], adding another layer of complexity to the process.

In this report we have shown that HDAC inhibitors prevent IL-1-induced mPGES-1 expression in human synovial fibroblasts (HSF). HDAC4 overexpression enhances, whereas HDAC4 knockdown abrogates, IL-1-mediated activation of the mPGES-1 promoter, suggesting that HDAC4 participates in mPGES-1 transcription. Accordingly, HDAC4 silencing reduces IL-1-induced mPGES-1 protein expression. Moreover, HDAC4 is recruited to the mPGES-1 promoter in the presence of IL-1. Surprisingly, this recruitment was not associated with local histone deacetylation of histones H3 and H4. We also show that HDAC4 overexpression enhances, whereas HDAC4 silencing reduces, Egr-1-mediated activation of the mPGES-1 promoter. These findings indicate that HDAC4 contributes to mPGES-1 expression via a mechanism involving enhancement of Egr-1 transcriptional activity.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

Human recombinant (rh) IL-1β was obtained from Genzyme (Cambridge, MA). TNF-α and IL-17 were purchased from R&D Systems (Minneapolis, MN). Trichostatin A (TSA), butyric acid (BA), valproic acid (VA), aprotinin, leupeptin, pepstatin, and phenylmethylsulphonyl fluoride (PMSF) were from Sigma-Aldrich Canada (Oakville, ON, Canada). Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin, fetal calf serum (FCS) and Trizol reagent were supplied by Invitrogen (Burlington, ON, Canada). Plasmid DNA was prepared using a kit from Qiagen (Mississauga, ON, Canada). FuGene 6 transfection reagent was from Roche Applied Science (Laval, QC, Canada). The luciferase reporter assay system was from Promega (Madison, WI). All other chemicals were purchased from either Sigma-Aldrich or Bio-Rad (Mississauga, ON, Canada). The following antibodies were used: anti-mPGES-1 (catalog no P9053-28; US Biological, Swampscott, MA); anti-cPGES (catalog no 160150; Cayman Chemical, Ann Arbor, MI); anti-Egr-1 (sc-110; Santa Cruz, Santa Cruz, CA); anti-β-actin (sc-7210; Santa Cruz); anti-Flag (catalog no F3165; Sigma); anti-HDAC1 (catalog no 06-720; Upstate/Millipore, Lake Placid, NY); anti-HDAC1 (catalog no 06-720; Upstate/Millipore); anti-HDAC2 (catalog no 05-814; Upstate/Millipore); anti-HDAC3 (catalog no 07-522; Upstate/Millipore); anti-HDAC4 (catalog no 2072; Cell Signaling); anti-HDAC5 (catalog no 2082; Cell Signaling); anti-HDAC6 (catalog no 2162; Cell Signaling). Anti-acetyl histone H3 antibodies (catalog no 06-599; Upstate/Millipore) were generated against the peptide ARTKQTAR[K*]STGG[K*]APRKQLC, and anti-acetyl histone H4 antibodies (catalog no 06-866; Upstate/Millipore) were generated against the peptide AGG[K*]GG[K*]GMG[K*]VGA[K*]RHSC where [K*] denotes acetylated lysine residues.

SPECIMEN SELECTION AND CELL CULTURE

HSF were isolated from synovial membranes obtained from osteoarthritic (OA) patients undergoing total knee replacement. All OA patients were evaluated by a certified rheumatologist and diagnosed based on criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA. Briefly, synovial fibroblasts were released by sequential enzymatic digestion with 1 mg/ml pronase (Roche Applied Science) for 1 h followed by a 6 h incubation with 2 mg/ml collagenase (Type IA, Sigma) at 37°C in DMEM supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were incubated for 1 h at 37°C in tissue culture flasks (Primaria 3824, Falcon) allowing the adherence of nonfibroblastic cells possibly present in the cell preparation. The cells were seeded in tissue culture flasks and cultured until confluence in DMEM supplemented with 10% FCS and antibiotics at 37°C in a humidified atmosphere of 5% CO_2/95% air. Only cells between passages 3 and 7 were used.

WESTERN BLOT ANALYSIS

Cells were lysed in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 μg/ml each of aprotinin, leupeptin and pepstatin, 1% NP-40, 1 mM sodium orthovanadate (Na_3VO_4), and 1 mM NaF). Lysates were sonicated on ice and centrifuged at 12,000 rpm for 15 min. The protein concentration of the supernatant was determined using the bicinchoninic acid method (Pierce). Twenty micrograms of cell lysate was subjected to SDS–polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose membrane (Bio-Rad). After blocking in 20 mM Tris–HCl, pH 7.5, containing 150 mM NaCl, 0.1% Tween-20, and 5% (w/v) nonfat dry milk, blots were incubated overnight at 4°C with primary antibodies and washed with wash buffer (TBS pH 7.5, with 0.1% Tween-20). The blots were then incubated with horseradish peroxidase-conjugated secondary antibody (Pierce), washed again, incubated with SuperSignal Ultra Chemiluminescent reagent (Pierce) and finally exposed to Kodak X-Omat film (Eastman Kodak Ltd).

PGE_2 ASSAYS

At the end of the incubation period, the culture medium was collected and stored at −80°C. Levels of PGE_2 were determined using a PGE_2 enzyme immunoassay kit from Cayman Chemical. The detection limit and sensitivity was 9 pg/ml. All assays were performed in duplicate.

RNA EXTRACTION AND CDNA SYNTHESIS

Total RNA was isolated from HSF using the TRIzol reagent (Invitrogen), and dissolved in 20 μl of diethylpyrocarbonate (DEPC)-treated-H_2O. One microgram of total RNA was treated with RNase-free DNase and reverse-transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Fermentas) as detailed in the manufacturer's guidelines. One-fifth of the reverse transcriptase reaction was analyzed by real-time PCR as described below. The following primers were used: mPGES-1: sense 5′-GAAGAAGGCCTTTGCCAAC-3′ and anti-sense 5′-GGAAGACCAG-GAAGTGCATC-3′; cPGES: sense 5′-GCAAAGTGGTACGATC-GAAGG-3′ and anti-sense 5′-TGTCCGTTCTTTTATGCTTGG-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): sense 5′-CAGAACATCATCCCTGCCTCT-3′ and anti-sense 5′-GCTTGA-CAAAGTGGTCGTTGAG-3′.

