

# Regulation of Nuclear Translocation of HDAC3 by I $\kappa$ B $\alpha$ Is Required for Tumor Necrosis Factor Inhibition of Peroxisome Proliferator-activated Receptor $\gamma$ Function\*

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**Inhibition of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) function by TNF- $\alpha$  contributes to glucose and fatty acid metabolic disorders in inflammation and cancer, although the molecular mechanism is not fully understood. In this study, we demonstrate that nuclear translocation of HDAC3 is regulated by TNF- $\alpha$ , and this event is required for inhibition of transcriptional activity of PPAR $\gamma$  by TNF- $\alpha$ . HDAC3 is associated with I $\kappa$ B $\alpha$  in the cytoplasm. After I $\kappa$ B $\alpha$  degradation in response to TNF- $\alpha$ , HDAC3 is subject to nuclear translocation, leading to an increase in HDAC3 activity in the nucleus. This event leads to subcellular redistribution of HDAC3. Knock-out of I $\kappa$ B $\alpha$ , but not p65 or p50, leads to disappearance of HDAC3 in the cytoplasm, which is associated with HDAC3 enrichment in the nucleus. These data suggest that inhibition of PPAR $\gamma$  by TNF- $\alpha$  is not associated with a reduction in the DNA binding activity of PPAR $\gamma$ . Rather, these results suggest that I $\kappa$ B $\alpha$ -dependent nuclear translocation of HDAC3 is responsible for PPAR $\gamma$  inhibition by TNF- $\alpha$ .**

PPAR $\gamma$  is a nuclear receptor in the family of peroxisome proliferator-activated receptor (PPAR)<sup>2</sup> that includes PPAR $\alpha$ , PPAR $\gamma$ , and PPAR $\delta$  (PPAR $\beta$ ) (reviewed in Refs. 1 and 2). PPAR $\gamma$  is a master transcriptional regulator of lipid and glucose metabolism (reviewed in Refs. 1–3). Inhibition of PPAR $\gamma$  function by inflammatory cytokines may contribute to the loss of insulin sensitivity in obese subjects and loss of fat storage in cancer patients under cachexia. Although TNF- $\alpha$  is known to inhibit the ligand-dependent transcriptional activity of PPAR $\gamma$ , the precise mechanism remains to be fully understood (4–8). In this study, we addressed this issue by analyzing the molecular mechanism of TNF- $\alpha$  action on PPAR $\gamma$ .

The transcriptional activity of PPAR $\gamma$  is controlled by DNA binding activity and nuclear receptor cofactors that include corepressors and coactivators. PPARs form heterodimers with the retinoid X receptor (RXR), which is activated by 9-*cis* retinoic acid (9). It is generally believed that the heterodimer is associated with the nuclear receptor

corepressor complex in the absence of PPAR $\gamma$  ligand. Upon activation by a ligand, the corepressor complex is replaced by coactivators leading to transcriptional initiation of target genes. The corepressor for PPAR $\gamma$  is a protein complex containing HDAC3 (histone deacetylase 3) and SMRT (silencing mediator for retinoic and thyroid hormone receptors) or NCoR (nuclear corepressor). RIP140 (receptor-interacting protein) may also be a component in the corepressor complex (10–13). The coactivators of PPAR $\gamma$  include well established cofactors such as p300/CBP, p160, and PGC-1 (PPAR $\gamma$  coactivator-1) (reviewed in Ref. 14), as well as the relatively new coactivators TRAP220 (thyroid hormone receptor-associated protein 220 or PBP, PPAR $\gamma$ -binding protein) (15, 16), ARA70 (androgen receptor-associated protein) (17), and PRIP (PPAR $\gamma$ -interacting protein, ASC-2/RAP250/TRBP/NRC) (18–21). The coactivator p160 has three isoforms: SRC-1 (steroid receptor coactivator 1, NCoA-1), SRC-2 (NCoA-2/TIF2/GRIP1), and SRC-3 (NCoA-3/pCIP/AIB-1/ACTR/RAC-3/TRAM-1) (22).

It has been well documented that PPAR $\gamma$  activity is inhibited by TNF- $\alpha$ . The inhibition can be divided into two types on the basis of PPAR $\gamma$  gene expression. First, PPAR $\gamma$  expression is reduced at the mRNA level (5, 6). This is observed in 3T3-L1 adipocytes treated with TNF- $\alpha$  for 24 h or longer. Second, PPAR $\gamma$  expression is not changed, and the inhibition is observed in cells transfected with a PPAR $\gamma$  expression vector (4, 7, 8). In the second model, the ligand-dependent transcriptional activity of PPAR $\gamma$  is reduced as a result of loss of DNA binding activity. However, both types of inhibition are dependent on activation of IKK/NF- $\kappa$ B pathway as the TNF- $\alpha$  activity was abolished by the superrepressor I $\kappa$ B $\alpha$  (inhibitor  $\kappa$ B $\alpha$ ) (6). NF- $\kappa$ B is a transcription factor that stays in the cytoplasm in the absence of activators. It is generally believed that I $\kappa$ B $\alpha$  inhibits NF- $\kappa$ B by maintaining NF- $\kappa$ B in the cytoplasm (reviewed in Ref. 23). I $\kappa$ B $\alpha$  degradation is controlled by a phosphorylation-mediated and proteasome-dependent mechanism that is initiated by activation of IKK2 (24). In the TNF- $\alpha$  signaling pathway, although ERK and JNK (c-Jun N-terminal kinase) were reported to inhibit the transcriptional activity of PPAR $\gamma$  through phosphorylation of serine residues in the PPAR $\gamma$  protein (25, 26), the role of these mitogen-activated protein kinases remains to be further characterized.

In this study, TNF-induced inhibition of the transcriptional activity of PPAR $\gamma$  is analyzed with a focus on I $\kappa$ B $\alpha$ . Our results demonstrate that I $\kappa$ B $\alpha$  controls the nuclear translocation of HDAC3, which is required for the suppression of PPAR $\gamma$  activity by TNF- $\alpha$ . This study supports a new mechanism by which TNF- $\alpha$  inhibits PPAR $\gamma$  activity by targeting the nuclear receptor corepressor.

## EXPERIMENTAL PROCEDURES

**Reagents**—The PPRE luciferase reporter was constructed utilizing the pGL3 basic luciferase vector. In this vector, the luciferase gene is driven by the thymidine kinase (TK) promoter (–105/+51) of herpes

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<sup>2</sup> The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; TNF, tumor necrosis factor; HDAC, histone deacetylase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; EMSA, electrophoretic mobility shift assay; HEK, human embryonic kidney; ChIP, chromatin immunoprecipitation assay; WT, wild type; GFP, green fluorescent protein; IL, interleukin;  $\text{ssI}\kappa\text{B}\alpha$ , supersuppressor I $\kappa$ B $\alpha$ ; RXR, retinoid X receptor; SMRT, silencing mediator for retinoic and thyroid hormone receptors; NCoR, nuclear corepressor; Trog, troglitazone; RT, reverse transcriptase.

