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ATF-2 regulates lipopolysaccharide-induced transcription in macrophage cells

Noriyuki Hirose [a,b], Toshio Maekawa [a], Toshie Shinagawa [a], Shunsuke Ishii [a,b,*]

[a] Laboratory of Molecular Genetics, RIKEN Tsukuba Institute, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan
[b] University of Tsukuba, Graduate School of Comprehensive Human Sciences, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8577, Japan

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ABSTRACT

The transcription factor ATF-2, a member of the ATF/CREB family, is a target of p38 that are involved in stress-induced apoptosis and in Toll-like receptor (TLR)-mediated signaling. Phosphorylation of ATF-2 at Thr-71 was enhanced by treating of RAW264.7 macrophage cells with either LPS, MALP-2, or CpG-ODN. LPS treatment enhanced the *trans*-activation capacity of ATF-2. Among multiple LPS-induced genes, the LPS-induced expression of *Socs-3* was significantly reduced by the treatment of RAW264.7 cells with an *Atf-2* siRNA. Transcription from the *Socs-3* promoter was synergistically stimulated by ATF-2 and LPS, whereas it was suppressed by *Atf-2* siRNA. Histone deacetylase 1 (HDAC1) interacted with ATF-2 after LPS treatment, but not before treatment. Treatment of RAW264.7 cells with trichostatin A, an inhibitor of HDAC, suppressed the LPS-induced *Socs-3* expression, suggesting that HDAC1 positively regulates the LPS-induced transcription of *Socs-3*. Thus, ATF-2 plays an important role in TLR-mediated transcriptional control in macrophage cells.

Introduction

The transcription factor ATF-2, is a member of the ATF family, has a b-ZIP type DNA-binding domain [1,2] and binds to the cyclic AMP response element (CRE: 5′-TGACGTCA-3′) as a homodimer or a heterodimer with c-Jun. Stress-activated protein kinases (SAPKs), including JNK (c-Jun N-terminal protein kinase) and p38, are activated by various extracellular stresses such as inflammatory cytokines and hypoxia and phosphorylate ATF-2 at Thr-69 and Thr-71 [3]. Upon phosphorylation, the *trans*-activating capacity of ATF-2 is enhanced and leads to induction of various target genes including the apoptosis-regulating genes [3,4].

Null *Atf-2* mutant mice die immediately after birth and exhibit severe respiratory defects like meconium aspiration syndrome due to the lungs filled with meconium [5]. This appeared to be correlated with the defects in cytotrophoblast growth of the mutant placenta and the reduced expression level of PDGF receptor-α gene, one of the ATF-2 target genes. *Atf-2*-defcient mouse embryonic fibroblasts (MEFs) are more resistant to hypoxia-induced apoptosis but not to apoptosis induced by other various stresses, including DNA damage and UV light [4]. In response to hypoxia, ATF-2 activates multiple target genes, including *Gadd45α,* which is a regulator of apoptosis. ATF-2 is also involved in the apoptosis at the high cell density, and *Atf-2*$^{-/-}$ and *Atf-2*$^{+/-}$ monolayers reach higher cell densities. In the absence of stress, ATF-2 activates a small number of genes such as *Maspin*, which was originally identified as a tumor suppressor in human breast cancers and is encodes a member of the serine protease inhibitor (serpin) family. Consistent with these observations, *Atf-2*$^{+/-}$ mice are highly prone to mammary tumors [4].

JNK and p38 are components of the signaling pathways of Toll-like receptors (TLRs), which play a critical role in the initiation of the innate immune response by sensing pathogen-specific molecular patterns [6]. p38 and its upstream kinases, MEKK3, ASK1, and TAK1, are required for the signaling pathway of multiple TLRs in various cell types including macrophages [7–9]. In response to bacterial lipopolysaccharide (LPS) exposure, macrophages activate the transcription of a large number of pro-inflammatory genes through TLR4 [10]. Ligation of the TLR4–MD2 complex induced by LPS results in recruitment of the adaptor molecule MyD88 and a serine/threonine kinase IL-1R-associated kinase (IRAK), which subsequently associates with TNF receptor-associated factor (TRAF)-6. This subsequently leads to the activation of two different pathways involving JNK/p38 and NF-κB [6]. However, it remains unknown whether ATF-2, which is one of the major phosphorylation targets of p38/JNK, is involved in the LPS-induced transcriptional regulation in macrophages. This is due to a lack of *Atf-2*-deficient macrophages, because null *Atf-2* mutant mice die immediately after birth [5] and the tissue-specific *Atf-2* knock-down mice are not available at present.