REAL-TIME PCR

Real-time PCR analysis was performed in a total volume of 50 μl containing cDNA template, 200 nM of sense and anti-sense primers and 25 μl of SYBR® Green master mix (Qiagen). Incorporation of SYBR® Green dye into PCR products was monitored in real time using a Gene Amp 5700 sequence detector (Applied Biosystems) allowing determination of the threshold cycle (C_T) at which exponential amplification of PCR products begins. After incubation at 95°C for 10 min to activate the AmpliTaq Gold enzyme, the mixtures were subjected to 40 amplification cycles (15 s at 95°C for denaturation and 1 min for annealing and extension at 60°C). After PCR, dissociation curves were generated with one peak indicating the specificity of the amplification. A threshold cycle (C_T value) was obtained from each amplification curve using the software provided by the manufacturer (Applied Biosystems). Data were expressed as fold changes relative to control conditions (unstimulated cells) using the $\Delta\Delta C_T$ method as detailed in the manufacturer's guidelines (Applied Biosystems). A ΔC_T value was first calculated by subtracting the C_T value for the housekeeping gene GAPDH from the C_T value for each sample. A $\Delta\Delta C_T$ value was then calculated by subtracting the ΔC_T value of the control from the ΔC_T value of each treatment. Fold changes compared with the control (unstimulated cells) were then determined by raising 2 to the $\Delta\Delta C_T$ power. Each PCR reaction generated only the expected specific amplicon as shown by the melting-temperature profiles of the final product and by gel electrophoresis of test PCR reactions. Each PCR was performed in triplicate on two separate occasions from at least three independent experiments.

TRANSIENT TRANSFECTION

The mPGES-1 promoter construct (−538/−28) was provided by Dr. Terry Smith (University of California, Los Angeles). Flag-tagged expression vectors for HDAC1, 5 and 6 were donated by Dr. Stuart Schreiber (Howard Hughes Medical Institute, Cambridge) and HDAC2 and 3 by Dr. Edward Seto (H. Lee Moffitt Cancer Center, Tampa). The flag-tagged expression vectors for wild-type HDAC4 and a mutant HDAC4 that lacks deacetylase activity were a kind gift from Dr. Xiang-Jiao Yang (McGill University, Montreal, Quebec, Canada). Egr-1 expression vector was provided by Dr. Yuqing Chen (Morehouse School of Medicine, Atlanta). A β-galactosidase reporter vector under the control of SV40 promoter (pSV40-β-gal) was from Promega. siRNA for HDAC1, 2, 3, 4, 5, 6, or scrambled control were obtained from Dharmacon.

Transient transfection experiments were performed using FuGene 6 transfection reagent (Roche Applied Science) according to the manufacturer's recommended protocol. Briefly, HSF were seeded 24 h prior to transfection at a density of 3×10^5 cells/well in 12-well plates and transiently transfected with 1 μg of the mPGES-1 promoter construct and 0.5 μg of the internal control pSV40-β-galactosidase. Six hours later, the medium was replaced with DMEM containing 1% FCS. At 1 day after transfection, the cells were treated or not with IL-1 in the absence or presence of TSA for 18 h. In the overexpression experiments, the amount of transfected DNA was kept constant by using the corresponding empty vector. In some assays, HSF were co-transfected with 200 nM of HDAC4 siRNA or scrambled control. At the end of the indicated treatment, the cells were washed twice in ice-cold PBS and extracts were prepared for luciferase reporter assay or Western blot analysis. Luciferase activity was normalized for transfection efficiency using the corresponding β-galactosidase activity [Cheng et al., 2004].

CHROMATIN IMMUNOPRECIPITATION (CHIP) ASSAY

The ChIP experiments were performed according to the ChIP protocol provided by Upstate/Millipore. Following treatment, the cells were cross-linked with 1% formaldehyde for 10 min at room temperature. The fixed cells were washed twice with ice-cold phosphate-buffered saline containing protease inhibitors and then lysed for 10 min at 1×10^6 cells/200 μl of SDS lysis buffer (50 mM Tris–Cl (pH 8.0), 0.5% SDS, 100 mM NaCl, 5 mM EDTA) plus protease inhibitors. The chromatin samples were sonicated to reduce DNA

length to 200–500 bp. Twenty microliters of the supernatant were saved as the input DNA and the remainder was diluted 1:10 in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA and 16.7 mM Tris–Cl) containing protease inhibitors. The chromatin samples were precleared with a salmon sperm DNA/protein A-agarose 50% gel slurry (Upstate Biotechnology, Inc.) for 3 h. The samples were then immunoprecipitated overnight at 4°C with antibodies specific for either HDAC4, acetylated H3, or acetylated H4 (Upstate Biotechnology, Inc.). As negative controls, cross-linked chromatin was incubated overnight with control Ig or in the absence of antibody. Immune complexes were recovered by addition of salmon sperm DNA/protein A-agarose slurry for 2 h at 4°C. The immune complexes were sequentially washed three times each (5 min on a rotating platform), with low salt, high salt, lithium chloride, and Tris/EDTA buffers, and eluted twice with 250 μl 1% SDS, 0.1 M $NaHCO_3$ for 15 min. The eluted material and the DNA input samples were heated for 4 h at 65°C to reverse cross-linking. The samples were treated with 40 μg/ml DNase-free proteinase K for 1 h at 45°C, extracted with phenol–chloroform–isoamyl alcohol and chloroform, and ethanol precipitated in the presence of 20 μg of glycogen. Pellets were suspended in 25–30 μl H_2O and subjected to PCR analysis. The primer sequences used were: mPGES-1 promoter sense 5′-CCCGGAGACTCTCTGCTTC-3′ and anti-sense 5′-TCAACTGTGGGTGTGATCAGC-3′; COX-2 sense 5′-AGAAAAG-ACATCTGGCGGAAAC-3′ and anti-sense 5-TATGACAATTGG-TCGCTAACCG-3′.

STATISTICAL ANALYSIS

All results were calculated as the mean ± SEM of independent experiments. Statistics were analyzed using Student's 2-tailed *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

HDAC INHIBITION PREVENTS IL-1-INDUCED mPGES-1 EXPRESSION

To examine the role of HDACs in IL-1-induced mPGES-1 expression in HSF, cells were stimulated with IL-1 in the absence or presence of increasing concentrations of TSA and the expression of mPGES-1 was evaluated by Western blotting. As shown in Figure 1A, TSA inhibited IL-1-induced mPGES-1 expression in a dose-dependent manner. In contrast, the expression of cPGES was not affected by these treatments. To rule out nonspecific effects of TSA, we tested two additional HDAC inhibitors, BA and VA. Treatment of HSF with BA and VA also prevented IL-1-induced mPGES-1 expression in a dose-dependent manner (Fig. 1B,C). To determine whether the inhibitory effect of HDAC inhibitors was specific to IL-1, we assessed their effects on TNF-α and IL-17-induced mPGES-1 expression. Interestingly, TSA prevented the induction of mPGES-1 expression by both TNF-α and IL-17 (Fig. 1D), indicating that its effect was not restricted to IL-1. Taken together, these results suggest that HDAC activity is required for induced mPGES-1 expression.