simplex virus. The PPAR $\gamma$ -specific reporter was generated by inserting three copies of the PPRE element of the rat acyl-CoA synthase gene (<sup>583</sup>C-CTTTCCCGAACGTGACCTTTGTCCTGGTCCCCTTTTGCT<sup>544</sup>) (27) at the upstream of TK promoter. Mammalian expression vectors for PPAR $\gamma$ 2, RXR $\alpha$ , and IKK2, have been described elsewhere (28–30). The supersuppressor I $\kappa$ B $\alpha$  expression vector, and I $\kappa$ B $\alpha$ <sup>-/-</sup> and p65<sup>-/-</sup> MEFs were originally obtained from Dr. Inder M. Verma (Salk Institute). p50<sup>-/-</sup> MEFs were made from p50 knock-out (p50<sup>-/-</sup>) embryo of 13 days. Antibodies to I $\kappa$ B $\alpha$  (sc-371), p65 (sc-8008), GLUT4 (sc-7938), PPAR $\gamma$  (sc-7373X), Sp-3 (sc-644X), and Pol II (sc-9001) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).  $\beta$ -Actin (ab6276), and HDAC3 (ab2379) antibodies were obtained from Abcam (Cambridge, UK). Antibodies to HDAC1 (H6287) and adiponectin (MAB3832) were from Sigma and Chemicon International, respectively. SMRT antibody (PAI-842) was from Affinity BioReagents (Golden, CO). RNAi expression vectors for SMRT, NCoR, HDAC1, HDAC2, and HDAC3 were described previously (31, 32).

**Transfection and Luciferase Assay**—Cell lines including HEK293 and 3T3-L1 are purchased from the American Type Culture Collection and maintained in cell culture according to the guidelines recommended by the provider. Transient transfection was conducted in triplicate in 12-well plates. Cells ( $1.5 \times 10^5$ /well) were plated for 16 h and transfected with plasmid DNA utilizing Lipofectamine 2000. The PPAR $\gamma$  reporter system was constituted utilizing 0.2  $\mu$ g each of PPRE (3 $\times$ )-luciferase, PPAR $\gamma$ 2, and RXR $\alpha$  in each well. In the cotransfection assay, 0.2  $\mu$ g of plasmid DNA was used unless indicated in the figure legend. The cells were treated with 1  $\mu$ M troglitazone (Trog) for 16 h to activate PPAR $\gamma$ 2 after transfection for 24 h. For TNF treatment, the cells were treated with troglitazone in serum-free medium for 16 h, followed by TNF treatment for 5 h. In all of the transient transfection experiments, the internal control was 0.1  $\mu$ g/well of SV40 *Renilla* luciferase reporter plasmid, and the total DNA concentration was corrected in each well with a control plasmid. The luciferase assay was conducted using the dual luciferase substrate system (Promega) with a 96-well luminometer. The luciferase activity was normalized with the internal control *Renilla* luciferase, and a mean value together with a standard error of the triplicate samples were used to determine the reporter activity. Each experiment was repeated at least three times.

**The Supersuppressor I $\kappa$ B $\alpha$  (ssI $\kappa$ B $\alpha$ ) Cell Line and 3T3-L1 Adipocytes**—To make the ssI $\kappa$ B $\alpha$  stable cell line, 3T3-L1 fibroblasts were infected with pBabe retrovirus that carries the FLAG-ssI $\kappa$ B $\alpha$  expression cassette. The positive clone was selected by culturing the infected cells in puromycin-containing medium for 2 days followed by a screening of the FLAG epitope in the whole cell lysate in an immunoblot. 3T3-L1 adipocytes were obtained by differentiation of the fibroblasts in the standard adipogenic mixture as described elsewhere (33). The control cells were made by infection of 3T3-L1 fibroblasts with the empty retrovirus.

**Immunoblotting and Coimmunoprecipitation**—The whole cell lysate, nuclear, and cytoplasmic extracts were made as described elsewhere (29). In immunoprecipitations, the conditions were as follows: the cell extract (400  $\mu$ g), 2–4  $\mu$ g of antibody, and 20  $\mu$ l of protein G-Sepharose beads (Amersham Biosciences) were incubated for 3–4 h at 4  $^{\circ}$ C. The immune complex was washed five times in a cell lysis buffer before immunoblotting. The product was resolved in 7% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane for immunoblotting. Blotting of the membrane was conducted in a milk buffer with the first antibody for 1–24 h and horseradish peroxidase-conjugated secondary antibody for 30 min. To detect multiple signals from one membrane, the membrane was treated with a stripping buffer (59 mM Tris-HCl, 2% SDS, 0.75% 2-mercaptoethanol) for 20 min at 37  $^{\circ}$ C before the next blotting.

**Cytoplasmic and Nuclear Extracts**—Cytoplasmic and nuclear proteins were prepared as described in an earlier study (34). Immunoblotting and coimmunoprecipitation were conducted according to protocols used previously (29).

**Electrophoretic Mobility Shift Assay (EMSA)**—HEK293 cells ( $2 \times 10^6$ ) were plated in the 100-mm cell culture plate, and transfected with PPAR $\gamma$ 2 plasmid DNA (1  $\mu$ g) with Lipofectamine 2000 as indicated. In cotransfection experiments, 1  $\mu$ g of each test plasmid was used unless otherwise indicated. The nuclear extract was made from 3T3-L1 adipocytes or HEK293 cells transfected with expression vectors for PPAR $\gamma$ 2 and RXR $\alpha$ . The assay was conducted as described elsewhere (34). The EMSA probes for PPAR $\gamma$  contains the PPRE element in the acyl-CoA synthase promoter. The probe was labeled with <sup>32</sup>P and used in EMSA with the nuclear extract. For oligonucleotide competition and antibody supershift experiments, a 50-fold excess of unlabeled oligonucleotide probe and 2  $\mu$ g of IgG were used at each point, respectively.

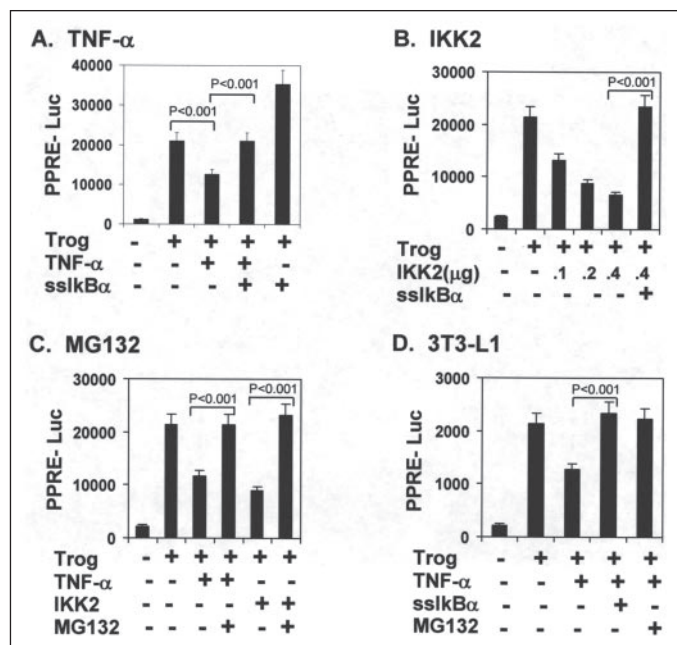
**Chromatin Immunoprecipitation (ChIP)**—The ChIP protocol was developed from a published study (35). Cells were maintained in a 100-mm cell culture plate, treated with TNF- $\alpha$  (10 ng/ml) after serum-starvation overnight, and collected after formaldehyde treatment. The chromatin DNA was extracted, broken into fragments of 400–1200 bp in length by sonication, and immunoprecipitated with antibodies to the target, such as PPAR $\gamma$ , HDAC3, SMRT, and Pol II. IgG was used in immunoprecipitation as a control for nonspecific signal. DNA in the immunoprecipitation product was amplified in PCR with the ChIP assay primers that cover the two PPRE sites (ARE6 and ARE7) in the *aP2* gene promoter (36): Forward, 5'-GGAATCAGGTAGCTG-GAGAATCGC-3'; reverse, 5'-GGCTTGATTGTTACAAGGCAAG-GAA-3'. This pair of primers has an annealing temperature of 59  $^{\circ}$ C, and yields a product of 280 bp. The image of the PCR product is presented in reversed black/white in which the DNA band is in black. The PCR products were quantitated for signal intensity.

