Specific cell type differentiation is driven by programmed regulation of gene expression, which is the result of coordinated modulation of the transcription machinery and chromatin-remodeling factors. We present evidence here that the down-regulation of histone deacetylases is an important process during adipocyte differentiation. In 3T3-L1 cells, histone hyperacetylation was selectively induced at the promoter regions of adipogenic genes during adipocyte differentiation. Interestingly, this was accompanied by a dramatic decrease in the expression level of several histone deacetylases including HDAC1, -2, and -5 and a reduction in overall histone deacetylase activity. Inhibition of histone deacetylase activity using sodium butyrate resulted in stimulation of adipogenic gene expression and adipocyte differentiation. Consistently, HDAC1 knock-down promoted adipogenesis whereas HDAC1 overexpression and adipocyte differentiation. Together, these results suggest that the regulation of not only adipogenic transcription factors, but also chromatin-modifying enzymes is crucial for the execution of bona fide adipogenesis.

The architecture of the eukaryotic chromatin is dynamically modulated by post-translational modifications of the histones, including acetylation, methylation, phosphorylation, and ubiquitination (1). The changes in nucleosome structure influence gene expression by modulating the accessibility of the promoter regions to specific transcription factors. In particular, acetylation of histones H3 (Lys4 and Lys14) and H4 (Lys8 and Lys12), mediated by histone acetyltransferases (HATs) such as p300, CBP, and P/CAB, is associated with transcriptional activation (2, 3). Methylation of H3 Lys4, H4 Arg3, and phosphorylation of H3 Ser10 also result in gene activation (4–7). In contrast, methylation of histone H3 Lys9 generally correlates with gene repression (8, 9).

Histone deacetylases (HDACs) repress gene expression by deacetylation of histones and other proteins, such as transcription factors (10, 11). Until now, five yeast and eleven human HDACs have been identified, which are classified into three classes (12). Class I HDACs consist of HDAC1, -2, -3, and -8. HDAC4, -5, -6, -7, -9, and -10 belong to the class II HDACs. Class III HDACs are members of the sirtuin family of HDACs (13–17). In addition, a new member of the HDAC family, HDAC11, has been recently identified (18). Class I HDACs are expressed ubiquitously, whereas class II HDACs are abundantly expressed in heart, skeletal muscle, and brain (12). The major function of HDACs is to remove acetyl groups from histones, which results in condensation of the chromatin structure. This, in turn, diminishes the access of transcription factors to the target DNA and ultimately leads to transcriptional repression.

Chromatin modifications, notably histone acetylation and deacetylation, are crucial for the regulation of gene expression and development in eukaryotes (19, 20). During tissue differentiation, early inductive processes that determine cell fate leave traces at particular genes in the form of histone modifications. HATs and HDACs have been shown to respond to signals that regulate cell differentiation and directly modulate tissue-specific gene expression. For example, studies of myogenesis have suggested a role for histone-modifying enzymes in coordinating the activation and repression of genes involved in differentiation processes (21–23). HDAC1 and members of the class II HDACs including HDAC4, -5, and -6 inhibit MyoD and Mef2, respectively, to block muscle cell differentiation (24–27).

Adipogenesis is the process by which undifferentiated precursor cells differentiate into adipocytes. In vitro studies using 3T3-L1 and 3T3-F442A cells have elucidated the complex transcriptional cascades that are essential for the execution of adipocyte differentiation (28, 29). CCAAT/enhancer-binding proteins (C/EBPα), peroxisome proliferator-activated receptor γ (PPARγ), and adipocyte determination- and differentiation-dependent factor 1/sterol response element-binding protein 1c (ADD1/SREBP1c) have been identified as the key transcription factors involved in adipogenesis (30, 31). When preadipocytes are exposed to differentiation conditions, a complex signaling cascade induces the expression of previously silent adipogenic genes. Although it is not clear how these genes are maintained quiescent in preadipocytes, it is possible to speculate that HDACs are involved.