HDAC INHIBITION PREVENTS IL-1-INDUCED mPGES-1 EXPRESSION AT THE TRANSCRIPTIONAL LEVEL

To understand the molecular mechanisms by which HDAC inhibition prevented IL-1-induced mPGES-1 expression we analyzed the effect of TSA on IL-1-induced mPGES-1 mRNA expression by real-time PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. Treatment with IL-1 increased mPGES-1 mRNA expression ~7-fold (Fig. 2A), an effect that was dose-dependently suppressed by TSA (Fig. 2A). These data suggest that TSA suppressed IL-1-induced mPGES-1 expression through a transcriptional mechanism. To verify this, we performed transient transfection experiments. HSF were transfected with the human

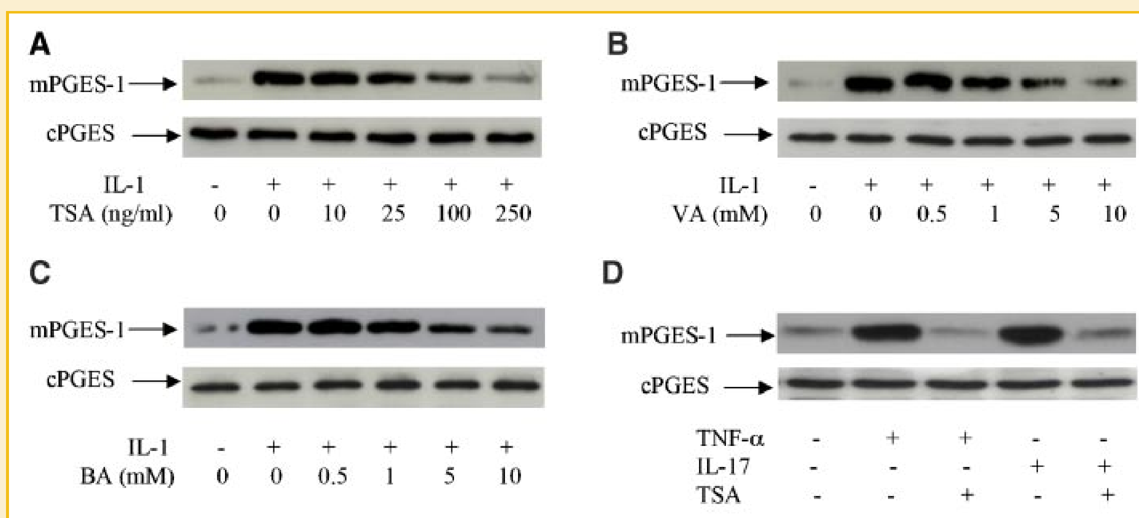

Fig. 1. HDAC inhibition suppressed IL-1-induced mPGES-1 expression. HSF were stimulated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of TSA (A), VA (B), or BA (C) for 20 h. D: HSF were treated with 1 ng/ml TNF-α or 100 ng/ml IL-17 in the absence or presence of TSA (250 ng/ml) for 20 h. Cell lysates were prepared and analyzed for mPGES-1 protein expression by Western blotting. In the lower panels the blots were stripped and reprobed with specific anti-cPGES antibody. The blots are representative of similar results obtained from four independent experiments.

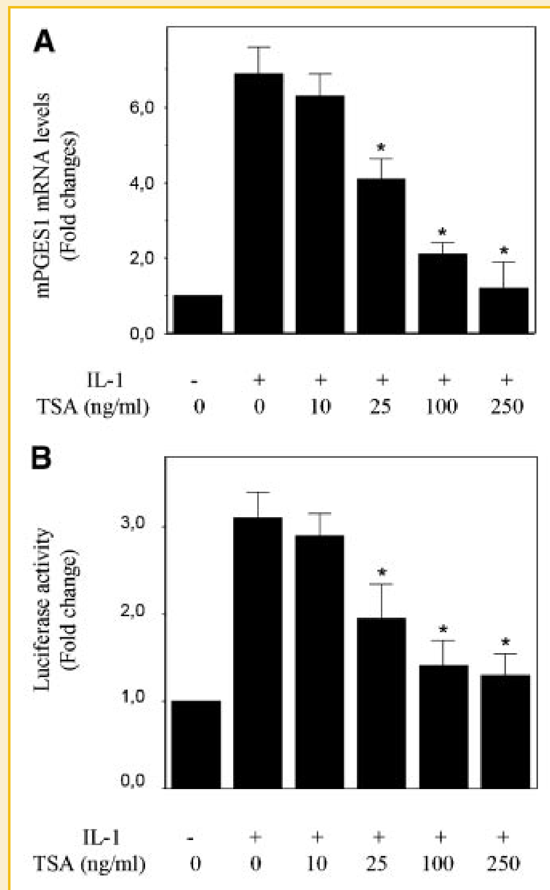

Fig. 2. TSA inhibited IL-1-induced mPGES-1 expression at the transcriptional level. A: HSF were stimulated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of TSA for 8 h. Total RNA was isolated, reverse transcribed into cDNA, and mPGES-1 levels were quantified using real-time PCR. GAPDH gene expression was used for normalization. All experiments were performed in triplicate, and negative controls without template RNA were included in each experiment. B: HSF were co-transfected with the human mPGES-1 promoter (1 μg/well) and the internal control pSV40-β-galactosidase (0.5 μg/well) using FuGene 6 transfection reagent. The next day, transfected cells were treated with IL-1 (100 pg/ml) in the absence or presence of increasing concentrations of TSA for 18 h. Luciferase activity values were determined and normalized to β-galactosidase activity. Results are expressed as fold changes, considering 1 as the value of untreated cells and represent the mean ± SEM of four independent experiments. $^{*}P < 0.05$ compared with cells treated with IL-1 alone (control).

mPGES-1 promoter-luciferase reporter gene and stimulated with IL-1 in the absence or presence of TSA. The results in Figure 2B show that IL-1 activates the mPGES-1 promoter and this activation was dose-dependently reduced by TSA. This suggests that the suppressive effect of TSA on IL-1-induced mPGES-1 takes place, at least in part, at the transcriptional level.