**Quantitative Real Time RT-PCR**—The mRNA level of aP2 (fatty acid-binding protein 4) was determined using Taqman quantitative real time RT-PCR. The total RNA was extracted using the TRIzol protocol. The real time RT-PCR reaction was conducted in triplicate using the Taqman probe and primers set for aP2 (Mm00445880\_m1, Applied Biosystems).  $\beta$ -Actin was used as a control for normalization of the aP2 signal. The mean value of the triplicates was used to indicate the mRNA level of aP2.

**Statistical Analysis**—Each experiment was conducted at least three times with consistent results. The representative gel or blot is presented in this article. In the PPAR $\gamma$  reporter assay, mean value and S.D. of the triplicates are used to represent the reporter activity. The data were analyzed using Student's *t* test with significance  $p < 0.05$ .

## RESULTS

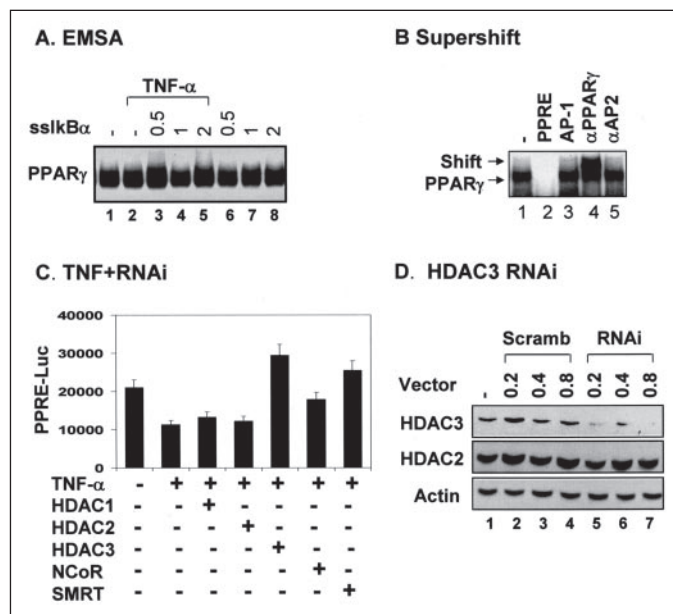
**ssI $\kappa$ B $\alpha$  Abolishes TNF Inhibition of PPAR $\gamma$  Function in Reporter Assay**—It is well known that TNF- $\alpha$  induces lipolysis in mature adipocytes and inhibits differentiation of preadipocytes. Because PPAR $\gamma$  controls transcription of a variety of genes for lipid biosynthesis and adipogenesis, PPAR $\gamma$  has been a target in the search for the molecular mechanism of TNF- $\alpha$  activity. In addition to the inhibition of PPAR $\gamma$  expression, TNF- $\alpha$  may reduce the transcriptional activity of PPAR $\gamma$  through two possible mechanisms: (a) phosphorylation of PPAR $\gamma$  by activation of serine kinases (ERK or JNK) (4, 25, 26, 37) and (b) suppression of DNA binding of PPAR $\gamma$  by activation of NF- $\kappa$ B (7). In the regulation of PPAR $\gamma$  function, TNF- $\alpha$  activity was investigated in this study with a focus on the role of I $\kappa$ B $\alpha$ . The initial observation was made using a PPAR $\gamma$  reporter system in the transient transfection of HEK293 cells,



**FIGURE 1. Inhibition of PPAR $\gamma$  reporter activity by TNF- $\alpha$ .** The transcriptional activity of PPAR $\gamma$  was analyzed in HEK293 cells and 3T3-L1 fibroblasts using the PPRE(3 $\times$ )-luciferase reporter system in the transient transfection. The transfection and data analysis were performed as stated under "Experimental Procedures." *A*, inhibition of TNF- $\alpha$  activity by sslk $\beta$  in HEK293 cells. The reporter activity was induced with Trog (1  $\mu$ M). TNF- $\alpha$  treatment was conducted at a final concentration of 10 ng/ml unless otherwise indicated. *B*, inhibition of IKK activity by sslk $\beta$  in HEK293 cells. *C*, inhibition of TNF- $\alpha$  and IKK activities by proteasome inhibitor MG-132 at a final concentration of 15  $\mu$ M. *D*, inhibition of TNF- $\alpha$  activity by sslk $\beta$  or MG-132 in 3T3-L1 adipocytes.

in which the reporter activity was induced by the synthetic ligand troglitazone (Fig. 1A). TNF- $\alpha$  led to 40% reduction in the PPAR $\gamma$  reporter. The reduction was completely blocked by expression of the sslk $\beta$ , a nondegradable mutant of I $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ <sup>S32A/S36A</sup>). A similar result was observed when TNF- $\alpha$  was replaced by IKK2 in the reporter system (Fig. 1B). Because sslk $\beta$  is not degradable, these results suggest that the TNF- $\alpha$  activity is dependent on degradation of the I $\kappa$ B $\alpha$  protein. In support of this possibility, the proteasome inhibitor MG132 that blocks I $\kappa$ B $\alpha$  degradation also eliminated activity of TNF- $\alpha$  or IKK2 (Fig. 1C). The experiment was repeated using 3T3-L1 cells, and a similar result was obtained (Fig. 1D), suggesting that the function of I $\kappa$ B $\alpha$  is not influenced by cell type. Of interest, sslk $\beta$  was able to enhance the reporter activity in the absence of TNF- $\alpha$  (Fig. 1A). Therefore, the molecular basis of sslk $\beta$  activity was further investigated in this study.