Here, we demonstrate that ATF-2 is phosphorylated via signaling through multiple TLRs. Especially ATF-2 regulates the LPS-induced *Socs-3* transcription. Unexpectedly, HDAC1, which

* Corresponding author. Address: Laboratory of Molecular Genetics, RIKEN Tsukuba Institute, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan. Fax: +81 298 836 9030.
E-mail address: sishii@rtc.riken.jp (S. Ishii).

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associates with ATF-2, positively regulates the LPS-induced *Socs-3* transcription.

Materials and methods

Western blotting. RAW264.7 cells were incubated with LPS from *Escherichia coli* 0111:B4 (1 μg/ml, Sigma), mycoplasmal macrophage-activating lipopeptide 2 (MALP-2; 100 ng/ml, Apotech), or CpG-ODN (10 μg/ml) for various times. The cells were then lysed in RIPA buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 2 mM Na_3VO_4, 0.1 μM okadaic acid, 25 mM β-glycerophosphate, and protease inhibitor mixture). After centrifugation, the supernatant was analyzed by 10% SDS–PAGE, followed by Western blotting using anti-phospho-ATF-2 (Thr-71) antibody (Cell Signaling) or anti-ATF-2 polyclonal antibody (N-96, Santa Cruz). To inhibit p38, JNK, and ERK, RAW264.7 cells were treated with SB202190 (Sigma), SP600125 (Sigma), and U0126 (Sigma), respectively, for 1 h before the treatment of LPS. To detect phosphorylated kinases, anti-p-ERK (E-4, Santa Cruz), anti-phospho-p38 (#9211, Cell Signaling), and anti-p-JNK (G-7, Santa Cruz) were used. To detect α-tubulin and GAPDH, anti-α-tubulin (DM1A, Sigma) and anti-GAPDH (FL-3, Santa Cruz) were used.

Luciferase reporter assays. RAW264.7 cells (2×10^5 or 8×10^5 cells per 60-mm dish) were transfected with a mixture containing 0.8 μg of a CRE-containing luciferase reporter plasmid [11] or a Socs-3 promoter-containing luciferase reporter plasmid (pGL3-SOCS3) [12], which contains the Socs-3 promoter region (from −273 to +160), 1 μg of the ATF-2 expression plasmid (pact-ATF-2), and the internal control phRL-TK(int-) (0.2 μg; Promega) using Lipofectamine 2000 (Invitrogen). The transfected cells were stimulated with LPS (1 μg/ml) for 16 h before lysate preparation. Forty-eight hours after transfection, luciferase activity was measured using the dual-luciferase reporter assay system (Promega). To evaluate the effect of Atf-2 siRNA on Socs-3 promoter activity, RAW264.7 cells were transfected with a mixture containing 0.8 μg of pGL3-Socs3, 0.2 μg of phRL-TK(int-), and Atf-2 siRNA or control siRNA (40 nM) using Lipofectamine 2000. Thirty-six hours after transfection, cells were treated with LPS (1 μg/ml) for 12 h and luciferase activity was then measured. To examine the effect of HDAC1 (Fig. 4C), RAW264.7 cells were transfected with a mixture containing 0.4 μg of pGL3-Socs3, 1 μg of pat-ATF-2, 1 μg of the HDAC1 expression vector (pCS2-HDAC1), and 0.1 μg of phRL-TK(int-). To examine the effect of HADAC1 and Atf-2 siRNA (Fig. 4D), RAW264.7 cells were transfected with a mixture containing 0.5 μg of pGL3-SOCS3, 0.8 μg of pCS2-HDAC1, and 0.1 μg of phRL-TK(int-) together with Atf-2 siRNA (60 nM). Thirty-six hours after transfection, cells were treated with LPS (1 μg/ml) for 12 h and luciferase activity was then measured.

RNAi. The *Atf-2* siRNA oligonucleotide was described previously [13]. siRNA specific for green fluorescent protein were used as controls. RAW264.7 cells (2×10^5 cells per 60-mm dish) were transfected with oligonucleotides (40 or 80 nM) using the Lipofectamine 2000 (Invitrogen), and 48 h later, the levels of endogenous ATF-2 proteins were assayed by Western blotting using an anti-ATF-2 (N-96, Santa Cruz Biotechnology, Santa Cruz, CA). To determine the effect of siRNA on the cytokine gene expression, total RNA was prepare at various times after LPS treatment and used for Northern blotting.