HDAC4 CONTRIBUTES TO IL-1-INDUCED mPGES-1 EXPRESSION

To define which HDAC is important in IL-1-induced mPGES-1 transcription, we examined the effect of elevated expression of individual HDAC on IL-1-mediated activation of the mPGES-1 promoter. HSF were co-transfected with the mPGES-1 promoter and increasing concentrations of expression vectors that encode HDAC1, 2, 3, 4, 5 or 6, and 24 h post-transfection the cells were stimulated with IL-1 for an additional 18 h. Overexpression of HDAC4 dose-dependently enhanced IL-1-mediated activation of the mPGES-1 promoter (Fig. 3A). In contrast, overexpression of HDAC1, 2, 3, 5, and 6 had no significant effect on mPGES-1 promoter activation by IL-1 (Fig. 3A). These data demonstrate that HDAC4 plays an essential role in IL-1-induced mPGES-1 promoter activation.

Next, we evaluated the impact of endogenous HDAC4 silencing by small interfering RNA (siRNA) on IL-1-mediated activation of the mPGES-1 promoter. HSF were co-transfected with the mPGES-1 promoter and scrambled control siRNA or siRNA for HDAC1, 2, 3, 4, 5, or 6 and after 24 h of transfection, the cells were stimulated with IL-1 for 18 h. As shown in Figure 3B, transfection with HDAC4 siRNA reduced IL-1-induced mPGES-1 promoter activity by more than 75%, whereas transfection with control scrambled siRNA or

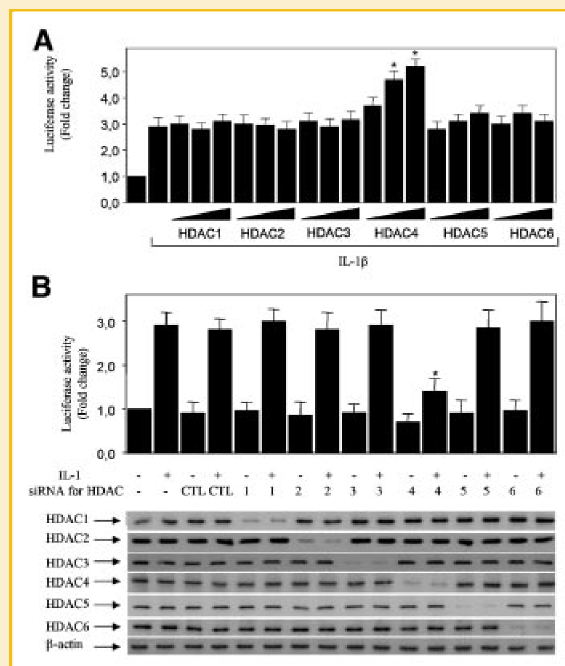

Fig. 3. HDAC4 contributes to IL-1-induced mPGES-1 promoter activation. A: HSF were co-transfected with the human mPGES-1 promoter (1 μg/well), the internal control pSV40-β-gal (0.5 μg/well) and increasing concentrations (0.01, 0.1, and 1 μg/well) of expression vectors for HDAC1, 2, 3, 4, 5, or 6. The total amount of transfected DNA was kept constant by the addition of the empty vector. The next day, transfected cells were stimulated with IL-1 (100 pg/ml) for 18 h. Cell extracts were prepared and analyzed for luciferase activity. Results are expressed as fold changes, considering 1 as the value of untreated cells and represent the mean ± SEM of four independent experiments. $^{*}P < 0.05$ compared with cells treated with IL-1 alone (control). B: HSF were co-transfected with the human mPGES-1 promoter (1 μg/ml), the internal control pSV40-β-gal (0.5 μg/well) together with 200 nM of control scrambled siRNA or siRNA for HDAC1, 2, 3, 4, 5, or 6. The next day, transfected cells were treated with IL-1 (100 pg/ml) for 18 h. Cell extracts were prepared and analyzed for luciferase activity. Specific knockdown of each HDAC isoform was confirmed by Western blotting using antibodies specific for HDAC1, 2, 3, 4, 5, or 6 (lower panel).

siRNA for HDAC1, 2, 3, 5, or 6 had no effect. Immunoblotting demonstrated that transfection with each siRNA resulted in a marked reduction in protein levels of the corresponding HDAC, but did not affect the levels of other HDACs (Fig. 3B, lower panel), indicating the specifcity and selectivity of the siRNA used. Together, these results indicate that HDAC4 is needed for IL-1-induced mPGES-1 transcription.

To determine whether the effect of HDAC4 requires its deacetylase activity, we tested a mutant version of HDAC4 that lacks deacetylase activity (HDAC4-H803L). Substitution of a histidine for a lysine residue at position 802 resulted in complete loss of deacetylase activity [Wang et al., 1999]. As shown in Figure 4A, HDAC4 increased IL-1-induced mPGES-1 promoter activity in a dose-dependent manner, whereas the deacetylase-deficient HDAC4 mutant had no significant effect on mPGES-1 promoter activity, suggesting that deacetylase activity is needed for HDAC4 to enhance IL-1-induced mPGES-1 promoter activation. Transfected HDAC4 and HDAC4-H803L were expressed at comparable levels as revealed by Western blotting (Fig. 4A, lower panel). Treatment with TSA dose-dependently attenuated the enhancing effect of HDAC4 on IL-1-mediated activation of the mPGES-1 promoter (Fig. 4B), confirming the requirement of deacetylase activity for the effect of HDAC4 on IL-1-induced mPGES-1 promoter activation.

To further characterize the role of HDAC4 in IL-1-induced mPGES-1 protein expression and activity, we analyzed the effect of HDAC4 siRNA on IL-1-induced mPGES-1 protein expression and PGE_2 production. HSF were transfected with scrambled control siRNA or siRNA for HDAC1, 2, 3, 4, 5, or 6 and then treated or not with IL-1 for 20 h. As shown in Figure 5, siRNA for HDAC4 reduced IL-1-induced mPGES-1 expression and PGE2 production by as much as 80–85%. In contrast, control siRNA or siRNA for HDAC1, 2, 3, 5, or 6 had no effect. Each siRNA reduced the level of the corresponding HDAC without affecting the levels of other HDACs (Fig. 5). Together, these data clearly show that HDAC4 is required for IL-1-induced mPGES-1 protein expression.