**Nuclear Corepressor Is Involved in TNF- $\alpha$  Activity**—Transactivation by PPAR $\gamma$  is regulated by events such as the association of nuclear receptor coactivator and the DNA binding activity of PPAR $\gamma$ . The coactivator association is determined by the corepressor that is composed of HDAC3/SMRT or HDAC3/NCoR. The corepressor is associated with PPAR $\gamma$  in the absence of PPAR $\gamma$  ligand (38, 39). To investigate how PPAR $\gamma$  function is repressed after I $\kappa$ B $\alpha$  degradation, the DNA binding activity of PPAR $\gamma$  was examined using the EMSA assay. The assay was done using the nuclear extract of 293 cells transfected with PPAR $\gamma$ . The results show that the PPAR $\gamma$  DNA binding activity was not reduced by either TNF- $\alpha$  or sslk $\beta$  (Fig. 2A), suggesting that the suppression of PPAR $\gamma$  activity by TNF- $\alpha$  is not a result of loss of DNA binding activity. Because sslk $\beta$  did not change the DNA binding activity, the enhancement of PPAR $\gamma$  activity by sslk $\beta$  in the absence of TNF- $\alpha$  may be achieved through a DNA-independent mechanism. These data suggest that TNF- $\alpha$  can modulate PPAR $\gamma$  function through a mechanism inde-

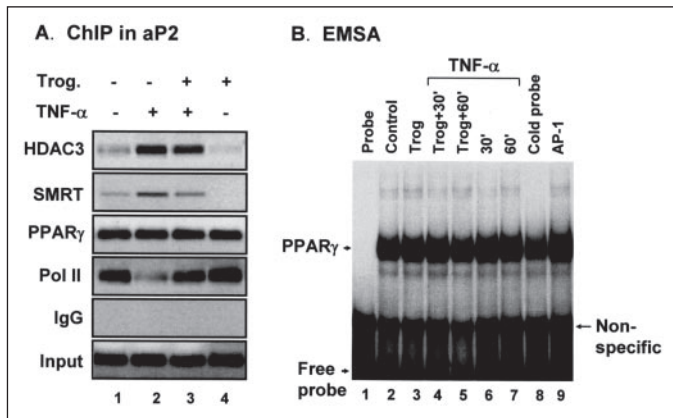


**FIGURE 2. HDAC3 and SMRT in TNF-induced PPAR $\gamma$  inhibition.** *A*, EMSA for DNA binding activity of PPAR $\gamma$ . The assay was done with the nuclear extract of HEK293 cells that were transfected with the PPAR $\gamma$ 2 expression vector. sslk $\beta$  expression vector was co-transfected at dosages ( $\mu$ g) indicated at the top of each lane. The nuclear extract was made after 2-h treatment with TNF- $\alpha$  (10 ng/ml). *B*, confirmation of PPAR $\gamma$ -DNA complex in supershift assay. *C*, analysis of corepressors with RNAi-mediated gene knock-down in the PPAR $\gamma$  reporter assay. RNAi expression vectors for HDAC1-3, NCoR, and SMRT were co-transfected with the reporter system into HEK293 cells at 0.2  $\mu$ g/point. The reporter was induced by Trog and inhibited by TNF- $\alpha$ . *D*, test of RNAi effect on HDAC3 expression in an immunoblot. HDAC3 protein level was determined in HEK293 cells transfected with control vector or RNAi expression vector. The dosage ( $\mu$ g) of each vector is shown at the top of each lane.

pendent of DNA binding. The nature of the PPAR $\gamma$ -DNA complex was confirmed in oligo competitions and supershift assays (Fig. 2B).

To examine the role of the corepressor complex, we tested HDAC1, HDAC2, HDAC3, SMRT, and NCoR with RNAi-mediated gene knock-down. The specificity and efficacy of the RNAi vectors used here were all confirmed in previous studies by determining the target protein in the whole cell lysate (31, 32, 40). The data for HDAC3 RNAi are presented here as an example to show that knock-down is significant in this experimental system (Fig. 2D). To save space, the knock-down results for HDAC1, HDAC2, SMRT, and NCoR are not shown here. We focused on HDAC3 and SMRT in this study because significant effects were observed with knock-down of these two molecules. Inhibition of either HDAC3 or SMRT led to abolishment of TNF- $\alpha$  activity in the suppression of PPAR $\gamma$  (Fig. 2C), suggesting that HDAC3 and SMRT are the major corepressor proteins for PPAR $\gamma$ . A decrease in NCoR also led to partial protection of PPAR $\gamma$  function. Under the same conditions, inhibition of HDAC1 and HDAC2 failed to generate the effect. Because inhibition of the corepressor protein HDAC3, SMRT, or NCoR led to protection of PPAR $\gamma$  activity, the data suggest that TNF-induced inhibition of PPAR $\gamma$  is dependent on the corepressor function.

**TNF- $\alpha$  Induces Recruitment of the Corepressor Proteins to PPAR $\gamma$** —It is believed that association of the corepressor with PPAR $\gamma$  is required for inhibition of the transcriptional activity of PPAR $\gamma$ . The above data suggest that TNF- $\alpha$  may act by enhancing recruitment of the corepressor to PPAR $\gamma$ . To test this possibility, the association of the corepressors with PPAR $\gamma$  was examined in the *ap2* (FABP-4) gene promoter using the ChIP assay. In mature 3T3-L1 adipocytes, the signal of HDAC3 was increased in the gene promoter after TNF treatment (Fig. 3A). This effect of TNF- $\alpha$  was reduced by Trog, which decreased the HDAC3 signal and increased the signal for RNA polymerase II (Pol II), an indi-

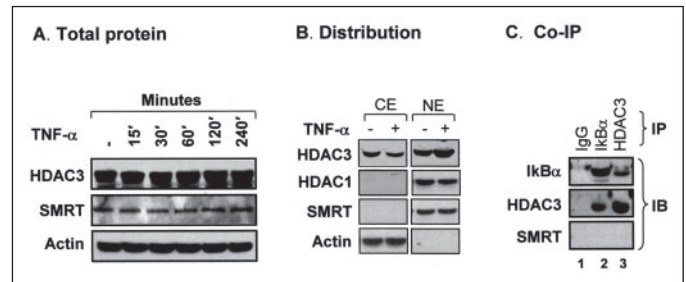


**FIGURE 3. HDAC3 interaction with PPAR $\gamma$  in adipocytes.** *A*, ChIP assay for the interaction of HDAC3 and PPAR $\gamma$  in the *aP2* gene promoter. The assay was conducted in 3T3-L1 mature adipocytes after TNF- $\alpha$  treatment (10 ng/ml, 30 min). *B*, DNA binding activity of PPAR $\gamma$  in EMSA. The nuclear extract was made from the mature 3T3-L1 adipocytes after TNF- $\alpha$  treatment at different times as indicated at the top of each lane. Unlabeled PPPE probe or AP-1 probe was used in oligo competition. In these two experiments, Trog was used at a final concentration of 1  $\mu$ M for 24 h of treatment.

erator of transcription initiation. Similarly, the SMRT signal was also increased by TNF- $\alpha$  (Fig. 3A), and the increase was also attenuated by Trog. This line of evidence suggests that TNF- $\alpha$  acts through increasing association of the corepressor HDAC3/SMRT with PPAR $\gamma$ . Although the corepressor association was modulated by TNF- $\alpha$ , the DNA binding activity of PPAR $\gamma$  was not changed before or after TNF treatment (Fig. 3A). In the EMSA assay, DNA binding activity of PPAR $\gamma$  was examined in the adipocyte nuclear extract. Similar to that observed in 293 cells, no significant reduction was observed for PPAR $\gamma$  (Fig. 3B). These data consistently support the notion that corepressors, but not DNA binding, are involved in PPAR $\gamma$  inhibition by TNF- $\alpha$ .