Past research on tissue differentiation has been mainly focused on understanding the regulatory roles of specific transcription factors. On the contrary, little is known about the functional roles of coregulators such as HATs and HDACs during differentiation processes. In this study we have discovered a novel role of HDACs during adipogenesis. Our results suggest that the down-regulation of HDACs at the early stages adipogenesis is a rate-limiting step during adipogenesis. These findings add to the list of the functional roles of coregulators in regulating cell differentiation and may provide a new target for discovering novel therapies for the treatment of fat-related disorders.
MATERIALS AND METHODS

Cell Culture—3T3-L1 and 3T3-F442A mouse fibroblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum (BCS, Invitrogen), with a change of medium every 2–3 days. 3T3-L1 cells were differentiated into adipocytes as previously described (32). 3T3-F442A cells were differentiated into adipocytes by replacing the medium with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS, Invitrogen) and 5 μg/ml insulin upon reaching confluence. BOSC and H29D retrovirus packaging cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS.

Chromatin Immunoprecipitation (ChIP) Assays—Chromatin was immunoprecipitated as described previously (33). Conditions for PCR reactions were as follows: 0.25 μM of each primer, 0.1 mM of each dNTP, 1 × PCR buffer, and 1 unit Nova Taq polymerase (Genemed). and 0.06 mCi/ml [α-32P]dCTP in 20 μl of reaction volume. PCR primers used were as follows: ADD1-P5, 5′-GGT TGG TAC CAC AGT GAC CG-3′; ADD1-P3, 5′-AAT GTG CAA TCC ATG CCT CGC TGG T-3′; ADPonectin-P5, 5′-GGT GCT GGG AAT TGA ACT CA-3′; ADPonectin-P3, 5′-CCT GTT TTC AGG CTC CCG GAG-3′; aP2-P5, 5′-TTG CAG GTG AGA AAG GCT GC-3′; aP2-P3, 5′-GTC CAG TGA TCA TTG AAT TGA ACT CA-3′; PPARγ, 5′-GTC CAG TGA TCA TTG AAT TGA ACT CA-3′; CBP, 5′-GTG CAG TGA TCA TTG AAT TGA ACT CA-3′. PCR primers were amplified using primers specific for the promoter regions of the indicated genes (see “Materials and Methods” for details).

Acid Extraction of Histones and Western Blotting—Histone proteins were isolated from 3T3-L1 cells at different stages of adipogenesis. Isolated DNA was immunoprecipitated with anti-acetylated histone H3 (K9, A), anti-acetylated histone H4 (K8, B), or anti-dimethylated histone H3 (K9, C) antibodies, and amplified using primers specific for the promoter regions of the indicated genes (see “Materials and Methods” for details).

HDAC Enzyme Activity Assays—Total cellular HDAC enzymatic activity was measured using an HDAC assay kit (Upstate Biotechnology) according to the manufacturer’s protocol. Briefly, 40 μg of total cell extracts from 3T3-L1 and 3T3-F442A preadipocytes and adipocytes were incubated with a fluorometric substrate in HDAC assay buffer for 45 min at 30 °C. An activator solution was then added to release the fluorophore from the deacetylated substrates, and the fluorescence was measured in a fluorescence plate reader.

mRNA Analysis—Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. For Northern blotting, 20 μg of RNA was denatured in formamide and formaldehyde, and separated by electrophoresis on formaldehyde-containing agarose gels. RNA was transferred to Nytran membranes (Schleicher and Schuell), and the membranes were cross-linked, hybridized, and washed as described by the manufacturers. Probes were labeled by the random priming method using the Klenow fragment of DNA polymerase I (Promega) and [α-32P]dCTP (Amersham Biosciences). PPARγ, aP2, CBP, and PPARγ were used as probes. For real-time PCR analysis, cDNA was synthesized by reverse transcription using oligodT and subjected to PCR amplification with gene-specific primers in the presence of Cybergreen (Bio-Rad). Relative abundance of mRNA was calculated after normalization to 18 S ribosomal RNA. Primer sequences for real-time PCR analyses are available upon request.