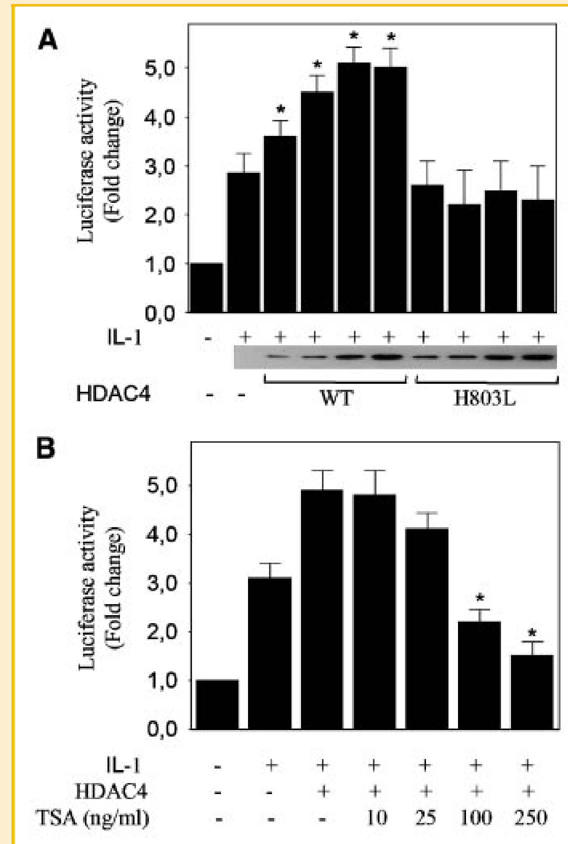

Fig. 4. A: HDAC4-mediated enhancement of IL-1-induced mPGES-1 promoter activation requires deacetylase activity. HSF were co-transfected with the human mPGES-1 promoter (1 μg/well), the internal control pSV40-β-gal (0.5 μg/well) and increasing concentrations (0.01, 0.1, 1, and 2 μg/well) of an expression vector for HDAC4 (HDAC4-WT) or HDAC mutant that lack deacetylase activity (HDAC4-H803L). The total amount of transfected DNA was kept constant by the addition of the empty vector. The next day, transfected cells were stimulated with IL-1 (100 pg/ml) for 18 h. Results are expressed as fold changes, considering 1 as the value of untreated cells and represent the mean ± SEM of four independent experiments. $^*P < 0.05$ compared with cells treated with IL-1 alone (control). The expression levels of HDAC4 and HDAC4-H803L were analyzed by Western blotting using an anti-Flag antibody (lower panel) B: HSF were co-transfected with 1 μg of the human mPGES-1 promoter, 0.5 μg of pSV40-β-gal, and 1 μg of an expression vector for HDAC4. The total amount of transfected DNA was kept constant by the addition of the empty vector. The next day, transfected cells were stimulated with IL-1 (100 pg/ml) in the absence or presence of increasing concentrations of TSA for 18 h. Results are expressed as fold changes, considering 1 as the value of untreated cells and represent the mean ± SEM of four independent experiments. $^*P < 0.05$ compared with cells transfected with Egr-1 and treated with IL-1 alone (control).

IL-1 INDUCES HDAC4 RECRUITMENT TO THE mPGES-1 PROMOTER WITHOUT CONCOMITANT DEACETYLATION OF HISTONES H3 AND H4

Next we performed ChIP assays to determine whether IL-1 promotes HDAC4 recruitment to the mPGES-1 promoter. As shown in Figure 6A, treatment with IL-1 induced the binding of HDAC4 to the proximal region of the mPGES-1 promoter. No PCR products were obtained with control rabbit IgG (data not shown). Thus, IL-1-induced HDAC4 recruitment to the mPGES-1 promoter in vivo. Given that histones H3 and H4 are important targets of HDAC, we assessed whether IL-1-induced HDAC4 recruitment to the mPGES-1 promoter correlates with local histone deacetylation. The status of histone acetylation at the mPGES promoter was analyzed using ChIP assays with antibodies against both acetylated histones H3 and H4. Acetylated histones H3 and H4 were readily detected at the mPGES-1 promoter. Unexpectedly, and despite the fact that IL-1-induced HDAC4 recruitment to the mPGES promoter, treatment with IL-1 had no effect on the level of acetylated histones H3 and H4 at the mPGES-1 promoter (Fig. 6B). As previously reported [Nie et al., 2003; Miao et al., 2004; Farrajota et al., 2005] we found that IL-1 enhances the levels of histones H3 and H4 acetylation at the COX-2 promoter (Fig. 6C), confirming the suitability of each antibody for ChIP assays. Together, these data indicate that the IL-1-induced mPGES-1 expression does not involve deacetylation of histones H3 and H4 at the analyzed region of this gene promoter and suggest that deacetylation of histones H3 and H4 is not the major mechanism by which HDAC4 contributes to mPGES-1 expression.

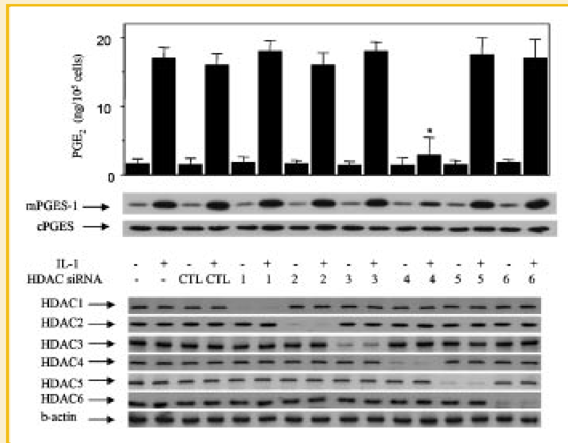

Fig. 5. HDAC4 is required for IL-1-induced mPGES-1 expression. HSF were transfected with 200 nM of control scrambled siRNA or siRNA for HDAC1, 2, 3, 4, 5, or 6. At 24 h post-transfection, cells were washed and left untreated or treated with IL-1 for 20 h. The culture media were collected and PGE_2 production was determined by EIA (upper panel). Data are expressed as mean ± SEM from four independent experiments. $^*P < 0.05$ compared with cells treated with IL-1β alone (control). Cell lysates were prepared and analyzed for the expression level of mPGES-1, cPGES (middle panel), HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC6 or β-actin (lower panel) proteins by Western blotting. The blots are representative of similar results obtained from four independent experiments.