**HDAC3 Is a Component of the I $\kappa$ B $\alpha$ -NF- $\kappa$ B Complex**—The data above suggest that the recruitment of HDAC3 and SMRT may be responsible for PPAR $\gamma$  inhibition by TNF- $\alpha$ . We hypothesized that the recruitment might be related to I $\kappa$ B $\alpha$  degradation because TNF- $\alpha$  activity was abolished by *ssI $\kappa$ B $\alpha$* . I $\kappa$ B $\alpha$  degradation may contribute to the enhanced recruitment of corepressors by changing total protein or intracellular distribution of HDAC3/SMRT. To test these possibilities, the total protein for HDAC3 and SMRT was determined in the whole cell lysate of 3T3-L1 adipocytes. Results do not support a change in the protein abundance after TNF- $\alpha$  treatment (Fig. 4A). The intracellular distribution of HDAC3 and SMRT was determined by examining their protein abundance in cytoplasmic and nuclear extracts. Before TNF treatment, the HDAC3 protein was detected in both cytoplasmic and nuclear extracts (Fig. 4B). With TNF treatment, the HDAC3 level was significantly decreased in the cytoplasm, but increased in nuclear extracts at 15 min (Fig. 4B). Such a quick and correlated HDAC3 change in the two different subcellular compartments suggested a role of nuclear translocation in the regulation of HDAC3 redistribution. The peak time of HDAC3 in the nucleus is correlated to that of HDAC3 recruitment in the *aP2* promoter, which was observed in the ChIP assay. In the experiment, HDAC1 and SMRT were only detected in the nucleus, and their subcellular distribution was not influenced by TNF- $\alpha$  (Fig. 4B). According to these data, we hypothesized that HDAC3 might be important in the inhibition of PPAR $\gamma$  by TNF- $\alpha$ .

HDAC3 translocation may be controlled by I $\kappa$ B $\alpha$  because TNF- $\alpha$  activity is dependent on I $\kappa$ B $\alpha$  degradation. To test this possibility, the study was focused on HDAC3-I $\kappa$ B $\alpha$  interaction, because the nuclear translocation may be a consequence of I $\kappa$ B $\alpha$  degradation. The association of HDAC3 with I $\kappa$ B $\alpha$  was investigated using coimmunoprecipita-

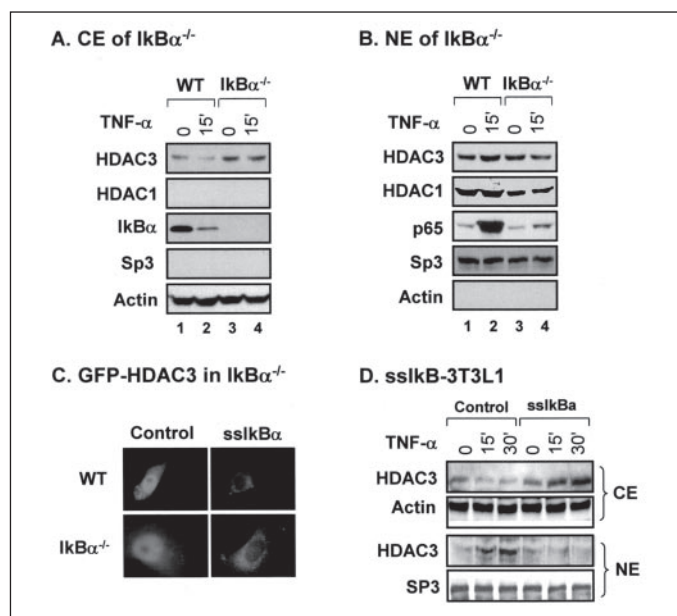


**FIGURE 4. Nuclear translocation of HDAC3 induced by TNF- $\alpha$ .** *A*, total protein of HDAC3 in 3T3-L1 adipocytes. The mature 3T3-L1 adipocytes were serum-starved overnight and treated with TNF- $\alpha$  for different times as indicated. The whole cell lysate was made and used in the immunoblot. *B*, immunoblot of the cytoplasmic and nuclear extracts of mature 3T3-L1 adipocytes. The cytoplasmic (CE) and nuclear (NE) extracts were made as described under "Experimental Procedures." The protein abundance of HDAC3 and SMRT was examined in an immunoblot. HDAC1 and actin were controls in the nuclear and cytoplasmic extracts, respectively. *C*, association of I $\kappa$ B $\alpha$  and HDAC3 in coimmunoprecipitation. The cytoplasmic extract was made from 3T3-L1 adipocytes and used in the coimmunoprecipitation. IgG was a control for nonspecific signal.

tion (Fig. 4C). The experiment was conducted using the cytoplasmic extract of 293 cells. As expected, HDAC3 was found in the immunoprecipitation product of I $\kappa$ B $\alpha$  antibody, and I $\kappa$ B $\alpha$  was identified in the immunoprecipitation product of HDAC3 antibody. These data suggest that I $\kappa$ B $\alpha$  and HDAC3 coexist in the same protein complex in cells. However, in this experiment, SMRT was not detected in the immunoprecipitation product of either I $\kappa$ B $\alpha$  or HDAC3, suggesting that HDAC3 does not associate with SMRT in the cytoplasm. This is consistent with the observation that SMRT was not detected in the cytoplasm (Fig. 4B).

**I $\kappa$ B $\alpha$  Controls Nuclear Translocation of HDAC3**—The functional significance of I $\kappa$ B $\alpha$ -HDAC3 association may be retention of HDAC3 in the cytoplasm. If this hypothesis is correct, HDAC3 should remain in the nucleus in the absence of I $\kappa$ B $\alpha$ . To test this possibility, I $\kappa$ B $\alpha$ <sup>-/-</sup> MEF cells were compared with the wild-type (WT) MEFs in this study. In the wild-type MEFs, the HDAC3 protein was found in both cytoplasmic and nuclear extracts by immunoblotting (Fig. 5, A and B). With TNF treatment, the decrease in cytoplasmic HDAC3 was associated with I $\kappa$ B $\alpha$  degradation (Fig. 5A). Corresponding to the decrease in the cytoplasm, HDAC3 was increased in the nucleus. This was accompanied by nuclear translocation of NF- $\kappa$ B p65 (Fig. 5B). As a control, HDAC1 was not detected in the cytoplasm, and its abundance was not changed in the nucleus after TNF- $\alpha$  treatment. In I $\kappa$ B $\alpha$ <sup>-/-</sup> MEF cells, the HDAC3 protein was not detectable in the cytoplasm (Fig. 5, A and B), but only detected in the nuclear extract. The nuclear abundance of HDAC3 was significantly higher than that of WT cells. In I $\kappa$ B $\alpha$ <sup>-/-</sup> cells, TNF- $\alpha$  was unable to increase the nuclear abundance of HDAC3. The data suggest that I $\kappa$ B $\alpha$  is required for the cytoplasmic localization of HDAC3. In the I $\kappa$ B $\alpha$ -null cells, HDAC1 abundance was reduced. This suggests that I $\kappa$ B $\alpha$  also regulates HDAC1 activity. Because HDAC1 has not been reported in the regulation of PPAR $\gamma$  function, HDAC1 was not further investigated here. The quality of the cytoplasmic and nuclear extracts is indicated by the control signals such as actin in the cytoplasm and Sp3 in the nucleus.

