Role of Krüppel-like Factor 15 (KLF15) in Transcriptional Regulation of Adipogenesis*

Received for publication, September 13, 2004, and in revised form, December 27, 2004
Published, JBC Papers in Press, January 20, 2005, DOI 10.1074/jbc.M410515200

Toshiyuki Morii‡, Hiroshi Sakaue‡§, Haruhisa Iguchi‡, Hideyuki Gomi‡, Yuko Okada‡, Yasuhiro Takashima‡, Takeshi Nakamura‡, Toshimasa Yamauchi‡, Naoto Kubota‡, Takashi Kadowaki‡, Yasushi Matsuki‡, Wataru Ogawa‡, Ryuji Hiramatsu‡, and Masato Kasugad

From the ‡Department of Clinical Molecular Medicine, Division of Diabetes and Digestive and Kidney Diseases, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan, the §Genomics Science Laboratories, Sumitomo Pharmaceuticals Co. Ltd., Takarazuka 665-0051, Japan, and the ¶Department of Metabolic Diseases, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655, Japan

Krüppel-like zinc finger transcription factors (KLFs) play diverse roles during cell differentiation and development in mammals. We have now shown by microarray analysis that expression of the KLF15 gene is markedly up-regulated during the differentiation of 3T3-L1 preadipocytes into adipocytes. Inhibition of the function of KLF15, either by expression of a dominant negative mutant or by RNA interference, both reduced the expression of peroxisome proliferator-activated receptor γ (PPARγ) and blocked adipogenesis in 3T3-L1 preadipocytes exposed to inducers of adipocyte differentiation. However, the dominant negative mutant of KLF15 did not affect the expression of CCAAT/enhancer-binding protein β (C/EBPβ) elicited by inducers of differentiation in 3T3-L1 preadipocytes. In addition, ectopic expression of KLF15 in NIH 3T3 or C2C12 cells triggered both lipid accumulation and the expression of PPARγ in the presence of inducers of adipocyte differentiation. Ectopic expression of C/EBPβ, C/EBPδ, or C/EBPα in NIH 3T3 cells also elicited the expression of KLF15 in the presence of inducers of adipocyte differentiation. Moreover, KLF15 and C/EBPα acted synergistically to increase the activity of the PPARγ gene promoter in 3T3-L1 adipocytes. Our observations thus demonstrate that KLF15 plays an essential role in adipogenesis in 3T3-L1 cells through its regulation of PPARγ expression.

The amount of adipose tissue in the body is an important determinant of energy homeostasis in animals and is altered in various physiological or pathological conditions (1). An increase in adipose tissue mass can arise through increases in cell size, cell number, or both (2). The size of adipocytes varies markedly and reflects largely the amount of stored triglyceride, whereas the number of adipocytes is thought to increase as a result of the proliferation of preadipocytes and their subsequent differentiation into mature adipocytes.

Murine preadipocyte cell lines, such as 3T3-L1 and 3T3-F442A, have been studied extensively to elucidate the mechanisms of growth and differentiation of preadipocytes (2, 3). In response to exposure to appropriate hormonal inducers (such as agents that increase the intracellular concentration of cyclic AMP, agonists of the insulin-like growth factor-1 receptor, glucocorticoids, and fetal bovine serum), these cells first undergo several rounds of mitosis, known as clonal expansion, and then become quiescent again, express adipocyte-specific proteins, and acquire biochemical and morphological characteristics of mature adipocytes (2, 3).

Both the proliferation and differentiation of preadipocytes are characterized by marked changes in the pattern of gene expression that are achieved by the sequential induction of transcription factors. Preadipocytes exposed to inducers of differentiation thus manifest an early and transient increase in the expression of the transcription factors CCAAT/enhancer-binding protein β (C/EBPβ)1 and C/EBPδ, which in turn appear to contribute to cell proliferation as well as to the subsequent increase in the expression of C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ) (4, 5). The latter two proteins are thought to act synergistically in the transcriptional activation of a variety of adipocyte-specific genes, with each also reciprocally activating the expression of the other (2–4).

Krüppel-like zinc finger transcription factors (KLFs) are DNA-binding transcriptional regulators that contain the C2H2 zinc finger motif and play diverse roles in the regulation of cell proliferation, cell differentiation, and development (6, 7). All 16 members of the KLF family identified to date bind to GC-rich sequences including GC boxes and GT boxes (also known as CACCC boxes) (7, 8). Certain KLF proteins have been implicated in adipogenesis. KLF2 has thus been shown to negatively regulate adipogenesis through inhibition of PPARγ gene expression (9). In addition, expression of KLF6 is transiently induced during adipogenesis in 3T3-L1 preadipocytes (10).