Northern blotting. RNA was prepared from RAW264.7 cells using the TRIzol reagent (Invitrogen). The blot was hybridized with digoxigenin-labeled mouse Socs-3, Il-6, and Rantes probes. The digoxigenin-labeled probes were generated with the PCR DIG Probe synthesis kit (Roche). The probes were detected with a mAb against digoxigenin coupled to alkaline phosphatase and CDP-STAR as a substrate (Roche).

Preparation of macrophages. Murine peritoneal macrophages from BALB/c mice were prepared as follows. The 8-week-old male mice were injected intraperitoneally with 3 ml of thioglycollate medium (Gibco BRL), and the resulting peritoneal exudate was harvested by lavage of the peritoneal cavities of mice with 8 ml of cold PBS 4 days later. The peritoneal cells were cultured in 10% FBS-DMEM (Gibco BRL) in a 100-mm-diameter plastic dish for 3 h, and the dish was then washed four times with PBS to remove non-adherent cells. The adherent cells were continuously cultured in the complete medium. The adherent macrophages, more than 95% of which appeared to be typical macrophages by light microscopy, were used for each experiment.

Coimmunoprecipitation. Nuclear extracts were prepared from RAW264.7 cells as described previously [14], except that the concentrations of NaCl and NP-40 were decreased to 105 mM and 0.025% by adding the three volumes of extraction buffer lacking NaCl and NP-40. Immunoprecipitation was performed using the anti-ATF-2 antibody (N-96, Santa Cruz) or control rabbit IgG. The immunocomplexes were washed six times with washing buffer and then separated in 10% SDS–PAGE gels and analyzed by Western blotting with an anti-Rbap48 polyclonal antibody (11G10, Genetex) and an anti-HDAC1 antibody (Upstate).

Results and discussion

ATF-2 phosphorylation induced by TLR ligands

RAW264.7 cells were treated with LPS, MALP-2, or CpG-ODN, which are recognized by TLR4 [10], TLR2 [15,16], and TLR9 [17], respectively. At various times after treatment, phosphorylated ATF-2 was detected using an anti-phospho-ATF-2 antibody that recognizes ATF-2 phosphorylated at Thr-71. Phosphorylated ATF-2 was detected within 15 min after treatment with LPS, MALP-2, or CpG-ODN, and its levels continued to increase through 30 min, after which they decreased (Fig. 1A). The total amount of ATF-2 was essentially not affected by these treatments, although it increased slightly at 15 and 30 min after treatment.

To determine whether p38, or JNK is the kinase that phosphorylates ATF-2 at Thr-71 in response to TLR activation, we examined the effect of inhibitors. Neither SP600125, an inhibitor of JNK, nor SB202190, an inhibitor of P38α and p38β, greatly affected the LPS-induced phosphorylation of ATF-2 (Fig. 1B). U0126, an inhibitor of ERK, also did not affect the LPS-induced phosphorylation of ATF-2 (Fig. 1B). In fact, SP600125 and U0126 reduced the levels of phosphorylated JNK and phosphorylated ERK induced by LPS, respectively (Fig. 1C). However, the levels of phosphorylated p38 induced by LPS were not significantly affected by SB202190. Because p38δ, which is not suppressed by SB202190, is abundant in macrophages [18], it is possible that p38δ phosphorylates ATF-2 at Thr-71 in response to LPS stimulation. Although a specific inhibitor of p38δ was previously reported, it is not commercially available and we therefore could not test this possibility.

ATF-2 stimulates the LPS-induced transcription from a CRE-containing promoter

When RAW264.7 cells were transfected with a luciferase reporter, in which the four tandem repeats of CRE were linked to the *thymidine kinase* promoter, LPS treatment enhanced the luciferase expression about 5.7-fold (Fig. 1D). Coexpression of ATF-2 with the CRE-containing luciferase reporter further enhanced the LPS induction about 2-fold. In contrast, the two ATF-2 mutants, in which the N-terminal region containing the transcriptional activation domain was deleted (Δ107) or three SAPK phosphorylation sites (Thr-69, Thr-71, and Ser-90) were mutated into Ala (Ala),