Distribution of HDAC3 was corrected in I $\kappa$ B $\alpha$ <sup>-/-</sup> MEFs by reconstitution of I $\kappa$ B $\alpha$ . Regulation of HDAC3 by I $\kappa$ B $\alpha$  was further examined using a GFP-HDAC3 fusion protein (Fig. 5C). In the wild-type MEFs, GFP-HDAC3 was detected in both cytoplasmic and nuclear compartments after transient transfection with a GFP-HDAC3 expression vector. GFP-HDAC3 was enriched in the cytoplasm by coexpression of *ssI $\kappa$ B $\alpha$* . In transient transfection of I $\kappa$ B $\alpha$ <sup>-/-</sup> MEFs, GFP-HDAC3 was concentrated in the nucleus and was not observed in the cytoplasm.



**FIGURE 5. Regulation of nuclear translocation of HDAC3 by I $\kappa$ B $\alpha$ .** *A*, immunoblot of the cytoplasmic extract of WT and I $\kappa$ B $\alpha$ <sup>-/-</sup> MEFs. The cells were treated with TNF- $\alpha$  for 15 min as indicated. HDAC3 protein was determined in the cytoplasmic extract in an immunoblot. HDAC1 and Sp3 were used as negative controls since they are nuclear proteins. I $\kappa$ B $\alpha$  and actin were positive controls in the cytoplasmic extract. *B*, immunoblot of the nuclear extract of wild type and I $\kappa$ B $\alpha$ <sup>-/-</sup> MEFs. HDAC1, p65, and Sp3 were positive controls in the nuclear extract. Actin was a negative control. *C*, intracellular distribution of GFP-HDAC3 in WT and I $\kappa$ B $\alpha$ <sup>-/-</sup> MEFs. The cells were transiently transfected with expression vectors for GFP-HDAC3 and ssI $\kappa$ B $\alpha$ . The photographs were taken 48 h later under a fluorescence microscope with oil lens ( $\times 100$ ). *D*, immunoblot of the cytoplasmic (CE) and nuclear extracts (NE) from the control and ssI $\kappa$ B $\alpha$  cell lines.

Coexpression of ssI $\kappa$ B $\alpha$  led to GFP-HDAC3 decrease in the nucleus, and an increase in the cytoplasm. These data further support the role of I $\kappa$ B $\alpha$  in the control of subcellular distribution of HDAC3. I $\kappa$ B $\alpha$  activity was also observed in 3T3-L1 cells that were engineered with FLAG-ssI $\kappa$ B $\alpha$ . The cytoplasmic abundance of HDAC3 was significantly increased in the FLAG-ssI $\kappa$ B $\alpha$  cells in comparison to the control 3T3-L1 cells (Fig. 5D). This change was associated with a decrease in nuclear HDAC3. As expected, TNF-induced nuclear translocation of HDAC3 was not detectable in this stable cell line. Collectively, the data consistently support that I $\kappa$ B $\alpha$  regulates nuclear translocation of HDAC3.

**ssI $\kappa$ B $\alpha$  Promotes Lipid Accumulation in 3T3-L1 Adipocytes**—The observations above suggest that ssI $\kappa$ B $\alpha$  should be able to promote PPAR $\gamma$  function by limiting HDAC3 access to PPAR $\gamma$ . If this is correct, the ssI $\kappa$ B $\alpha$ -3T3-L1 cells should exhibit an accelerated lipid accumulation during adipogenesis, and become resistant to the inhibitory activity of TNF- $\alpha$ . To test these possibilities, adipogenesis was induced in the control and ssI $\kappa$ B $\alpha$  cells (3T3-L1) in the absence or presence of TNF- $\alpha$ . Without TNF- $\alpha$ , both cell lines are well differentiated into adipocytes after exposure to the adipogenic cocktail (Fig. 6A). However, the lipid droplets were much bigger in the ssI $\kappa$ B $\alpha$  cells as indicated in the photograph under the microscope. In the presence of TNF- $\alpha$ , differentiation of ssI $\kappa$ B $\alpha$  cells was not inhibited, as indicated by the expression of molecular markers of adipocytes, such as adiponectin, aP2, PPAR $\gamma$ , and GLUT4 (Fig. 6B). Expression of these markers was significantly reduced by TNF- $\alpha$  in control cells, but not in ssI $\kappa$ B $\alpha$  cells, suggesting that ssI $\kappa$ B $\alpha$  cells are resistant to TNF- $\alpha$ . In this study, cells were exposed to TNF- $\alpha$  along the course of adipogenesis for 7 days. This chronic treatment led to inhibition of PPAR $\gamma$  protein expression as indicated by its protein level (Fig. 6B, PPAR $\gamma$ ), suggesting that TNF- $\alpha$  may block differentiation of preadipocytes into adipocytes through suppression of PPAR $\gamma$  expres-

sion. This activity of TNF- $\alpha$  might be dependent on activation of the NF- $\kappa$ B pathway because inhibition of the NF- $\kappa$ B pathway by ssI $\kappa$ B $\alpha$  leads to resistance to TNF- $\alpha$ . HDAC3 translocation may contribute to this inhibition.

To test TNF- $\alpha$  inhibition of the ligand-dependent activity of PPAR $\gamma$ , the interaction of HDAC3 and PPAR $\gamma$  was examined in 3T3-L1 adipocytes using the ChIP assay. In the absence of TNF- $\alpha$ , the interaction was detectable in the control adipocytes, but significantly reduced in the ssI $\kappa$ B $\alpha$  adipocytes (Fig. 6C). In the presence of TNF- $\alpha$ , the interaction was significantly enhanced by TNF- $\alpha$  in control cells (Fig. 6C). In the ssI $\kappa$ B $\alpha$  cells, the TNF-mediated enhancement was remarkably reduced. The change in HDAC3-PPAR $\gamma$  interaction was correlated to the Pol II signal that was reduced in control cells, but not in ssI $\kappa$ B $\alpha$  cells. The DNA binding activity of PPAR $\gamma$  was not changed by TNF- $\alpha$  in either cell line as PPAR $\gamma$  signal was not reduced. Because nuclear translocation of HDAC3 was blocked by ssI $\kappa$ B $\alpha$ , these results suggest that in adipocytes, HDAC3 translocation is responsible for the enhanced interaction between HDAC3 and PPAR $\gamma$ , and ssI $\kappa$ B $\alpha$  is able to block the translocation induced by TNF- $\alpha$ .

