Biochemical and Biophysical Research Communications 379 (2009) 616–620

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

HDAC3 influences phosphorylation of STAT3 at serine 727 by interacting with PP2A

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ARTICLE INFO

Article history:
Received 16 December 2008
Available online 1 January 2009

Keywords:
STAT3
PP2A
HDAC3
Dephosphorylation
Transcriptional regulation

ABSTRACT

Signal transducer and activator of transcription 3 (STAT3), which mediates biological actions in many physiological processes, is activated by cytokines and growth factors, and has been reported to be involved in the pathogenesis of various human diseases. Here, we show that treatment of HeLa cells with a histone deacetylase (HDAC) inhibitor, trichostatin A, or small-interfering RNA (siRNA)-mediated repression of HDAC3, enhances phosphorylation of STAT3 at Ser727. Furthermore, dephosphorylation of STAT3 at Ser727 by protein phosphatase 2A (PP2A) was restored by treatment of cells with HDAC3 siRNA. We further found that formation of a complex between STAT3 and PP2A was enhanced in the presence of HDAC3. Importantly, small-interfering RNA-mediated repression of both HDAC3 and PP2A effectively enhanced leukemia inhibitory factor (LIF)-induced STAT3 activation. These results indicate that HDAC3 may act as a scaffold protein for PP2A to regulate the LIF/STAT3-mediated signaling pathway.

Signal transducer and activator of transcription 3 (STAT3) was originally cloned as an acute-phase response factor activated by interleukin (IL)-6 in the mouse liver, and based on its homology with STAT1 [1,2]. Growth factors, such as epidermal growth factor and platelet-derived growth factor, can also stimulate STAT3 activity [3]. STAT3 is known to play crucial roles in early embryonic development as well as in other biological responses, including cell growth and apoptosis [4–6]. STAT3 is constitutively activated in v-src- or v-abl-transformed cells and various primary tumors and cell lines [7,8]. Moreover, STAT3 itself acts as an oncogene in NIH-3T3 cells [9]. Since dysregulation of the STAT3-mediated signaling pathway is frequently detected in clinical tumor samples, understanding the mechanisms underlying STAT3 regulation of cell survival may lead to successful strategies for targeting STAT3 in cancer therapy [5,7,8].

A single tyrosine residue in all STATs is phosphorylated as a consequence of cytokine or growth factor stimulation and this phosphorylation is essential for the activation of these STATs. In addition, all STATs, except for STAT2 and STAT6, are phosphorylated on serine residues in response to ligand stimulation [10]. In the case of STAT3, phosphorylation of a single serine residue (Ser727) in the transcriptional activation domain is required for maximal transcriptional activation [11]. A STAT3 S727A mutant, in which Ser727 is replaced with an alanine, exhibited a marked reduction in transcriptional activation in vivo [12]. Serine phosphorylation most likely increases STAT3 activity by increasing its association with other cofactors, such as p300 [13]. Several different kinases, including ZIP kinase, have been implicated in serine phosphorylation, implying an interaction between STAT3 signaling and serine kinase signaling pathways [10,14]. However, the regulatory mechanisms underlying Ser727-phosphorylation of STAT3 have not yet been identified.

A recent study demonstrated that STAT3 is also acetylated on a single lysine residue, Lys685 [15]. Histone acetyltransferase p300-mediated STAT3 acetylation on Lys685 was restored by type I histone deacetylase (HDAC). This effect was reversible by treatment of cells with trichostatin A (TSA), a broad inhibitor of HDACs. STAT3 interacted with HDAC1, HDAC2 and HDAC3. Among them, HDAC3 displayed the strongest inhibitory effect on STAT3 deacetylase activity and bound to STAT3 through its C-terminal region, which plays a regulatory role in HDAC catalytic activity.

In the present study, we found that treatment of HeLa cells with TSA, or small-interfering RNA (siRNA)-mediated repression of HDAC3, enhances phosphorylation of STAT3 at Ser727. Furthermore, dephosphorylation of STAT3 at Ser727 by protein phosphatase 2A (PP2A) was restored by treatment of HeLa cells with HDAC3 siRNA. We further found that formation of a complex between STAT3 and PP2A was enhanced in the presence of HDAC3. Moreover, siRNA-mediated repression of both HDAC3 and PP2A effectively enhanced leukemia inhibitory factor (LIF)-induced STAT3 activation. These results indicate that HDAC3 regulates both the phosphorylation and acetylation of STAT3 in the STAT3-mediated signaling pathway.

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doi:10.1016/j.bbrc.2008.12.132

Materials and methods

Reagents and antibodies. Trichostatin A (TSA) was purchased from Wako Pure Chemicals (Osaka, Japan). Recombinant human LIF was purchased from INTERGEN (Purchase, NY). Expression vectors for STAT3, HDAC3, PP2A and STAT3-LUC were described previously [16,17]. Anti-Myc, anti-STAT3 and anti-HDAC3 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-FLAG antibody from Sigma–Aldrich (St. Louis, MO); anti-pSTAT3(Tyr705), anti-pSTAT3(Ser727) and anti-PP2A antibodies from Cell Signaling Technologies (Beverly, MA); anti-actin antibody from Chemicon International (Temecula, CA).