The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; PPARγ, peroxisome proliferator-activated receptor γ; KLF, Krüppel-like zinc finger transcription factor; CREB, cyclic AMP response element-binding protein; IBMX, isobutylmethylxanthine; MEF, mouse embryonic fibroblast; RT, reverse transcription; GFP, green fluorescent protein; RNAi, RNA interference; siRNA, small interfering RNA.
thermore, overexpression of KLF15 induces adipocyte matura-
tion and GLUT4 expression (11), although the physiological
significance of KLF15 in the induction or maintenance of ter-

minal differentiation has remained unclear. We now provide
evidence that KLF15 promotes maintenance of the biochemical
and morphological characteristics of mature adipocytes
through direct induction of PPARγ expression in cooperation
with C/EBPα.

**EXPERIMENTAL PROCEDURES**

**Antibodies, Reagents, and Cells—**Antibodies to C/EBPα, C/EBPβ, and C/EBPδ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to cyclic AMP response element-binding protein (CREB) were from Cell Signaling Technology (Beverly, MA). Antibodies to PPARγ (12) and aP2 (13) were kindly provided by B. M. Spiegelman (Harvard Medical School, Boston, MA) and D. Bernlohr (University of Minnesota, Minneapolis, MN), respectively. Troglitazone was kindly provided by Sankyo Pharmaceutical Co. (Tokyo, Japan). Blasticidin S hydrochloride, hygromycin B, and puromycin dihydro-
chloride were obtained from Invitrogen (Carlsbad, CA), Wako (Osaka, Japan), and Sigma, respectively; hexadimethrine bromide was also
from Sigma. 3T3-L1, NIH 3T3, and C2C12 cells were obtained from American Type Culture Collection (Manassas, VA), and Plat-E retrovi-
rnal packaging cells (14) were kindly provided by T. Kitamura (Univer-
sity of Tokyo, Tokyo, Japan).

**Cell Culture and Staining—**3T3-L1 preadipocytes were maintained
as described previously (15). Their differentiation into adipocytes was
induced at 6 days of treatment of confluent cells first for 2 days with insulin (5
μg/ml), 0.25 μM dexamethasone, and 0.5 mM isobutylmethylxanthine
(IBMX) in Dulbecco’s modified Eagle’s medium supplemented with 10%
fetal bovine serum and then for 2 days with insulin (5 μg/ml) alone in
the same medium. The cells were then returned to the basal medium,
which was replenished every other day.

NIH 3T3 or C2C12 cells were maintained in Dulbecco’s modified
Eagle’s medium supplemented with 10% fetal bovine serum and cul-
tured to confluence. Adipogenesis in transfected cells was induced by
exposure to inducers of differentiation were subjected to Northern blot
analysis of the indicated mRNAs. The 28 S rRNA bands on the ethidium
bromide-stained gel are also shown. All data are representative of
least three independent experiments.

**Knockout Mice—**To produce retroviral vectors for PPARγ knock-
out mice or their wild-type littermates were immortalized by the 3T3
protocol (16) and exposed to inducers of adipocyte differentiation as
described above for NIH 3T3 cells. Cells were stained with oil red O as
described (17).

**Microarray Analysis—**Total RNA was isolated from
3T3-L1 preadipocytes or fully differentiated 3T3-L1 adipocytes (8 days
after the induction of differentiation) and was processed with RNeasy
columns (Qiagen, Hilden, Germany). Portions (10 μg) of the RNA were
then used for synthesis of biotin-labeled cRNA, which in turn was used
to prepare cDNA probes (Affymetrix, Santa Clara, CA). After washing and staining, the arrays were scanned with a Hewlett Packard confocal laser scanner and visualized with Affymetrix GeneChip 3.1 software. Finally, the results were analyzed with Gene-
chip Analysis Suite software version 4.0 (Affymetrix), and the fold
changes in hybridization intensity between samples from undiffer-
entiated 3T3-L1 preadipocytes and fully differentiated 3T3-L1 adipocytes
were determined.

**Expression Plasmids—**A mammalian expression vector for KLF15
(pcDNA3.1/KLF15) was constructed by inserting the products of reverse
transcription (RT) and the PCR obtained from rat adipose tissue RNA into pcDNA3.1 (Invitrogen). To construct an expression vector for a deletion
mutant of KLF15 that lacks the NH2-terminal 318 amino acids (∆318), we
amplified a DNA fragment encoding ∆318 by PCR with the sense primer
5′-CTGCCATGCACAATGGCCTATTAC-3′ and the antisense primer
5′-TTCCAGGTGTAGGGCCGCTAC-3′ and then inserted the PCR product
into the PPT-blue T vector (Takara, Japan). The sequence of the inserted
fragment was verified and then subcloned into pcDNA3.1. Mammalian expression vectors for C/EBPα or C/EBPδ were constructed by
subcloning the corresponding mouse cDNAs (kindly provided by S. Akira,
Osaka University, Osaka, Japan) into pcDNA3.1.