Retroviral Infection—BOSC or H29D cells were transfected with either pBabe, pBabe-HDAC1, pSUPER-retro (Oligoengine), or pSUPER-retro-HDAC1 RNAi (5′-GCA GCG TCT TGA GAA C-3′) using Lipofectamine (Invitrogen). 48 h after transfection, the medium containing retroviruses was harvested, filtered, and transferred to 3T3-L1 target cells with polybrene (1 μg/ml). Infected cells were selected with 5 μg/ml puromycin (Sigma) for 7 days.

Transient Transfections and Luciferase Assays—HEK293 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% bovine calf serum. A luciferase reporter driven by multiple PRP1s (DR1- luc) was cotransfected with PPARγ and HDAC1 expression vectors using the calcium phosphate method (32). The cells were lysed and assayed for luciferase activity.

Protein Interaction Analyses—GST-pulldown assays were performed as previously described (34). Briefly, GST and GST-PPARγ fusion proteins were bound to glutathione beads and incubated with 35S-labeled HDAC1 protein. The beads were washed with TBS and analyzed by autoradiography after SDS-PAGE. Protein-protein interaction in cells was also analyzed by co-immunoprecipitation experiments. HDAC1 and PPARγ expression vectors were transfected in HEK293 cells using the calcium phosphate method. After transfection, whole cell extracts were prepared and incubated with HDAC1 antibody overnight at 4 °C. The immunocomplexes were isolated with protein A-Sepharose beads.
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(Amersham Biosciences) and washed three times with TBS. The immunoprecipitates were separated by SDS-PAGE and immunoblotted using antibodies against PPARγ.

RESULTS

Histone Modification at the Promoters of Adipogenic Genes during Adipocyte Differentiation—The dramatic changes in gene expression profiles during adipocyte differentiation have been previously described (35, 36). Notably, the expression of adipogenic genes such as PPARγ, C/EBPα, and ADD1/SREBP1c increased (28), whereas that of preadipocyte marker genes including preadipocyte factor-1 (Pref-1) and GATA2 is conversely decreased (37, 38). Although many transcription factors that participate in adipocyte differentiation have been identified, the roles of histone-modifying enzymes and chromatin-remodeling factors during this process have not been thoroughly investigated.

To determine if histone modification is involved in adipogenesis, we examined the level of histone acetylation and methylation at the promoter regions of adipocyte marker genes. The degree of histone H3 Lys9 acetylation at the promoters of ADD1/SREBP1c, adiponectin, aP2, C/EBPα, and PPARγ was increased upon differentiation (Fig. 1A). Similarly, histone H4 Lys8 acetylation was increased at the promoters of ADD1/SREBP1c and adiponectin (Fig. 1B). In contrast, histone acetylation gradually decreased at the promoter of Pref-1, consistent with the fact that it is down-regulated during adipogenesis (37). On the other hand, the level of histone H3 Lys9 methylation displayed opposite patterns, decreasing at the promoters of ADD1/SREBP1c and C/EBPα and marginally increasing at the Pref-1 promoter (Fig. 1C). These results indicate that the up-regulation of adipogenic genes during adipogenesis is tightly associated with the selective induction of histone hyperacetylation at the promoter regions of these genes.

Changes of Histone Modification and Expression of HDACs during Adipogenesis—Because the steady state of histone acetylation is determined by the balance between HATs and HDACs, we next examined the protein levels of several histone-modifying enzymes before and after differentiation. In 3T3-L1 cells, the expression levels of several HDACs including HDAC1, -2, -5, and -6 were drastically reduced during differentiation (Fig. 2A) whereas HDAC3, -4, -7, and -8 were either slightly increased or unchanged (supplemental Fig. S1). The protein levels of HDACs, such as CBP or p300, were shown to be unchanged or slightly increased, respectively (Fig. 2A). A similar reduction in HDAC1, -2, -3, -5, -7, and -8 was observed in 3T3-F442A cells, another adipogenic cell line (Fig. 2B). C/EBPα and PPARγ were used as control to confirm proper differentiation.