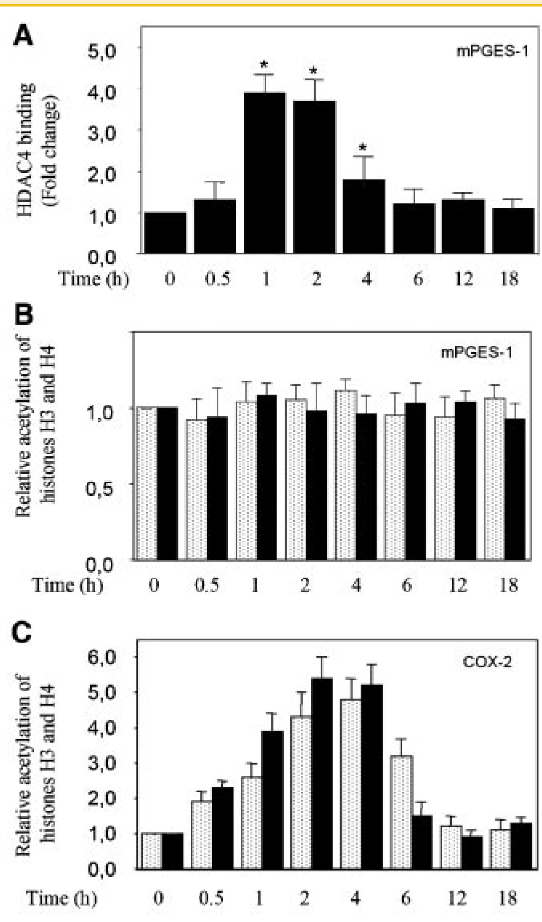

Fig. 6. Effect of IL-1 on HDAC4 recruitment and histone acetylation at the mPGES-1 promoter. A: Confluent HSF were treated with 100 pg/ml IL-1 for the indicated time periods. ChIP assays, coupled with real-time PCR, were performed using a specific anti-HDAC4 antibody. The results are expressed as fold changes of HDAC4 binding to the mPGES-1 promoter relative to untreated cells (upper panel) and represent the mean ± SEM of four independent experiments. No PCR product was detected when a control IgG was used for immunoprecipitation. B,C: Confluent HSF were treated as in (A) and ChIP assays were performed using antibodies specific to acetylated histones H3 and H4. The results are expressed as fold changes of histones H3 and H4 acetylation at the mPGES-1 (B) or the COX-2 promoter (C) over untreated cells and represent the mean ± SEM of four independent experiments. For each ChIP assay, the immunoprecipitated DNA was quantitated in triplicate on two separate occasions. $^*P < 0.05$ compared with untreated cells.

HDAC4 IS REQUIRED FOR EGR-1-MEDIATED ACTIVATION OF THE mPGES-1 PROMOTER

We have previously shown that IL-1 induces Egr-1 expression in HSF [Cheng et al., 2004]. Moreover, Egr-1 is known to play a crucial role in mPGES-1 transcription [Naraba et al., 2002; Subbaramaiah et al., 2004]. To evaluate the involvement of this transcription factor in IL-1-induced mPGES-1 expression in HSF, cells were transfected with a control or Egr-1 siRNA and then treated with IL-1 for 1 or 20 h. As shown in Figure 7, Egr-1 siRNA almost completely blocked IL-1-induced Egr-1 and mPGES-1 expression as well as PGE_2 production. These results clearly show that Egr-1 is crucial for IL-1-induced mPGES-1 expression in HSF.

HDACs are also known to regulate transcription by modulating the activity of transcription factors [Nusinzon and Horvath, 2003; Xu et al., 2003; Kato et al., 2004; Klampfer et al., 2004; Qian et al., 2006; Qiu et al., 2006]. Therefore, we hypothesized that HDAC4 may contribute to IL-1-induced mPGES-1 expression by enhancing Egr-1 activity. To test this hypothesis, HSF were co-transfected with the mPGES-1 promoter and an expression vector for Egr-1 together with increasing concentrations of vectors encoding for HDAC1, 2, 3, 4, 5, or 6. As shown in Figure 8A, overexpression of Egr-1 caused a strong increase in the mPGES-1 promoter activity. Interestingly, co-transfection with HDAC4 further enhanced Egr-1-mediated activation of the mPGES-1 promoter. In contrast, overexpression of HDAC1, 2, 3, 5, and 6 had no effect on Egr-1-mediated activation of the mPGES-1 promoter. These results strongly implicate HDAC4 as a critical positive coactivator of Egr-1-dependent activation of the mPGES-1 promoter. This effect of HDAC4 appears to require its deacetylase activity since TSA dose-dependently suppressed the enhancing effect of HDAC4 (Fig. 8B) and the deacetylase-deficient HDAC4 mutant failed to enhance Egr-1-mediated activation of the mPGES-1 promoter (Fig. 8C).

Next, we examined the effect of silencing endogenous HDAC4 on Egr-1-dependent activation of the mPGES-1 promoter. HSF were co-transfected with the mPGES-1 promoter and an expression vector for Egr-1 together with HDAC4 siRNA or scrambled control siRNA. As shown in Figure 8D, overexpression of Egr-1 strongly activated the mPGES-1 promoter and this effect of Egr-1 was reduced by ~75% in the presence of HDAC4 siRNA. In contrast, transfection with control siRNA did not affect Egr-1-induced

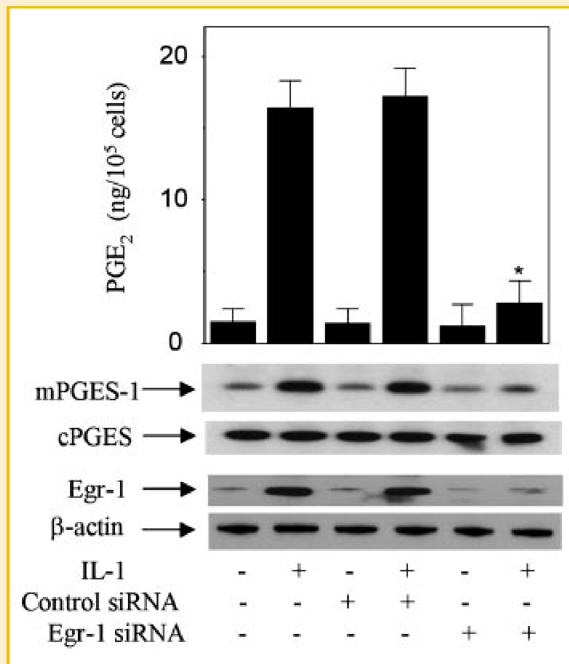

Fig. 7. Effect of Egr-1 siRNA on IL-1-induced mPGES-1 expression in HSF. HSF were transfected with 200 nM of control scrambled siRNA or Egr-1 siRNA. At 24 h post-transfection, cells were washed and treated with IL-1 for 1 or 20 h. Cell lysates were prepared and analyzed for Egr-1, β-actin (1 h treatment) mPGES-1 and cPGES (20 h treatment) protein expression by Western blotting. The blots are representative of similar results obtained from three independent experiments. The upper graph shows the production of PGE2 in the cultured media of cells transfected with Egr-1 siRNA and treated with IL-1 for 20 h. Data are expressed as mean ± SEM from four independent experiments. $^*P < 0.05$ compared with cells treated with IL-1 alone (control).

mPGES-1 promoter activation. The effectiveness of HDAC4 siRNA in down-regulating HDAC4 expression was documented by densitometry analysis showing that HDAC4 levels were reduced by as much as 70% in cells transfected with HDAC4 siRNA when compared to cells transfected with control siRNA. These findings suggest that HDAC4 is required for Egr-1-mediated mPGES-1 promoter activation.