**Retroviral Vectors and Infection—**To produce retroviral vectors for
green fluorescent protein (GFP), C/EBPα, C/EBPβ, C/EBPδ, KLF6, KLF9, KLF15, or ∆318, we subcloned cDNAs for GFP, mouse C/EBPα,
mouse C/EBPβ (kindly provided by S. Akira), mouse KLF6 (RT-PCR product of mouse adipose tissue RNA), mouse KLF9
(RT-PCR product of mouse adipose tissue RNA), rat KLF15, or ∆318 into
pWZL containing blasticidin or hygromycin resistance genes
(kindly provided by K. Nakamura, Stanford University, Stanford, CA). To construct a retroviral vector for Plat-E packaging cells were trans-
fected with the retroviral vector with the use of FuGENE 6 (Roche
Applied Science, Indianapolis, IN). After 24 h, the cells were incubated
for an additional 24 h in fresh medium to obtain retrovirus-containing
superantennas. NIH 3T3 cells (50 to 60% confluence) were then incu-
bated with these supernatants in the presence of hexadimethrine bromide
(4 μg/ml) for 12 h. Cells expressing KLF15 were selected on the
basis of their resistance to blasticidin (10 μg/ml) or hygromycin B
(500 μg/ml).

NIH 3T3 cells stably expressing other ectopic proteins with the exception of PPARγ-2, 3T3-L1 cells stably expressing GFP (3T3-L1/GFP
cells), KLF15, or ∆318; and C2C12 cells stably expressing GFP (C2C122/
FIG. 2. Effect of a dominant negative mutant of KLF15 on adipocytic differentiation of 3T3-L1 preadipocytes. A, NIH 3T3 cells were transfected with a GLUT4 promoter-luciferase gene reporter plasmid (or pcDNA3.1-basic as a control), a β-galactosidase expression plasmid, and expression vectors for KLF15, the Δ318 mutant, or both KLF15 and Δ318 (or the pcDNA3.1 empty vector). The cells were subsequently assayed for luciferase and β-galactosidase activities, and the former was normalized by the latter. Data represent normalized luciferase activity expressed relative to the value for cells transfected with pcDNA3.1-basic and pcDNA3.1; data are mean ± S.D. of triplicates from a representative experiment. B, 3T3-L1 preadipocytes stably expressing GFP, KLF15, or Δ318, as indicated, were subjected to Northern blot analysis of KLF15 mRNA. Control 3T3-L1 cells before or 8 days after exposure to inducers of differentiation were similarly analyzed. C, 3T3-L1 preadipocytes stably expressing GFP, KLF15, Δ318, or both Δ318 and PPARγ were stained with oil red O 8 days after exposure to inducers of differentiation. Macroscopic (upper panel) and microscopic (×100, lower panel) views are shown. D, 3T3-L1 preadipocytes stably expressing GFP, KLF15, Δ318, or both Δ318 and PPARγ were subjected to immunoblot analysis with antibodies to PPARγ, C/EBPα, aP2, or CREB (control) 8 days after exposure to inducers of differentiation. E and F, 3T3-L1 adipocytes 8 days after exposure to inducers of differentiation were infected with adenovirus vector AxCA-lacZ or AxCA-Klf15 at the indicted multiplicity of infection. 48 h after adenovirus infection (MOI), the cells were subjected to Northern blot analysis of KLF15 mRNA (E) or subjected to immunoblot analysis with antibodies to PPARγ, C/EBPα, or CREB (control) (F). Data in B–F are representative of at least three independent experiments.

FIG. 3. Effect of a dominant negative mutant of KLF15 on the expression of C/EBPβ or PPARγ during adipocyte differentiation in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes stably expressing GFP, KLF15, or Δ318 were exposed to inducers of differentiation for the indicated times and then subjected to immunoblot analysis with antibodies to C/EBPβ, PPARγ, or CREB. Data are representative of at least three independent experiments.
solution provided with Nucleofector Kit V (Amaza, Cologne, Germany). The plasmid DNA was then introduced into the cells by electroporation with the use of a Nucleofector instrument (program U-28). After electroporation, the cells were resuspended in culture medium and replated on culture dishes.