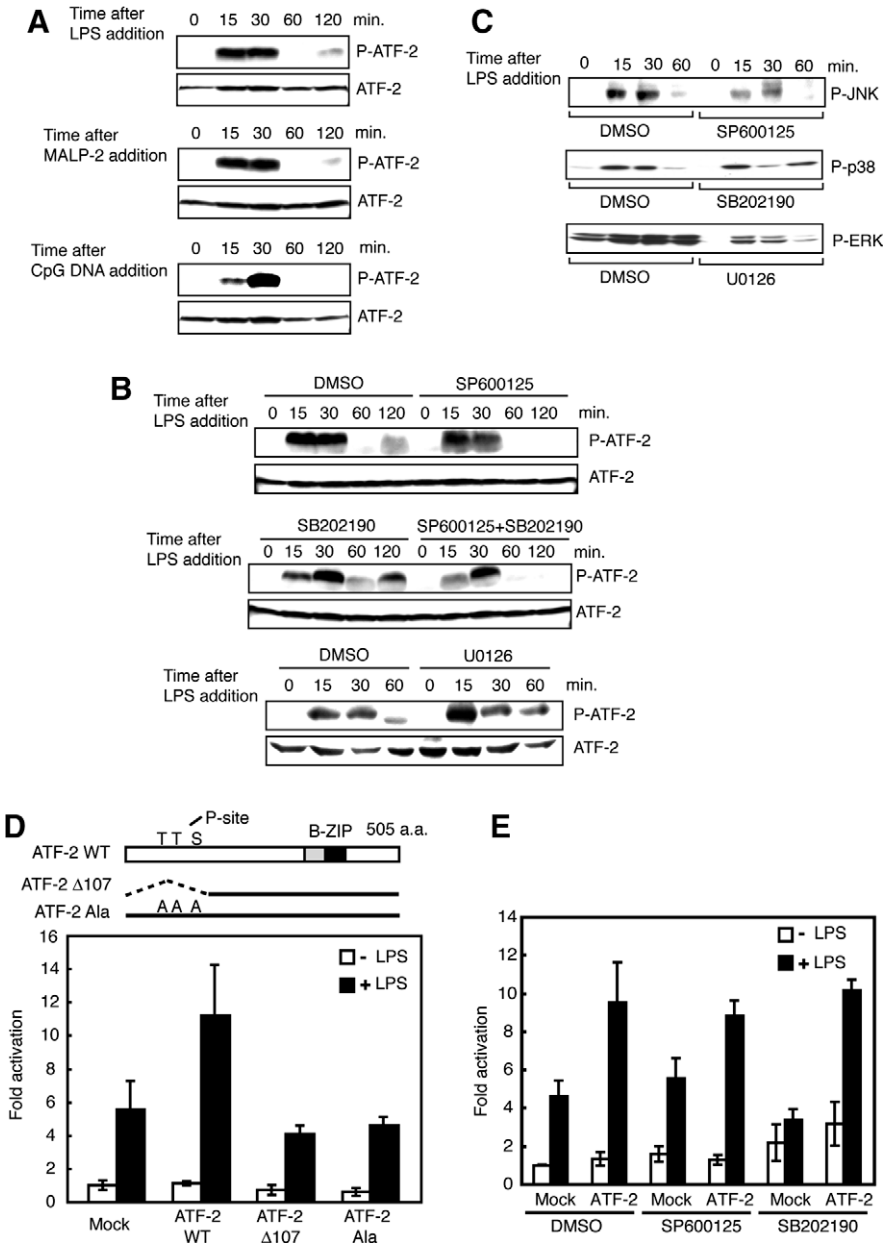

Fig. 1. Activation of ATF-2 by TLR stimulation. (A) Phosphorylation of ATF-2. RAW264.7 cells were treated with LPS, MALP-2, or CpG-ODN for the indicated time periods. Cell lysates were prepared and used for Western blotting with antibodies specific for phosphorylated ATF-2 (P-ATF-2) or non-phosphorylated ATF-2. (B) Effects of kinase inhibitors on LPS-induced phosphorylation of ATF-2. RAW264.7 cells were pretreated with the JNK inhibitor SP600125, the p38 inhibitor SB202190, a mixture of SP600125 and SB202190, or the ERK inhibitor U0126 (each 10 μM) for 1 h, and then were treated with LPS (1 μg/ml) for the indicated time periods. DMSO was used as a vehicle control. ATF-2 and P-ATF-2 were detected as described above. (C) Effects of kinase inhibitors on LPS-induced phosphorylation of kinases. RAW264.7 cells were pretreated with the indicated inhibitors, and then were treated with LPS. Cell lysates were prepared and used for Western blotting. (D) Enhancement of LPS-induced transcription from the CRE-containing promoter by ATF-2. Top, schematic of the wild-type (WT) and mutant forms of ATF-2. P-site, phosphorylation site; LZ, leucine-zipper; Δ107, an N-terminal truncated mutant; Ala, mutant with SAPK phosphorylation sites replaced with Ala. RAW264.7 cells were transfected with a mixture containing the CRE-containing luciferase reporter, the plasmid to express the WT ATF-2 or the Δ107 or the Ala mutants, and an internal control plasmid. Mock, cells transfected with the control plasmid alone. In some cases, the transfected cells were treated with LPS for 16 h before lysates preparation. The luciferase activity was measured, and the relative luciferase activity compared to that without effector is indicated. Means of three experiments ± SD are shown. (E) Effects of JNK and p38 inhibitors on LPS-induced *trans*-activation by ATF-2. RAW264.7 cells were transfected with the CRE-containing reporter, the ATF-2 expression plasmid or the control plasmid (Mock). Thirty-two hours after transfection, the cells were treated with SP600125 or SB202190 for 1 h, and then stimulated with LPS for 16 h. The luciferase activity was measured as described above.

did not enhance the LPS-induced expression from the CRE-containing promoter. Thus, ATF-2 enhances the LPS-induced transcription from the CRE-containing promoter.