The finding that the expression of several HDACs is severely decreased prompted us to examine whether the overall balance would be shifted in favor of histone acetylation. Indeed, the degree of overall histone acetylation was increased in adipocytes compared with undifferentiated preadipocytes (Fig. 3A). Methylation of histone H3 lysine 9, which usually displays an opposite pattern to acetylation, was decreased in adipocytes. To determine whether histone deacetylase activity is actually regulated during differentiation, we directly measured HDAC enzyme activity using the same total cell extracts. As shown in Fig. 3B, total cellular HDAC activity was reduced to ~50~60% in both 3T3-L1 and 3T3-F442A cells upon differentiation (Fig. 3B). On the contrary, total cellular HAT activity was slightly increased in differentiated adipocytes (data not shown). Thus, it is likely that the increase of histone acetylation during adipogenesis is the result of a combination of decreased HDAC activity, and to some extent, increased HAT activity. Such modification of histone tails would probably contribute to the overall increase of gene expression in adipocytes, which have been reported to express a larger number of genes than preadipocytes (36).

Effects of HDAC Inhibition on Adipocyte Differentiation—Next, to determine whether the down-regulation of HDAC activity indeed affects adipogenesis, we treated 3T3-L1 cells with an inhibitor of HDACs, sodium butyrate (NaB). Butyrate is a short chain fatty acid that noncompetitively inhibits the activity of most HDAC isoforms (39, 40).
As shown in Fig. 4A chronic treatment of NaB accelerated differentiation and increased lipid accumulation assessed by Oil Red-O staining. Northern blot analysis revealed that the expression of adipogenic genes was induced at an earlier time point (Fig. 4B). A similar effect was observed in C3H10T1/2 cells, a murine mesenchymal fibroblast cell line that can also be induced to differentiate into adipocytes (supplemental Fig. S2).

To investigate the time periods at which HDAC inhibition exhibits the most critical effect on adipogenesis, we treated the cells with NaB for different periods during adipocyte differentiation. Cells treated with NaB throughout the entire differentiation period clearly exhibited stimulated adipocyte differentiation compared with control cells (Fig. 4C, panels II and VIII). Notably, inhibition of the HDACs at the early stage of adipogenesis (the first 1 or 2 days) seemed to be sufficient to stimulate adipogenesis (Fig. 4C, panels IV and V), while treatment of NaB at later periods had little or no effect (Fig. 4C, panels VI and VII). Taken together, these results indicate that suppression of HDAC activity at the early stage of adipogenesis may be crucial for adipocyte differentiation.

The Role of HDAC1 during Adipogenesis—Among many HDAC isoforms, HDAC1 was most consistently and dramatically down-regulated during adipogenesis (Fig. 2, A and B). To determine if HDAC1 plays an important role in adipogenic gene expression, we determined the recruitment of HDAC1 to the promoter regions of adipogenic genes using ChIP assays. As expected, recruitment of HDAC1 decreased upon differentiation in most adipogenic genes including adiponectin, ADD1/SREBP1c, aP2, C/EBPα, and PPARγ, indicating that HDAC1 may be involved in regulating the expression of these genes (Fig. 5A).

To directly determine whether HDAC1 is specifically involved in adipocyte differentiation, we generated 3T3-L1 preadipocytes retrovirologically overexpressing HDAC1. These cells expressed higher amounts of HDAC1 (−2-fold) compared with mock-infected cells (Fig. 5B). Under differentiation conditions, the HDAC1-overexpressing cells exhibited substantially attenuated adipocyte differentiation (Fig. 5C). In addition, the expression of several adipogenic genes is also decreased in HDAC1-overexpressing cells, suggesting that HDAC1 suppresses adipogenesis by down-regulating the expression of adipogenic genes (Fig. 5D).