**Luciferase Reporter Assays**—For GLUT4 gene promoter assays, NIH 3T3 cells plated in 24-well dishes (50 to 60% confluence) were transiently transfected with 500 ng of the reporter plasmid pGL3-basic (Promega, Madison, WI) containing the human GLUT4 gene promoter (nucleotides − 894 to +84 relative to the transcriptional start site), 500 ng of the indicated expression plasmids, and 100 ng of pCMV encoding β-galactosidase (pCMV/β-gal) with the use of the Lipofectamine reagent (Invitrogen). After incubation for 48 to 72 h, the cells were lysed in 100 µl of 1 times passive lysis buffer (Promega), and portions of the lysate were subjected to assays for firefly luciferase (Promega) and β-galactosidase (Clontech, Palo Alto, CA) activities. Promoter activity was determined as the ratio of luciferase to β-galactosidase activities for PPARγ2 gene promoter assays, 3T3-L1 adipocytes (~3 × 10⁶) were transfected with 500 ng of pGL3-basic containing the mouse PPARγ2 gene promoter (nucleotides −626 to +60), 500 ng of the indicated expression plasmids, and 100 ng of pCMV/β-gal by electroporation and were subsequently assayed for firefly luciferase and β-galactosidase activities as described for the GLUT4 gene promoter assay.

**RNA Interference**—The target sequences of the KLF15 gene selected for RNA interference (RNAi) comprised nucleotides 300 to 319 and nucleotides 1112 to 1131 relative to the transcriptional start site. To construct mammalian expression vectors for each of the corresponding small interfering RNAs (siRNAs), designated si300 and si1112, respectively, we amplified by PCR a DNA fragment including the mouse U6 (mU6) promoter and encoding the sense sequence of the siRNA, a short hairpin sequence, the antisense sequence of the siRNA, and a poly(A) terminator. PCR was performed for 35 cycles of 95 °C for 30 s, 45 °C for 30 s, and 72 °C for 30 s with specific primers, the pMXs/mU6 plasmid (kindly provided by K. I. Nakayama, Kyushu University, Fukuoka, Japan) as the template, and Ex-taq polymerase (Takara). The PCR primers were 5′-ACCGAAGGAG-3′ (sense) and 5′-ACAAGGCTTTTCTCCAAG-3′ (antisense) for si300 and 5′-ACCGAACCGAGGAGGAG-3′ (sense) and 5′-ACTAAAGTGCGAACAAAGAGCATCTCTTGATGAGGCGCAGAGGACCGAAGGCGCTTTTCTCCAAG-3′ (antisense) for si1112. The PCR products were subjected to Northern blot analysis with probes for the corresponding mRNAs (left panel). Cells stably expressing KLF6, KLF9, or KLF15 were also stained with oil red O at 10 days after exposure to inducers of adipocyte differentiation; microscopic views (~×100) of the stained cells are shown (right panel). B, before or 10 days after exposure to inducers of adipocyte differentiation, NIH 3T3 cells stably expressing KLF6, KLF9, or KLF15 were subjected to Northern blot analysis of PPARγ, aP2, C/EBPα, and GLUT4 mRNAs. 3T3-L1 cells before or 8 days after exposure to differentiation inducers were similarly analyzed for comparison. C, C2C12 cells infected with retrovectors for GFP or KLF15 were subjected to Northern blot analysis of PPARγ and aP2 mRNAs at 10 days after exposure to inducers of adipocyte differentiation; microscopic views (~×100) are shown (lower panel). D, C2C12 cells stably expressing GFP or KLF15 were subjected to Northern blot analysis of PPARγ and aP2 mRNAs at 10 days after exposure to inducers of adipocyte differentiation. All data are representative of at least three independent experiments.

**RESULTS**

Up-regulation of KLF15 Gene Expression during Adipocyte Differentiation in 3T3-L1 Cells—We first profiled genes whose level of expression changed in association with the differentiation of 3T3-L1 preadipocytes induced by IBMX, dexametha-
KLF15 and Adipocyte Differentiation

Inhibition of KLF15 Function by a Dominant Negative Mutant in 3T3-L1 Cells—To determine whether KLF15 is required for adipocyte differentiation in 3T3-L1 cells, we constructed an expression vector for a putative dominant negative mutant (Δ318) that lacks the NH₂-terminal 318 amino acids of KLF15, which contain the transactivation domain, leaving the DNA binding domain intact. To verify that the Δ318 mutant was able to inhibit KLF15 function, we examined the effect of its expression on the activity of the GLUT4 gene promoter, a target of KLF15 action (11). We transiently cotransfected NIH 3T3 cells with a GLUT4 promoter-luciferase gene reporter construct together with an expression plasmid either for full-length KLF15 or for the Δ318 mutant. The activity of the GLUT4 gene promoter was increased by ~50% by expression of wild-type KLF15 and was reduced by ~50% by expression of Δ318 (Fig. 2A). In addition, Δ318 prevented the increase in GLUT4 gene promoter activity induced by the full-length protein, confirming that Δ318 acts in a dominant negative manner.