DISCUSSION

In this report we have shown that deacetylase inhibition prevents IL-1-induced mPGES-1 expression in HSF. HDAC4 overexpression enhanced, whereas HDAC4 silencing by siRNA reduced, IL-1-mediated activation of the mPGES-1 promoter. IL-1-induced mPGES-1 protein expression was also prevented by HDAC4 siRNA. Using ChIP analysis, we demonstrated that HDAC4 is recruited to the mPGES-1 promoter in the presence of IL-1, but without concomitant local deacetylation of histones H3 and H4. HDAC4 overexpression enhances, whereas HDAC4 knockdown, reduces Egr-1-mediated activation of the mPGES-1 promoter. Together, these data indicate that HDAC4 contributes to IL-1-induced mPGES-1 expression through a mechanism that involves up-regulation of Egr-1 transcriptional activity.

Our current data show that inhibitors of HDAC activity, including TSA, BA and VA, prevent IL-1-induced mPGES-1 protein expression, suggesting that a deacetylase activity is required for IL-1-induced mPGES-1 expression. The repressive effect of TSA on IL-1-induced mPGES-1 expression occurred at the transcriptional level, as determined by real-time RT-PCR analysis and transient transfection assays. Interestingly, the induction of mPGES-1 expression by TNF-α and IL-17 was also inhibited by HDAC inhibitors, suggesting that the requirement for deacetylase activity might be a general mechanism involved in mPGES-1 expression.

Analysis of individual HDAC family members revealed that overexpression of HDAC4 enhanced IL-1-induced mPGES-1 promoter activation, while HDAC1, 2, 3, 5, and 6 had little or no effect. Moreover, siRNA-mediated HDAC4 silencing reduced IL-1-induced mPGES-1 promoter activation, suggesting that HDAC4 contributes to IL-1-induced mPGES-1 expression. Indeed, HDAC4 siRNA strongly reduced IL-1-induced mPGES-1 protein expression. These data suggest that HDACs can function not only as transcription repressors, but also as transcription activators. This is supported by the current finding as well as data from several studies. For example, treatment with HDAC inhibitors prevents the expression of several genes including IL-1, IL-2, IL-8, IL-12, TNF-α, Id-1, and a number of IFNα and γ responsive genes [Huang et al., 1997; Koyama et al., 2000; Leoni et al., 2002, 2005; Nusinzon and Horvath, 2003; Xu et al., 2003; Klampfer et al., 2004; Chang and Pikaard, 2005]. Furthermore, gene profiling studies have identified many genes that are activated by overexpression of individual HDACs in yeast [Bernstein et al., 2000], Drosophila [Cho et al., 2005], and mammalian cells [Zupkovitz et al., 2006]. Therefore, HDACs, besides their well-documented role as transcription repressors, may also function as positive regulators of gene expression.

Furthermore, we showed by ChIP assays that HDAC4 is recruited to the mPGES-1 promoter when the cells are stimulated with IL-1. Surprisingly, HDAC4 recruitment does not correlate with deacetylation of histones H3 or H4 at the mPGES-1 promoter, suggesting that local deacetylation of histones H3 and H4 is not the primary mechanism by which HDAC4 contributes to IL-1-induced mPGES-1 expression.

In addition to histones, HDACs have been shown to modulate gene expression by enhancing the activity of several transcription factors such as C/EBPβ [Xu et al., 2003], HIF-α, [Kato et al., 2004; Qian et al., 2006], GR [Qiu et al., 2006], STAT1 [Klampfer et al., 2004], STAT2 [Nusinzon and Horvath, 2003], and STAT5 [Xu et al., 2003]. Therefore, it is possible that HDAC4 contributes to IL-1-induced mPGES-1 expression by increasing Egr-1-dependent transcriptional activity. Indeed, overexpression of HDAC4 enhanced, whereas silencing of endogenous HDAC4 by siRNA, reduced Egr-1-mediated activation of the mPGES-1 promoter. This is the first evidence that HDAC4 up-regulates Egr-1 transcriptional activity in HSF. Taken together, these data suggest that HDAC4 contributes to IL-1-induced mPGES-1 expression through a histone deacetylation-independent mechanism that involves up-regulation of Egr-1 transcriptional activity.