Cell culture, transfection,small-interfering RNA (siRNA) and luciferase assays. Human cervix carcinoma cell line HeLa and human embryonic kidney carcinoma cell line 293T were maintained in DMEM containing 10% FCS. 293T cells were transfected with the standard calcium precipitation protocol [18]. siRNAs targeting human HDAC3 and PP2A (catalytic subunit) used in this study were as follows: HDAC3, 5′-GCCGGUUAUCAACCAGGUATT-3′; PP2A, 5′-GAUACAAAUUACUUGUUUATT-3′. HeLa cells were plated on a 24-well plate at 2×10^4 cells/well, and then incubated with an siRNA-Lipofectamine 2000 (Invitrogen, Carlsbad, CA) mixture at 37 °C for 4 h, followed by addition of fresh medium containing 10% FCS [19]. Twenty-four hrs after transfection, the cells were harvested and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. HeLa cells were then transfected with STAT3-LUC using jetPEI (PolyPlus-transfection, Strasbourg, France) according to the manufacturer's instruction. The cells were harvested 36 h after transfection and lysed in Reporter Lysis Buffer (Promega) and assayed for luciferase and β-galactosidase activities according to the manufacturer's instructions. Three or more independent experiments were carried out for each assay.

Immunoprecipitation and immunoblotting. The immunoprecipitation and Western blotting assays were performed as described previously [20]. The immunoprecipitates from cell lysates were resolved on SDS–PAGE and transferred to PVDF transfer membrane (PerkinElmer, Boston, MA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore, Bedford, MA).

Results and discussion

HDAC3 influences phosphorylation of STAT3 at Ser727

It has recently been reported that HDAC3 interacts with and deacetylates STAT3 at Lys685, and that treatment of cells with TSA enhances STAT3-mediated transcriptional activity [15]. These findings led us to examine whether HDAC3 is also involved in the regulation of phosphorylation of STAT3. To examine the involvement of HDAC3 in the phosphorylation of STAT3, we first tested the effects of TSA on the phosphorylation of STAT3. 293T or HeLa cells were pretreated with TSA for 12 h, stimulated with LIF for 30 min and analyzed for their levels of endogenous STAT3 phosphorylation. As shown in Fig. 1A and B, Ser727-phosphorylation, but not Tyr705-phosphorylation, was markedly enhanced by TSA pretreatment in both types of cells, suggesting that type I HDAC may be involved in regulating Ser727-phosphorylation, but not Tyr705-phosphorylation, of STAT3. We further investigated whether HDAC3 is involved in the regulation of STAT3 Ser727-phosphorylation using an siRNA to reduce endogenous HDAC3 expression in HeLa cells. HeLa cells were transfected with a specific siRNA against HDAC3, or a control siRNA, and aliquots of cell lysates were analyzed by Western blotting to confirm reductions in HDAC3 protein levels (Fig. 1C). Importantly, siRNA-mediated reduced expression of HDAC3 significantly enhanced Ser727-phosphorylation, but not Tyr705-phosphorylation, of STAT3 (Fig. 1C), further indicating that HDAC3 regulates Ser727-phosphorylation, but not Tyr705-phosphorylation, of STAT3.

HDAC3 affects phosphorylation of STAT3 at Ser727 by interacting with PP2A

In a previous study, inhibition of a major cellular serine/threonine-specific protein phosphatase, PP2A was found to induce serine phosphorylation and activation of STAT3 [21]. Furthermore, expression of PP2A antisense constructs in human hepatoma Hep3B cells was shown to enhance IL-6-induced transcription

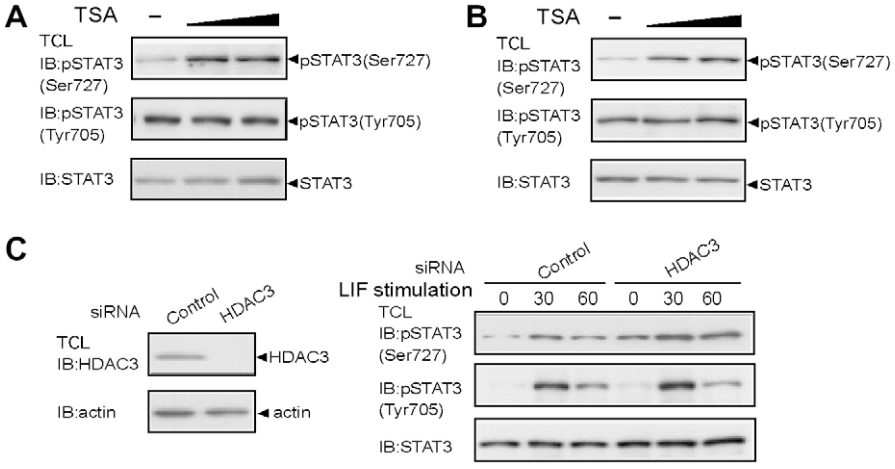

Fig. 1. HDAC3 influences phosphorylation of STAT3 at Ser727. (A) 293T cells in a 12-well plate were pretreated with TSA (100 and 200 ng/ml) for 12 h, cells were stimulated with LIF (100 ng/ml) for an additional 30 min. The cells were lysed, and an aliquot of total cell lysates (TCL) was analyzed by immunoblotting using anti-pSTAT3 (Ser727) or anti-pSTAT3 (Tyr705) or anti-STAT3 antibody. (B) HeLa cells in a 12-well plate were pretreated with TSA (100 and 200 ng/ml) for 12 h, cells were stimulated with LIF (100 ng/ml) for an additional 30 min. The cells were lysed, and an aliquot of TCL was analyzed by immunoblotting using anti-pSTAT3 (Ser727) or anti-pSTAT3 (Tyr705) or anti-STAT3 antibody. (C) HeLa cells in a 24-well plate were treated with control or HDAC3 siRNA, and cells were lysed, and an aliquot of TCL was analyzed by immunoblotting using anti-HDAC3 or anti-actin antibody. HeLa cells treated with control or HDAC3 siRNA were also stimulated with LIF (100 ng/ml) for the indicated periods. The cells were lysed, and an aliquot of TCL was analyzed by immunoblotting using anti-pSTAT3 (Ser727) or anti-pSTAT3 (Tyr705) or anti-STAT3 antibody.