We next constructed retroviral vectors encoding Δ318, wild-type KLF15, and GFP for expression of these proteins in 3T3-L1 preadipocytes (Fig. 2B). Expression of Δ318 in these cells greatly inhibited both the lipid accumulation (Fig. 2C) and the induction of PPARγ, C/EBPα, and aP2 (Fig. 2D) normally apparent 8 days after exposure of the cells to inducers of differentiation. Expression of GFP or of wild-type KLF15 did not affect these aspects of adipocyte differentiation. The amount of exogenous KLF15 mRNA by a retrovirus vector was one-half of that of endogenous KLF15 in 3T3-L1 adipocytes (Fig. 2B). We have therefore investigated the effect of overexpression of KLF15 by an adenovirus vector on the induction of PPARγ and C/EBPα. Although the abundance of KLF15 mRNA in 3T3-L1 adipocytes infected with an adenovirus vector for this protein at a multiplicity of infection of 30 plaque-forming units (pfu)/cell was eight times to that of endogenous KLF15 mRNA in 3T3-L1 adipocytes (Fig. 2E), this overexpression of KLF15 did not affect the amount of PPARγ and C/EBPα protein (Fig. 2F) in 3T3-L1 adipocytes. To examine whether this
inhibitory effect of Δ318 on adipogenesis was prevented by coexpression of PPARγ2, we infected 3T3-L1 cells both with a Δ318-encoding retroviral vector containing a basic domain resistance gene and with a PPARγ2-encoding retroviral vector containing a puromycin resistance gene. The infected cells were then subjected to selection in the presence of both basic domain S and puromycin. The inhibitory effect of Δ318 on adipogenesis, as monitored by both lipid accumulation (Fig. 2C) and the expression of C/EBPβ and aP2 (Fig. 2D), was indeed prevented by coexpression of PPARγ2, indicating that it was attributable to the prevention of PPARγ induction.

Lack of Effect of Δ318 on the Expression of C/EBPβ in 3T3-L1 Cells—The increase in the amount of KLF15 mRNA during adipocytic differentiation of 3T3-L1 cells occurred slightly later than did that in the amount of PPARγ mRNA (Fig. 1B). PPARγ mRNA was thus detected 2 days after exposure of cells to inducers of differentiation, whereas KLF15 mRNA was not apparent at this time. The transient increase in the abundance of the mRNAs for C/EBPβ and C/EBPα, both of which directly induce the expression of PPARγ (20, 21), was maximal 2 days after induction of differentiation. Furthermore, MEFs that lack C/EBPβ and C/EBPα are unable to differentiate into adipocytes (22). These two proteins thus likely induce the expression of PPARγ before the induction of C/EBPα or KLF15 during adipogenesis in 3T3-L1 cells. We next investigated whether the expression of the dominant negative mutant of KLF15 (Δ318) affected the induction of C/EBPβ or PPARγ during the 4 days of exposure of 3T3-L1 cells to inducers of differentiation, a period when the levels of C/EBPβ and C/EBPα are increased. Immunoblot analysis revealed that expression of Δ318 in 3T3-L1 cells inhibited the expression of PPARγ on day 4 (Fig. 3) as it did on day 8 (Fig. 2D); it did not affect PPARγ abundance on day 2 (Fig. 3), however, a time before induction of KLF15 gene expression is apparent. The up-regulation of C/EBPβ expression, which was maximal 1 and 2 days after exposure to the inducers of differentiation, was not inhibited by Δ318 (Fig. 3). Moreover, overexpression of wild-type KLF15 slightly increased the abundance of PPARγ at 1 or 2 days after the induction of differentiation (Fig. 3), consistent with the previous observation that KLF15 promoted adipocyte differentiation (9).