We also examined the effect of inhibitors of kinases in the luciferase reporter assays. Neither SP600125, an inhibitor of JNK, nor SB202190, an inhibitor of p38α and p38β, significantly abrogated the stimulation of LPS-induced transcription by ATF-2 (Fig. 1E). This is consistent with the previous results that neither of these inhibitors inhibited the LPS-induced phosphorylation of ATF-2.

ATF-2 is required for LPS-induced Socs-3 expression

To identify the ATF-2 target genes, we reduced the ATF-2 protein level in RAW264.7 cells using siRNA. Transfection of RAW264.7 cells with *Atf-2* siRNA more greatly reduced the ATF-2 levels compared to control siRNA (Fig. 2A). Northern blotting using RNAs from RAW264.7 cells transfected with siRNA indicated that the LPS-induced level of *Socs-3* mRNA was reduced by *Atf-2* siRNA, but *Il-1β*, *Il-6*, and *Rantes* mRNA were not affected (Fig. 2B). We

have also examined the effect *Atf-2* siRNA on the LPS-induced *Socs-3* expression using primary macrophages. Transfection of macrophages with *Atf-2* siRNA reduced the ATF-2 levels (Fig. 2C). Northern blotting indicated that the LPS-induced level of *Socs-3* mRNA was reduced by *Atf-2* siRNA at 3 and 5 h after LPS treatment (Fig. 2D).

We then performed luciferase reporter assays using a reporter containing the *Socs-3* promoter. LPS enhanced the luciferase expression from the *Socs-3* promoter about 3.8-fold, and coexpression of ATF-2 further enhanced the induction by LPS to about 6.5-fold over baseline (Fig. 3A). In contrast, the N-terminally truncated mutant (Δ107) and the SAPK phosphorylation sites mutant (Ala) did not enhance the LPS-induced expression from the *Socs-3* promoter. In contrast, ATF-2 did not affect the LPS-induced luciferase expression from the *Il-6* promoter (Fig. 3B). Treatment of transfected cells with *Atf-2* siRNA reduced the induction of luciferase expression from the *Socs-3* promoter by LPS (Fig. 3C). In innate responses, *Socs1*-deficient mice are highly sensitive to LPS, indicating that SOCS1 is a negative regulator of TLR4 signaling [19]. SOCS-3 is also a negative regulator of the LPS-induced TNF-α and NO production in macrophages [20]. Therefore, ATF-2 may negatively regulate TLR4 signaling via induction of SOCS-3 in macrophages.