In a reciprocal experiment, we examined the effect of HDAC1 down-regulation via siRNA. To test whether the HDAC1-siRNA effectively reduced HDAC1 expression, HEK293 cells were co-transfected with FLAG-tagged HDAC1 and HDAC1-siRNA vectors. As expected, over-expression of HDAC1-siRNA efficiently repressed HDAC1 expression (Fig. 6A). Next, 3T3-L1 cells overexpressing the retroviral HDAC1-siRNA were tested for their ability to differentiate into adipocytes. Consistent with the above observations, HDAC1 knock-down 3T3-L1 cells clearly displayed accelerated differentiation (Fig. 6B) and stimulated expression of ADD1/SREBP1c, aP2, and PPARγ (Fig. 6C). Furthermore, HDAC1 knockdown led to the increase of histone acetylation at H3 Lys9, H4 Lys8, and H4 Lys12 residues. Conversely, the extent of histone H3 Lys9 methylation decreased (Fig. 6D). These results provide strong evidence that HDAC1 controls adipocyte differentiation by regulating the acetylation of histones and implicating that a threshold level of HDAC activity is probably required to maintain preadipocytes by silencing adipogenic genes in the undifferentiated state.

Regulation of PPARγ Activity by HDAC1—To understand how HDAC1 affects adipocyte differentiation, we examined whether HDAC1 could directly regulate the transcriptional activity of PPARγ, a master adipogenic transcription factor. In transient transfection assays, HDAC1 repressed the transcriptional activity PPARγ in a dose-dependent manner (Fig. 7A). On the other hand, the activity of ADD1/SREBP1c was not altered by HDAC1 (data not shown). To see if HDAC1 physically associates with PPARγ, we performed GST-pulldown assays using recombinant GST-PPARγ fusion proteins and in vitro translated HDAC1. HDAC1 bound specifically with purified GST-PPARγ but not GST, suggesting that HDAC1 may directly interact with PPARγ (Fig. 7B). To investigate whether this interaction occurs in cells, we isolated whole cell lysates of HEK293 cells after transfection with plasmids encoding PPARγ and HDAC1. Immunoprecipitation with anti-HDAC1 antibody followed by Western blot assays using anti-PPARγ antibody
demonstrates that HDAC1 indeed interacts with PPARγ in vivo (Fig. 7C). It is therefore possible to speculate that HDAC1 could modulate adipocyte differentiation by repressing PPARγ activity.

DISCUSSION

Recent studies have demonstrated the involvement of chromatin remodeling factors in the differentiation of specific cell types including muscle, neurons, and T cells (41–43). Although it is well known that certain transcription factors or cofactors interact with HATs or HDACs to regulate their target gene expression, there are few reports that the overall level of histone modifications or expression of histone-modifying enzymes such as HDACs is changed during bona fide differentiation. Furthermore, little is known about the regulatory role of chromatin remodeling such as histone modifications in adipocyte differentiation.

In the present study, we sought to identify the regulatory function of histone modifications in adipocyte differentiation. ChIP assays revealed that histones are dynamically modified at the promoter regions of adipocyte marker genes indicating its involvement in adipogenesis. More importantly, we discovered that the protein level of class I and class II HDACs significantly decreased upon differentiation (Fig. 2, A and B). Indeed, the endogenous HDAC enzyme activity measured in adipocytes was shown to be only half that of preadipocytes (Fig. 3B). Inversely, total HAT activity of adipocytes was slightly elevated upon differentiation (data not shown). Therefore, it seems that histone hyperacetylation in adipocytes, as a result of decreased HDAC activity and increased HAT activity, leads to enhanced adipogenic gene expression. This increase of histone acetylation is most likely preferentially targeted to adipogenic genes by tissue-specific transcription factors and cofactors. This idea is strongly supported by the fact that PPARγ, a master adipogenic transcription factor, interacts with several coactivators such as p300, CBP, TRAP, p160, and PGC1α and corepressors like HDAC3 to selectively regulate the expression of its target genes during adipogenesis (44–48). Because these cofactors are able to interact with specific transcription factors in a highly specific manner, decondensation of chromatin is tightly controlled so that it only takes place in a subset of genes in the appropriate tissues and at the appropriate times.