Inhibition of Adipogenesis by RNAi-mediated Depletion of KLF15 in 3T3-L1 Cells—It was possible that expression of a dominant negative mutant (Δ318) of KLF15 that contains the DNA binding domain of the protein affected transactivation by other KLF isoforms. To exclude this possibility, we designed plasmid constructs that encode two different siRNAs (siRNA300 and siRNA1112) and are controlled by the mU6 promoter (23) to deplete KLF15 from 3T3-L1 cells. Cotransfection with the mU6/siRNA1112 plasmid resulted in a marked reduction in the amount of KLF15 mRNA in NIH 3T3 or 293 cells also transfected with an expression vector for KLF15, whereas cotransfection with the mU6/siRNA300 plasmid had no such effect (data not shown). We therefore transfected 3T3-L1 preadipocytes with the mU6/siRNA1112 plasmid, a plasmid containing only the mU6 promoter, or the corresponding empty plasmid by electroporation. Similar electroporation with an expression vector for β-galactosidase revealed that >70% of 3T3-L1 preadipocytes expressed the exogenous gene (19), indicative of a high efficiency of transfection. Transfection of 3T3-L1 cells with the mU6/siRNA1112 plasmid resulted in marked inhibition both of lipid accumulation (Fig. 4A) and of the expression of KLF15, PPARγ, C/EBPα, aP2, and GLUT4 at the mRNA (Fig. 4B) or protein (Fig. 4C) levels apparent 8 days after exposure of the cells to inducers of differentiation. Transfection with either the mU6 plasmid or the empty plasmid had no such effects. These data thus confirmed that induction of KLF15, rather than that of other KLFs, is essential for adipocytic differentiation of 3T3-L1 cells.

Induction of Adipogenesis by Ectopic Expression of KLF15 in NIH 3T3 and C2C12 Cells—To examine whether KLF15 is able to induce adipogenesis, we next determined the effect of ectopic expression of this protein on adipocyte differentiation in NIH 3T3 cells, which, unlike 3T3-L1 preadipocytes, are not committed to the adipocyte lineage and do not express PPARγ or C/EBPα in response to treatment with IBMX, dexamethasone, insulin, and synthetic PPARγ ligands such as ciglitazone (24). NIH 3T3 cells were infected with retroviral vectors for KLF15 or for KLF6 or KLF9, changes in the expression of which also accompanied adipocyte differentiation in 3T3-L1 cells (Fig. 1). The infected cells were then exposed to IBMX, dexamethasone, insulin, and the synthetic PPARγ ligand troglitazone. Such treatment did not induce lipid accumulation (Fig. 5A) or the expression of PPARγ, aP2, C/EBPα, or GLUT4 genes (Fig. 5B) in cells infected with the KLF6 or KLF9 vectors. In contrast, the cells infected with the KLF15 vector exhibited marked lipid accumulation (Fig. 5A) and expression of PPARγ and aP2 genes (Fig. 5B). Induction of the C/EBPα gene was not apparent in the KLF15-overexpressing cells, however, suggesting that adipogenesis mediated by KLF15 was dependent largely on the induction of PPARγ. Induction of GLUT4 gene expression was induced by KLF15 even in the absence of differentiation inducers, consistent with the previous observation that KLF15 binds to and transactivates the GLUT4 gene promoter (11). These effects of KLF15 on adipocyte differentiation were also observed in the multipotent cell line C2C12, which possesses the ability to differentiate into myocytes (25) or osteoblastic cells (26). Treatment with IBMX, dexamethasone, insulin, and tro-
glitazone thus induced both lipid accumulation (Fig. 5C) and PPARγ and aP2 gene expression (Fig. 5D) in C2C12 cells infected with a retroviral vector for KLF15 but not in those infected with a control vector for GFP. These data indicated that KLF15 possesses the ability to execute adipogenesis through the induction of PPARγ in NIH 3T3 and C2C12 cells, neither of which are programmed to express PPARγ.

Dependence of Adipogenesis Induced by KLF15 on the Expression of PPARγ—To confirm that adipogenesis induced by ectopic expression of KLF15 in NIH 3T3 or C2C12 cells was dependent on the induction of PPARγ, we investigated the effect of KLF15 on adipocyte differentiation in MEFs derived from PPARγ-knockout (PPARγ−/−) mice (27). Wild-type (PPARγ+/+) and PPARγ−/− MEFs isolated from littersmates at embryonic day 13.5 were immortalized, infected with retroviral vectors for GFP, KLF15, or PPARγ (Fig. 6A), and exposed to inducers of adipocyte differentiation (IBMX, dexamethasone, insulin, and troglitazone). Lipid accumulation (Fig. 6B) and up-regulation of aP2 mRNA (Fig. 6C) were apparent in both PPARγ+/+ and PPARγ−/− MEFs overexpressing PPARγ2 but only in PPARγ−/+ MEFs (not PPARγ−/− cells) overexpressing KLF15 and not in either type of MEFs expressing GFP.