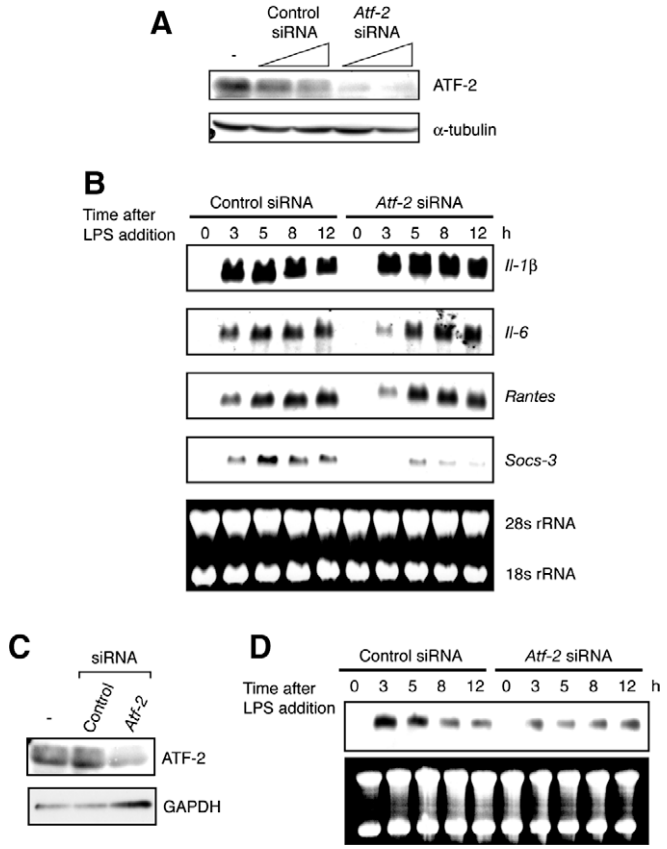

Fig. 2. ATF-2 is required for LPS-induced *Socs-3* expression. (A) ATF-2 levels are decreased by siRNA treatment. RAW264.7 cells were transfected with 40 or 80 nM *Atf-2* siRNA or control siRNA as indicated, and cell lysates were used for Western blotting with anti-ATF-2 or anti-α-tubulin antibodies. (B) RAW264.7 cells were transfected with 80 nM *Atf-2* siRNA or control siRNA as indicated. Forty-eight hours later, the cells were stimulated with LPS for the indicated periods. Total RNA was prepared and analyzed for expression of *Il-1β*, *Il-6*, *Rantes* and *Socs-3* by Northern blotting. (C) The peritoneal macrophages were treated with 80 nM *Atf-2* siRNA or control siRNA. Whole-cell lysates were used for Western blotting with anti-ATF-2 or anti-α-GAPDH antibodies. (D) Macrophages were transfected with 80 nM *Atf-2* siRNA or control siRNA. Forty-eight hours later, the cells were stimulated with LPS for the indicated periods. Total RNA was prepared and analyzed by Northern blotting.