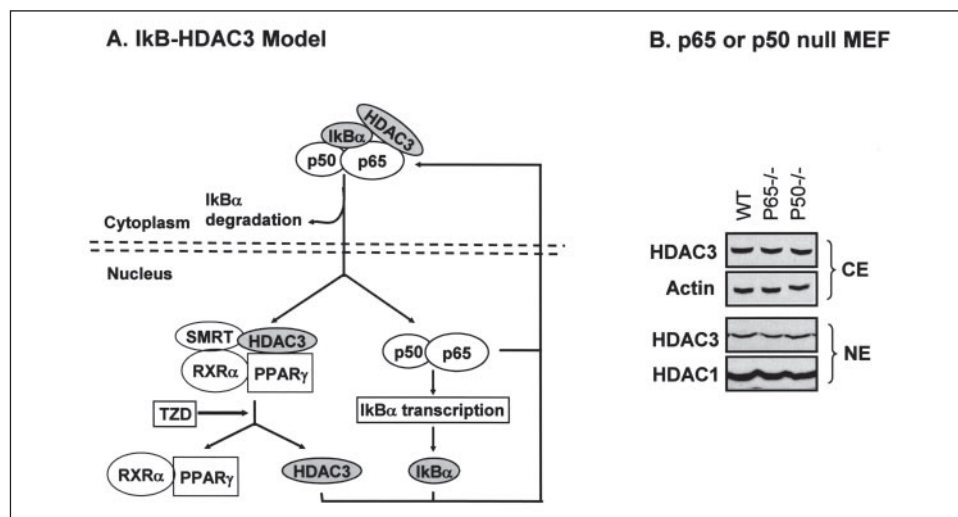
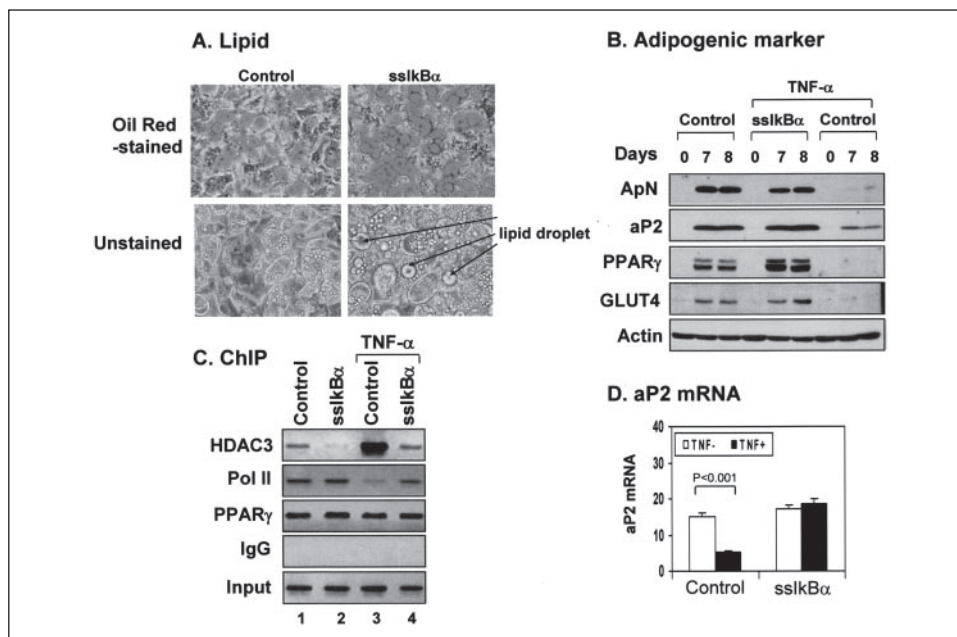
The interaction of HDAC3 and PPAR $\gamma$  is reflected in the transcriptional suppression of the PPAR $\gamma$  target gene. *aP2* is a well established target gene for PPAR $\gamma$ . *aP2* expression was reduced by TNF- $\alpha$  in the control adipocytes, but not in the ssI $\kappa$ B $\alpha$  adipocytes (Fig. 6D), suggesting that HDAC3 is required for TNF-mediated inhibition of PPAR $\gamma$  target gene expression. Collectively, these data suggest that the TNF- $\alpha$  uses two different mechanisms in the inhibition of adipogenesis and adipocyte de-differentiation. In the inhibition of adipogenesis, TNF- $\alpha$  blocks gene expression of PPAR $\gamma$  leading to a low level of PPAR $\gamma$  protein. In the mature adipocytes, TNF- $\alpha$  induces de-differentiation through inhibition of the ligand-dependent PPAR $\gamma$  activity. Nuclear translocation of HDAC3 is required for the latter activity of TNF- $\alpha$ . HDAC3 translocation may be involved in both mechanisms because I $\kappa$ B $\alpha$  degradation is required in either case.

## DISCUSSION

IKK is a major kinase mediating TNF regulation of PPAR $\gamma$  function. TNF- $\alpha$  is able to activate several serine kinases that have been reported to regulate the ligand-dependent activity of PPAR $\gamma$ . Three of the kinases (IKK, ERK, and JNK) were shown to inhibit (4, 7, 8, 25, 26, 37), but one (p38) to enhance the function of PPAR $\gamma$  (41–44). Although all of the four serine kinases are activated by TNF- $\alpha$  (45), results from this and other studies suggest that IKK is a dominant kinase in the regulation of PPAR $\gamma$  function by TNF- $\alpha$  (7, 8). The key evidence is that inhibition of PPAR $\gamma$  function by TNF- $\alpha$  is completely blocked by inactivation of IKK or its downstream event. The IKK/NF- $\kappa$ B signaling pathway also represents a major avenue for IL-1 inhibition of PPAR $\gamma$  function (5, 7, 8, 46). ERK and JNK were shown to inhibit PPAR $\gamma$  function by a direct phosphorylation of serine residues in PPAR $\gamma$  (4, 25, 26, 37), such as Ser<sup>112</sup> in PPAR $\gamma$ 2 (4). Because ERK and JNK are the major kinases in the signaling pathways of EGF (epidermal growth factor) and FGF (fibroblast growth factor) (47), these kinases may play an important role in the inhibition of adipogenesis by EGF and FGF (48–50).

HDAC3 is required for the inhibition of ligand-dependent activity of PPAR $\gamma$  by TNF- $\alpha$ . Our data support that TNF- $\alpha$  is able to inhibit PPAR $\gamma$  activity at two different levels. In the chronic (>16 h) treatment, TNF- $\alpha$  reduces expression of PPAR $\gamma$  in adipocytes. In the acute treatment, TNF- $\alpha$  inhibits the ligand-dependent activity without decreasing PPAR $\gamma$  expression or its DNA binding activity. Results from this and other studies suggest that both chronic and acute inhibition are dependent on the IKK/NF- $\kappa$ B pathway (6, 8). Regarding the acute effect, NF- $\kappa$ B

**FIGURE 6. Inhibition of TNF- $\alpha$  activity by *sslk*B $\alpha$  in 3T3-L1 adipocytes.** A, lipid droplet in adipocytes from control and *sslk*B $\alpha$  cells. The photo was made at day 7 of differentiation under a microscope at  $\times 400$  magnification. The large lipid droplets are highlighted by arrows. B, molecular markers of adipocytes in immunoblots. The cells were treated with TNF- $\alpha$  during adipogenesis. Adipogenesis was determined by the adipocyte-specific markers in the whole cell lysate at days 7 and 8 of differentiation. The markers include adiponectin (*ApN*), fatty acid-binding protein 4 (*aP2*), PPAR $\gamma$  and glucose transporter 4 (*GLUT4*). C, HDAC3-PPAR $\gamma$  interaction in adipocytes. Mature adipocytes were obtained by differentiation of 3T3-L1 fibroblasts in the presence of adipogenic cocktail for 7 days. The CHIP assay was conducted to determine the interaction in adipocytes after TNF- $\alpha$  treatment for 30 min. The cells were serum-starved for 4 h before exposure to TNF- $\alpha$  (10 ng/ml). D, inhibition of *aP2* mRNA expression by TNF- $\alpha$  in mature adipocytes. *aP2* mRNA was determined by quantitative real time RT-PCR in 3T3-L1 adipocytes after overnight TNF- $\alpha$  treatment.