We next tested whether PPARγ was able to induce the expression of KLF15 in NIH 3T3 cells. The abundance of PPARγ2 mRNA in NIH 3T3 cells infected with a retroviral vector for this protein was similar to that of endogenous PPARγ mRNA in 3T3-L1 adipocytes (Fig. 6D). Overexpression of PPARγ2 elicited adipogenesis in NIH 3T3 cells exposed to inducers of adipocyte differentiation (Fig. 6E), but it did not affect the amount of KLF15 mRNA in these cells (Fig. 6F). These results suggested that PPARγ is a downstream effector in the induction of adipocyte differentiation by KLF15.

Induction of KLF15 by Ectopic Expression of C/EBPβ or C/EBPδ in NIH 3T3 Cells—C/EBPβ and C/EBPδ are early participants in the adipogenic differentiation of 3T3-L1 cells (Fig. 1B), and ectopic expression of these proteins induces the differentiation of NIH 3T3 cells into adipocytes (20, 21). We therefore examined whether C/EBPβ or C/EBPδ were able to induce the expression of KLF15 in NIH 3T3 cells. Immunoblot analysis revealed that the abundance of C/EBPβ or C/EBPδ in NIH 3T3 cells infected with retroviral vectors for these proteins was similar to that of the endogenous proteins in 3T3-L1 adipocytes at 2 days or 6 h, respectively, after exposure to inducers of differentiation (Fig. 7A). Overexpression of C/EBPβ or C/EBPδ induced
lipid accumulation (data not shown) and the up-regulation of both PPARγ and KLF15 mRNAs (Fig. 7B) in NIH 3T3 cells exposed to inducers of adipocyte differentiation, suggesting that KLF15 is a downstream effector of C/EBPα or C/EBPβ in the induction of PPARγ expression during adipocyte differentiation.

Synergistic Induction of Adipocyte Differentiation by KLF15 and C/EBPα—The time course of the induction of KLF15 gene expression during adipogenic differentiation of 3T3-L1 cells was similar to that of the up-regulation of C/EBPα mRNA (Fig. 1B). We therefore examined whether C/EBPα was able to induce the expression of KLF15 in NIH 3T3 cells. Immunoblot analysis revealed that the abundance of C/EBPα in NIH 3T3 cells infected with retroviral vectors for these proteins was similar to that of the endogenous proteins in 3T3-L1 adipocytes at 8 days, after exposure to inducers of differentiation (Fig. 8A). Overexpression of C/EBPα induced lipid accumulation (Fig. 8D) and the up-regulation of both PPARγ and KLF15 mRNAs (Fig. 8B) in NIH 3T3 cells exposed to inducers of adipocyte differentiation, suggesting that KLF15 is also a downstream effector of C/EBPα in the induction of PPARγ expression during adipocyte differentiation.

We next investigated whether KLF15 promotes adipocyte differentiation in cooperation with C/EBPα. We infected NIH 3T3 cells both with a C/EBPα retroviral vector that contained a blasticidin resistance gene and with a KLF15 retroviral vector that contained a hygromycin resistance gene, and then subjected the cells to selection with both blasticidin S and hygromycin B (Fig. 8C). Whereas overexpression of KLF15 or C/EBPα alone each induced lipid accumulation (Fig. 8D) and up-regulation of PPARγ and aP2 mRNAs (Fig. 8E) in NIH 3T3 cells exposed to inducers of adipocyte differentiation, the coexpression of these proteins had an even more pronounced effect on adipocyte differentiation. The amount of GLUT4 mRNA was increased in cells overexpressing KLF15 but not in those overexpressing C/EBPα alone (Fig. 8F). Finally, we examined the effect of overexpression of C/EBPα, C/EBPβ, or KLF15 on the activity of a PPARγ2 promoter-luciferase gene reporter construct in 3T3-L1 adipocytes. The activity of the PPARγ2 promoter was increased 5- and 3.5-fold by overexpression of C/EBPα or KLF15, respectively, but was not affected by that of C/EBPβ (Fig. 8F). In addition, coexpression of KLF15 and C/EBPα induced a 14-fold increase in the activity of the PPARγ2 gene promoter (Fig. 8F), consistent with the notion that KLF15 and C/EBPα act synergistically to promote adipocyte differentiation.