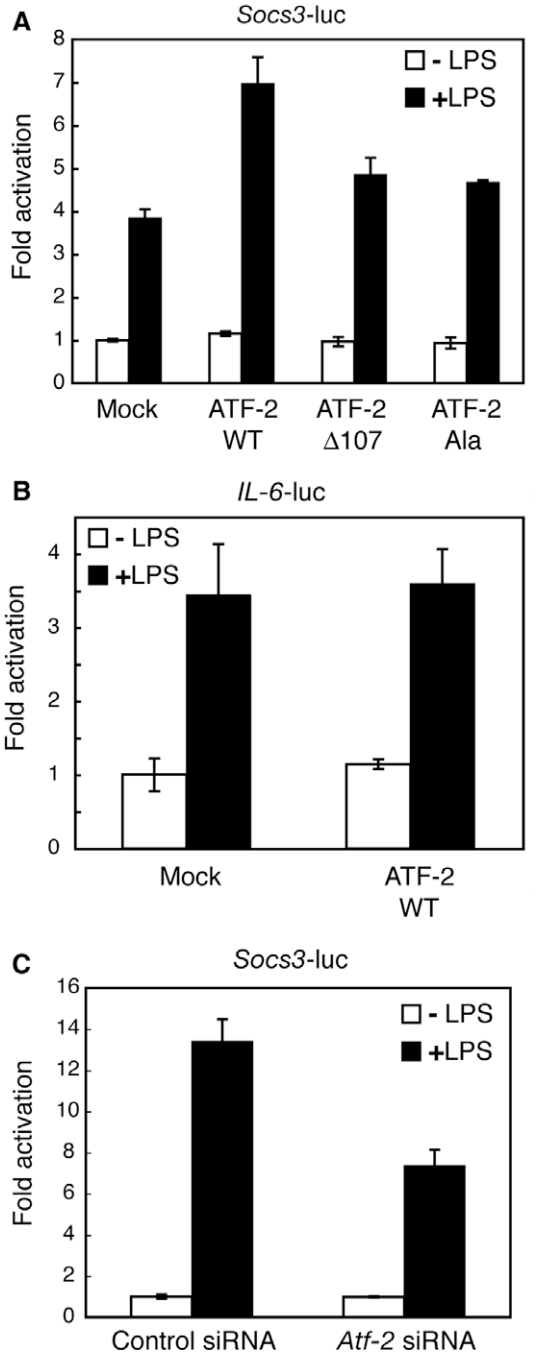

Fig. 3. Regulation of *Socs-3* transcription by ATF-2. (A) Activation of the *Socs-3* promoter by LPS and ATF-2. The *Socs-3* promoter-luc reporter plasmid was cotransfected into RAW264.7 cells with a plasmid expressing wild-type (WT) or mutant (Δ107, Ala) ATF-2. Thirty-two hours later, the cells were stimulated with LPS for 16 h, and the luciferase activity was measured. Means of three experiments ± SD are shown. (B) Effect of ATF-2 on the *Il-6* promoter. The *Il-6* promoter-luc reporter plasmid was cotransfected into RAW264.7 cells with the ATF-2 expression plasmid or the control empty vector, and the luciferase activity was measured as described above. (C) Effect of *Atf-2* siRNA on *Socs-3* promoter activity. RAW264.7 cells were cotransfected with the *Socs-3* promoter-luc reporter plasmid and *Atf-2* siRNA or control siRNA. Thirty-two hours later, the cells were stimulated with LPS for 16 h, and the luciferase activity was measured. Note that degree of LPS-dependent induction is higher than that shown in (A), possibly due to a difference in the amounts of plasmid DNA and/or siRNA.

HDAC activity positively regulates LPS-induced Socs-3 expression

To understand the mechanism by which ATF-2 regulates the LPS-induced *Socs-3* transcription, we purified the ATF-2 complex,

and identified RbAp48 (data not shown). Since RbAp48 is known to interact with HDAC [21], we investigated whether ATF-2 associates with RbAp48 and HDAC1. RbAp48 was coprecipitated with ATF-2 both before and after LPS treatment. On the other hand, HDAC1 was unexpectedly coprecipitated with ATF-2 only after LPS stimulation (Fig. 4A). We then investigated the effect of trichostatin A (TSA), an inhibitor of HDAC, on the LPS-induced expression of *Socs-3*. TSA suppressed the LPS-induced *Socs-3* expression level, whereas it enhanced the LPS-induced *Rantes* expression (Fig. 4B). Since *Atf-2* siRNA did not affect the *Rantes* expression (Fig. 2B), this data suggests that HDAC negatively regulates *Rantes* expression by interacting with transcription factor(s) other than ATF-2. This is not surprising, because many transcription factors utilize HDAC to suppress transcription. Further overexpression of HDAC1 enhanced the LPS-induced luciferase expression from the *Socs-3* promoter (Fig. 4C). However, overexpression of HDAC1 together with the *Atf-2* siRNA did not stimulate the *Socs-3* promoter activity (Fig. 4D). These results suggest that HDAC activity positively regulates the LPS-induced transcription of *Socs-3* via interaction with ATF-2. Although HDACs are generally thought to be involved in transcriptional repression, several reports showed that HDAC activity can positively regulate transcription. Rpd3, the yeast HDAC1 homologue, plays a positive role in the activation of osmo-responsive promoters [22]. In mammalian cells, HDAC1 activity is required for transcription of interferon-responsive genes, such as *Irf1* and *Gbp2* [23]. Although the molecular mechanism by which HDAC positively regulates these genes remains elusive, HDAC may acetylates factors other than histones.