**FIGURE 7. Regulation of HDAC3 cytoplasm-nucleus shuttling by I $\kappa$ B $\alpha$ .** A, I $\kappa$ B-HDAC3 model. HDAC3 stays in the cytoplasm through association with I $\kappa$ B $\alpha$ . After I $\kappa$ B $\alpha$  degradation, HDAC3 enters the nucleus where it inhibits the transcriptional activity of PPAR $\gamma$  through histone deacetylation. When the newly synthesized I $\kappa$ B $\alpha$  is available, it associates with nuclear HDAC3 and transfers HDAC3 into the cytoplasm. HDAC3 may enter and leave the nucleus through this mechanism. In this model, the role of I $\kappa$ B $\alpha$  is highlighted in the control of cytoplasm-nucleus shuttling of HDAC3. B, HDAC3 distribution in *p65*<sup>-/-</sup> or *p50*<sup>-/-</sup> MEFs. HDAC3 abundance was determined in the cytoplasmic and nuclear extracts of the wild-type and the knock-out MEFs in an immunoblot.

was reported to reduce the DNA binding activity of PPAR $\gamma$  (7). Protein-protein interaction between NF- $\kappa$ B and PPAR $\gamma$  was proposed to mediate the inhibition, and PGC-2 was shown to be required for the NF $\kappa$ B-PPAR $\gamma$  interaction. Our data from ChIP and EMSA assays demonstrated that the DNA binding activity of PPAR $\gamma$  was not changed by TNF- $\alpha$  in the acute treatment. The data are consistent in adipocytes and 293 cells. The only change induced by TNF- $\alpha$  was an increased association of PPAR $\gamma$  with HDAC3/SMRT. When this change was blocked by *sslk*B $\alpha$ , TNF- $\alpha$  lost its inhibitory activity in PPAR $\gamma$ . In 293 cells, knock-down of either HDAC3 or SMRT led to abolishment of TNF- $\alpha$  activity. These data suggest that the nuclear receptor corepressor is the target of TNF- $\alpha$ . Because TNF- $\alpha$  induces nuclear translocation of HDAC3, TNF- $\alpha$  may regulate the corepressor function through HDAC3. Recently, IKK has been shown to modify SMRT activity through direct phosphorylation of SMRT protein (51). This event may also contribute to TNF inhibition of PPAR $\gamma$ , but our data suggest that HDAC3 nuclear translocation is required for the inhibition. We observed that the TNF activity was completely blocked by *sslk*B $\alpha$ , which should not influence IKK activity if phosphorylation of SMRT is

indeed induced by IKK. It is not clear if TNF- $\alpha$  can modify the phosphorylation status of HDAC3. If this does happen, nuclear translocation of HDAC3 is still necessary. The role of nuclear corepressor is also supported by our observation that the TNF- $\alpha$  inhibition was attenuated by overexpression of the nuclear receptor coactivators.<sup>3</sup> Overexpression of the coactivators is able to rescue PPAR $\gamma$  function in the presence of TNF- $\alpha$ . It is known that coactivator is able to antagonize corepressor activity through increasing histone acetylation.

Nuclear translocation of HDAC3 is controlled by I $\kappa$ B $\alpha$  in the I $\kappa$ B-HDAC3 model (Fig. 7A). HDAC3 contains both the nuclear export signal (180–313 amino acids in the central portion) and the nuclear localization signal (312–428 amino acids in the C terminus) (52). The protein structure suggests that HDAC3 may shuttle between the cytoplasm and nucleus by itself. However, the molecular events that initiate the shuttling are not clear. Our data suggest that I $\kappa$ B $\alpha$  is important in the control of HDAC3 shuttling. When I $\kappa$ B $\alpha$  is degraded, HDAC3

<sup>3</sup> Z. Gao and J. Ye, unpublished data.

enters the nucleus; when newly synthesized I $\kappa$ B $\alpha$  is available in the nucleus, HDAC3 is bound to I $\kappa$ B $\alpha$  and subject to nuclear export. This molecular model is supported by four lines of evidence: (a) Nuclear translocation of HDAC3 is coupled with I $\kappa$ B $\alpha$  degradation; (b) in I $\kappa$ B $\alpha$ <sup>-/-</sup> cells, HDAC3 is exclusively located in the nucleus; (c) ssI $\kappa$ B $\alpha$  retains HDAC3 in the cytoplasm and reduces the nuclear abundance of HDAC3; (d) HDAC3 associates with I $\kappa$ B $\alpha$  through the ankyrin repeat domain of I $\kappa$ B $\alpha$  (53). Because HDAC3 is able to regulate transcription of a variety of genes (54, 55), the I $\kappa$ B-HDAC3 model may provide new explanation to many phenomena that have been reported for I $\kappa$ B $\alpha$ , such as death of newborn mice with I $\kappa$ B $\alpha$  knock-out (I $\kappa$ B $\alpha$ <sup>-/-</sup>) (56), inhibition of limb development by I $\kappa$ B $\alpha$  in *Drosophila* (57), and enhancement of transcriptional activity of non- $\kappa$ B transcription factors by I $\kappa$ B $\alpha$  (53, 58).

The role of transcription factor NF- $\kappa$ B in the inhibition of PPAR $\gamma$  activity remains to be investigated. HDAC3 was reported to form a complex with NF- $\kappa$ B p65, and this leads to inhibition of the transcriptional activity of NF- $\kappa$ B through deacetylation (40, 59–61). However, it is not clear what mediates the HDAC3-p65 association (59, 61). Our data suggest that I $\kappa$ B $\alpha$  may be required for the association since I $\kappa$ B $\alpha$  is able to interact with both proteins. This raises a possibility about control of HDAC3 nuclear translocation by p65. If this is true, we would observe a change in HDAC3 distribution in the absence of p65. However, our data do not support this possibility. The pattern of intracellular distribution of HDAC3 was not changed in p65<sup>-/-</sup> MEFs (Fig. 7B). However, p65 may contribute to PPAR $\gamma$  inhibition by blocking I $\kappa$ B $\alpha$ -HDAC3 association in the nucleus (53). The pattern of intracellular distribution of HDAC3 was not changed in p50<sup>-/-</sup> MEFs (Fig. 7B). These data suggest that p65 and p50 are not required for HDAC3 localization in cells.

In summary, TNF- $\alpha$  inhibits PPAR $\gamma$  activity through two mechanisms. One is dependent on inhibition of PPAR $\gamma$  expression. The other is suppression of the transcriptional activity of PPAR $\gamma$  by nuclear corepressor. I $\kappa$ B $\alpha$  regulation of corepressor function may be required in both mechanisms. In this study, TNF- $\alpha$  is shown to inhibit the transcriptional activity of PPAR $\gamma$  through a pathway in which DNA binding activity of PPAR $\gamma$  is not reduced. I $\kappa$ B $\alpha$  plays an important role in this pathway by regulating nuclear translocation of HDAC3. Our observation also supports a new function for I $\kappa$ B $\alpha$ .

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