Stimulation of adipogenesis and adipogenic gene expression by NaB suggests that the down-regulation of histone deacetylases might be one
of the rate-limiting steps during adipocyte differentiation (Fig. 4, A and B). Interestingly, cells treated with NaB for the first 2 or 3 days, but not at later stages of differentiation, showed a remarkable stimulation of adipogenesis (Fig. 3C). These results implicate that a critical determination step for adipogenesis occurs through histone modification at an early phase of the differentiation process. This crucial event is probably associated with tissue- or developmentally specific transcription factors and/or cofactors responsible for triggering tissue-specific gene expression with down-regulation of HDAC activity. Similar results have been obtained with Swiss 3T3 cells (49) and was also observed in C3H 10T1/2 cells (supplemental Fig. S2). These cell lines are less determined to the adipogenic lineage, suggesting that this phenomenon is not restricted to a specific cell line.

A remarkable drop in the expression of HDAC1 suggested that this specific isoform may play a major role in adipogenesis. This idea was supported by the observation that the amounts of HDAC1 localized to the promoters of adipogenic genes were clearly decreased after differentiation (Fig. 5A). Gain-of-function and loss-of function studies in 3T3-L1 cells also confirmed the critical role of HDAC1 in adipogenesis. While we were preparing this article, Wiper-Bergeron et al. (50) demonstrated that HDAC1, which forms a complex with mSin3A and C/EBPβ, is able to attenuate differentiation by repressing C/EBPα expression. In another recent report, Fajas et al. (48) demonstrated that HDAC3 can suppress PPARγ by forming a PPARγRb-HD3 complex. It is also known that HD3 is a component of a corepressor complex with NCoR/SMRT that represses PPARγ. Interestingly, HDAC1 interacted with PPARγ and repressed its transcriptional activity (Fig. 7). Even though many HDACs are known to bind to transcription factors as part of a corepressor complex, results from GST-pulldown and co-immunoprecipitation assays suggest that HDAC1 may bind directly to PPARγ. In addition, it should be noted that PPARγ and HDAC1 display reciprocal expression patterns during adipogenesis (Fig. 2A). Thus, it appears that activation of PPARγ might be regulated by two steps during adipocyte differentiation. First, PPARγ activity is stimulated by increases in protein levels and its endogenous ligands. Second, the down-regulation of HDAC1 would further promote PPARγ activity by relieving it from repression. Therefore, in addition to the previously described role as a modulator of C/EBPα, HDAC1 would also regulate adipocyte differentiation by controlling PPARγ.

In addition to these findings, which suggest that HDACs interact with several adipogenic transcription factors to modulate their activities, our results implicate the importance of the regulation of HDAC expression during adipocyte differentiation. Although the exact mechanism by which adipogenic stimuli decrease the level of HDACs is yet to be determined, it appears that the lowering of HDAC expression results in decondensation of the chromatin structure around the promoter regions of adipogenic genes, thereby facilitating the binding of transcription factors to their binding sites (supplemental Fig. S3).
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of colon cancer by altering gene expression and arresting cell division or to induce apoptosis in human leukemic lymphoblasts (53). Furthermore, HDAC inhibitors induce the expression of cell cycle kinase inhibitor p21, causing growth arrest in transformed cells (54, 55). It is interesting to note that expression of p21 is induced by HDAC inhibitors since p21 is induced at a very early stage of adipocyte differentiation to modulate clonal expansion (56). Although the precise mechanisms by which HDAC inhibitors induce cell cycle arrest via p21 expression during adipogenesis remain to be elucidated, it might shed light in understanding how histone acetylation and deacetylation are possibly linked to adipocyte differentiation.

In summary, our results show that the down-regulation of HDAC expression and the resulting increase of histone acetylation are important for adipogenesis. The substantial decrease in HDAC activity results in preferential histone hyperacetylation at the promoter regions of adipocyte marker genes. It is therefore likely that regulation of HDAC expression would be an essential process in adipocyte differentiation and that preadipocytes could initiate the differentiation process only when cellular level of HDACs falls below a certain threshold level and also that HDACs are involved in maintaining the preadipocyte phenotype. This is a novel viewpoint concerning the regulation of adipogenesis since previous studies have mainly focused on adipogenic transcription factors, such as PPARγ and C/EBPα and their ability to recruit coregulators to adipogenic gene promoters. Our findings could also provide a novel means of treating obesity-related diseases. Further studies will reveal whether this is a general mechanism controlling the differentiation of diverse cell types.

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