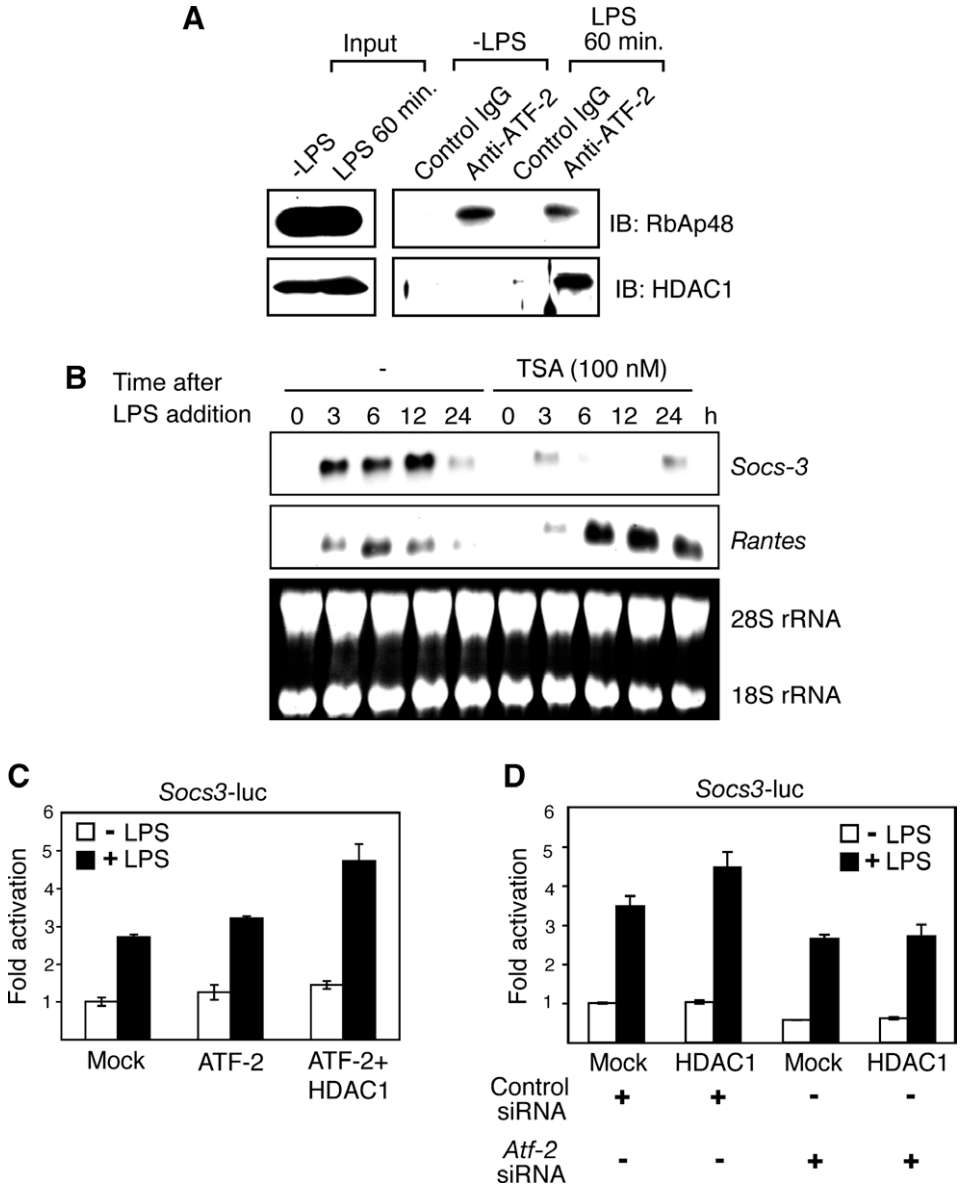

Fig. 4. HDAC activity is required for the LPS-induced *Socs-3* expression. (A) Coimmunoprecipitation of ATF-2 and HDAC1. RAW264.7 cells were treated with LPS for the indicated periods, and nuclear extracts were prepared. Nuclear extracts were precipitated with the anti-ATF-2 antibody or a control IgG, and the immunocomplexes were analyzed by Western blotting with anti-RbAp48 and anti-HDAC1 antibodies. (B) Effects of TSA in LPS-induced *Socs-3* expression. RAW264.7 cells were pretreated with TSA (100 nM) for 1 h, and then were treated with LPS for the indicated time periods. Total RNA was prepared, and analyzed by Northern blotting. 28S and 18S rRNA were used as loading controls. (C) HDAC1 stimulates the *Socs-3* promoter activity. The *Socs-3* promoter-luc reporter plasmid was cotransfected into RAW264.7 cells with a plasmid expressing ATF-2 and HDAC1. The luciferase activity was measured. Note that degree of ATF-2-dependent enhancement is lower than that shown in Fig. 3A is due to a difference in the amounts of plasmid DNA. (D) ATF-2 is required for the HDAC1-dependent stimulation of the *Socs-3* promoter activity. RAW264.7 cells were transfected with a mixture of the *Socs-3* promoter-luc reporter plasmid, the HDAC1 expression plasmid, and the *Atf-2* or control siRNA. The luciferase activity was measured.

Acknowledgments

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