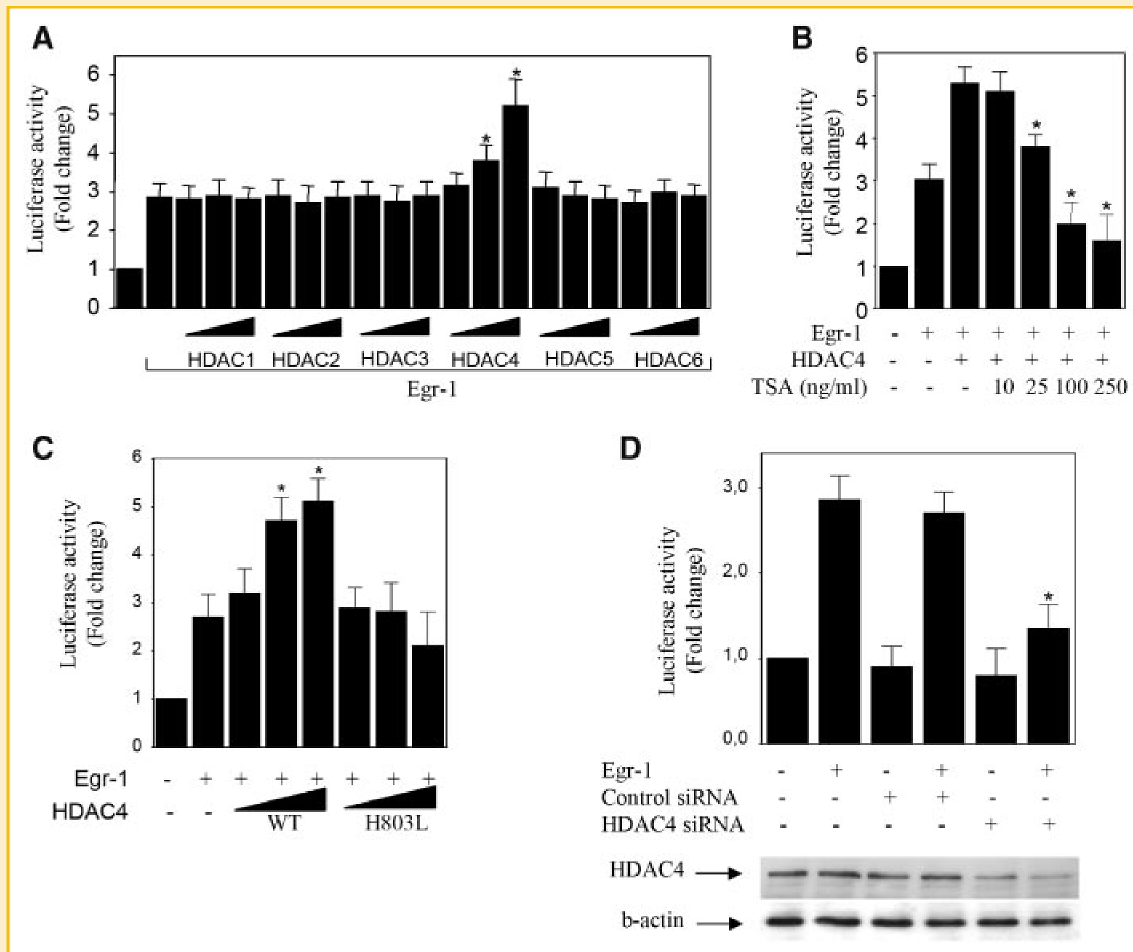

Fig. 8. HDAC4 is required for Egr-1-mediated mPGES-1 promoter activation. A: HSF were co-transfected with 1 μg of the human mPGES-1 promoter, 0.5 μg of pSV40-β-gal, 100 ng of the Egr-1 expression vector and increasing concentrations (0.1, 0.5, and 1 μg/ml) of expression vectors for HDAC1, 2, 3, 4, 5, or 6. The total amount of transfected DNA was kept constant by addition of the empty vector. Total cell extracts were prepared 40 h after transfection and analyzed for luciferase activity. Results are expressed as fold changes, considering 1 as the value of untreated cells and represent the mean ± SEM of four independent experiments. $^{*}P < 0.05$ compared with cells transfected with Egr-1 alone (control). B: HSF were co-transfected with 1 μg of the human mPGES-1 promoter, 0.5 μg of pSV40-β-gal, 100 ng of the Egr-1 expression vector and 1 μg of an expression vector for HDAC4. The total amount of transfected DNA was kept constant by the addition of the empty vector. The cells were then left untreated or treated with increasing concentrations of TSA for 40 h. Total cell extracts were prepared and analyzed for luciferase activity as indicated above. Results are expressed as fold changes, considering 1 as the value of untreated cells and represent the mean ± SEM of four independent experiments. $^{*}P < 0.05$ compared with cells transfected with Egr-1 and HDAC4 (control). C: HSF were co-transfected with 1 μg of the human mPGES-1 promoter, 0.5 μg of pSV40-β-gal, 100 ng of the Egr-1 expression vector and increasing concentrations (0.01, 0.1, and 1 μg/well) of an expression vector for HDAC4 (HDAC4-WT) or HDAC mutant that lack deacetylase activity (HDAC4-H803L). The total amount of transfected DNA was kept constant by the addition of the empty vector. Total cell extracts were prepared 40 h after transfection and analyzed for luciferase activity. Results are expressed as fold changes, considering 1 as the value of untransfected cells and represent the mean ± SEM of four independent experiments. $^{*}P < 0.05$ compared with cells transfected with Egr-1 alone (control). D: HSF were co-transfected with the human mPGES-1 promoter (1 μg/ml), 0.5 μg of pSV40-β-gal, 100 ng of the Egr-1 expression vector, together with 200 nM of control scrambled siRNA or HDAC4 siRNA. Total cell extracts were prepared 40 h after transfection for luciferase activity analysis or for Western blot analysis to evaluate HDAC4 levels.

The molecular mechanism by which HDAC4 promotes transcriptional activity of Egr-1 remains undefined but it may include modulation of the acetylation level of Egr-1 itself. Indeed, Yu et al. reported that the acetylation status of Egr-1 appears to modulate its activity. Acetylated Egr-1 activates growth and survival genes, while nonacetylated Egr-1 activates genes involved in apoptosis [Yu et al., 2004]. Egr-1 transcriptional activity involves structural and functional cooperativity (positive and negative) with numerous transcription or regulatory factors [Svaren et al., 1996; Tsai et al., 2000; Barthel et al., 2003; Mouillet et al., 2004]. Therefore, it is also possible that HDAC4 regulates Egr-1 activity through deacetylation of co-factors involved in Egr-1 transcriptional response. Additional molecular and biochemical studies are needed to understand the detailed mechanism by which HDAC4 regulates Egr-1 transcriptional activity.

The realization that HDAC4 contributes to mPGES-1 expression has potentially important implications for a variety of human disorders associated with increased mPGES-1 levels, such as arthritis [Westman et al., 2004; Li et al., 2005] inflammatory bowel disease [Subbaramaiah et al., 2004] atherosclerosis [Gomez-Hernandez

et al., 2006] and tumorogenesis [Kamei et al., 2003]. Thus, strategies to suppress mPGES-1 expression by inhibiting HDAC4 activity would be predicted to have therapeutic values.

ACKNOWLEDGMENTS

HF is a Research Scholar of the Fonds de Recherche en Santé du Québec (FRSQ). We would like to thank Dr. T. J. Smith (University of California, Los Angeles) for the mPGES-1 promoter, Dr. S. Schreiber (Howard Hughes Medical Institute, Cambridge) for the HDAC1, 5, and 6 expression plasmids, Dr. E. Seto (H. Lee Moffitt Cancer Center, Tampa) for HDAC2 and 3 expression plasmids, Dr. X-J Yang (McGill University, Montreal) for the WT and mutant HDAC4 and Dr. Y. E. Chen (Morehouse School of Medicine, Atlanta) for Egr-1 expression vector.

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