**DISCUSSION**

We have shown here that up-regulation of transcription factor KLF15 plays an essential role in the differentiation of 3T3-L1 preadipocytes into adipocytes, and that this action of KLF15 is mediated, at least in part, through induction of PPARγ expression. Ectopic expression of KLF15 thus induced the expression of PPARγ in both NIH 3T3 and C2C12 cells. In addition, PPARγ expression was found to be required for KLF15-induced adipocyte differentiation in MEFs. These data indicate that KLF15 possesses the ability to mediate adipogenesis through the induction of PPARγ expression. KLF15 was originally identified on the basis of its ability to bind to a GA element in the promoter of the CLC-K1 gene, which encodes a kidney-specific member of the CLC family of Cl− channels (28). The gene for the insulin-sensitive glucose transporter GLUT4 in adipose or muscle tissue and the acetyl-CoA synthetase 2 gene in muscle are also targets of these genes (11, 29). The PPARγ gene contains two tandem KLF binding sites (shown in bold) that bind KLF2 (9) within the sequence 5′-CCACCTCTCCCCA-3′ (nucleotides −82 to −70 relative to the transcriptional start site). However, the activity of the PPARγ gene promoter was increased 3.5-fold by overexpression of KLF15 alone (Fig. 8F), but was not affected by the deletion of the CACC elements of PPARγ gene promoter.

---

2 T. Mori, H. Sakaue, and M. Kasuga, unpublished observations.
ments in the promoter of the PPARγ2 by using chromatin immunoprecipitation assay. These data suggest that KLF15 binds to different sites from these CACC elements and thereby increases the promoter activity of the PPARγ2 gene, but they do not exclude the possibility that KLF15 may function through interaction with other transcription factors. The exact nature of KLF15-mediated activation of PPARγ2 transcription warrants further investigation.

Inhibition of KLF15 function by expression of a dominant negative mutant (Δ318) or by RNAi during exposure of 3T3-L1 preadipocytes to inducers of differentiation resulted in inhibition of the expression of PPARγ measured on day 8, but Δ318 did not inhibit the expression of PPARγ measured on day 2 (before the onset of KLF15 expression). The abundance of C/EBPβ mRNA and protein increased transiently during adipocytic differentiation of 3T3-L1 cells, reaching a maximum within 2 days and returning to basal levels on day 4, at which time KLF15 mRNA was detected. The initial induction of PPARγ expression is thus likely mediated by C/EBPβ, rather than by KLF15, during adipogenesis in 3T3-L1 cells, and this effect of C/EBPβ is likely the direct result of its binding to C/EBP recognition sites in the PPARγ gene promoter (30, 31). We also demonstrated that C/EBPβ and C/EBPδ were each able to induce the expression of KLF15 as well as that of PPARγ in NIH 3T3 cells. The expression of KLF15 induced by C/EBPβ and C/EBPδ during adipocyte differentiation in 3T3-L1 cells is thus likely responsible for that of PPARγ after the abundance of C/EBPβ and C/EBPδ has decreased on day 4. In addition, we found that KLF15 and C/EBPδ acted synergistically to activate transcription of the PPARγ2 gene in 3T3-L1 adipocytes. KLF15 might thus function to maintain the differentiated state by mediating the persistent expression of PPARγ in cooperation with C/EBPδ.

We showed that ectopic expression of KLF15 in NIH 3T3 cells induced lipid accumulation and the expression of PPARγ after exposure of the cells to IBMX, dexamethasone, insulin, and the synthetic PPARγ ligand troglitazone. The omission of troglitazone resulted in a similar extent of PPARγ induction but largely prevented lipid accumulation (data not shown). These results suggest that production of the cellular ligand for PPARγ is not regulated by KLF15. Previous studies have suggested a role for C/EBPβ (32) or ADD1 (SREBP-1) (33) in the transient production of the cellular PPARγ ligand during an early phase of adipocyte differentiation in 3T3-L1 cells (34).

In conclusion, our study has demonstrated an essential role for KLF15 in the complex regulation of gene transcription during adipogenesis. A model for the transcriptional control of adipogenesis in 3T3-L1 cells that takes into account both our present data and those of previous studies (2, 20, 21, 35, 36) is shown in Fig. 9. A transient increase in the expression of C/EBPβ and C/EBPδ in response to inducers of differentiation results in up-regulation of PPARγ expression (20, 21, 37). C/EBPβ and C/EBPδ also activate transcription of the KLF15 and C/EBPα genes, the proteins encoded by which then act synergistically to maintain the increase in PPARγ expression after the levels of C/EBPβ and C/EBPδ have decreased. PPARγ is also able to increase expression of C/EBPα, thereby establishing a positive feedback loop between PPARγ and C/EBPα (36, 38, 39). In addition, KLF15 directly induces the expression of adipocyte-specific genes such as that for GLUT4 (11). The cooperative effects of KLF15, PPARγ, and C/EBPα are thus responsible for the full range of adipocyte-specific gene expression.