The Krüppel-like Factor KLF2 Inhibits Peroxisome Proliferator-activated Receptor-γ Expression and Adipogenesis*

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Sucharita Sen Banerjee, Mark W. Feinberg, Masaumi Watanabe, Susan Gray, Richard L. Haspel, Diane J. Denkinger, Rodney Kawahara, Hans Hauner, and Mukesh K. Jain

From the Cardiovascular Division, Brigham and Women’s Hospital, Boston, Massachusetts 02115, Department of Pharmacology, University of Nebraska Medical Center, Omaha, Nebraska 68198-6260, and Deutsches Diabetes-Forschungsinstitut Auf’m Hennekamp 65, 40255 Düsseldorf, Germany

Obesity is an important public health problem associated with a number of disease states such as diabetes and arteriosclerosis. As such, an understanding of the mechanisms governing adipose tissue differentiation and function is of considerable importance. We recently reported that the Krüppel-like zinc finger transcription factor KLF15 can induce adipocyte maturation and GLUT4 expression. In this study, we identify that a second family member, KLF2/Lung Krüppel-like factor (LKLF), as a negative regulator of adipocyte differentiation. KLF2 is highly expressed in adipose tissue, and studies in cell lines and primary cells demonstrate that KLF2 is expressed in preadipocytes but not mature adipocytes. Constitutive overexpression of KLF2 but not KLF15 potently inhibits peroxisome proliferator-activated receptor-γ (PPARγ) expression with no effect on the upstream regulators C/EBPβ and C/EBPδ. However, the expression of C/EBPα and SREBP1c/ADD1 (adipocyte determination and differentiation factor-1/sterol regulatory element-binding protein-1), two factors that feedback in a positive manner to enhance PPARγ function, was also markedly reduced. In addition, transient transfection studies show that KLF2 directly inhibits PPARγ2 promoter activity (70% inhibition; p < 0.001). Using a combination of promoter mutational analysis and gel mobility shift assays, we have identified a binding site within the PPARγ promoter, which mediates this inhibitory effect. These data identify a novel role for KLF2 as a negative regulator of adipogenesis.

Obesity is recognized as a worldwide public health problem that contributes to a wide range of disease conditions (1, 2). Accumulating evidence derived from both clinical and experimental observations highlights the association of obesity with a number of chronic diseases such as type II diabetes, atherosclerosis, restrictive lung disease, cancer, and degenerative arthritis. Furthermore, there is growing recognition that a greater understanding of mechanisms regulating fat cell differentiation and the modulation of specific genes in the adipocyte may allow for novel strategies to limit obesity and its attendant consequences (1, 2).

Current models of the transcriptional basis for adipocyte differentiation highlight an interplay among members of several major families, the CCAAT/enhancer-binding protein (C/EBP), the peroxisome proliferator-activated receptor (PPAR) family, and the basic-helix-loop-helix family, and the basic-helix-loop-helix protein ADD1/SREBP1c (reviewed in Refs. 3 and 4). Studies to date suggest that C/EBPβ and C/EBPδ induce PPARγ, which in turn initiates the adipogenic program (3, 4). Both gain and loss of function experiments argue strongly that PPARγ is necessary and sufficient to promote fat cell differentiation (5, 6). C/EBPα has also been shown to play a critical role in certain aspects of adipogenesis (7, 8). Recent studies using C/EBPα null fibroblasts suggest that the critical role of this factor is to induce and maintain PPARγ levels as well as to confer insulin sensitivity to adipocytes (7). Finally, ADD1/SREBP1c can also promote adipogenesis. This probably occurs through several mechanisms including direct stimulation of PPARγ expression as well as production of the endogenous ligand that activates PPARγ (9–11).

Krüppel-like factors are zinc finger proteins that constitute an important class of transcriptional regulators. Members of this family are characterized by multiple zinc fingers containing regions with conserved sequences CX3CX2FX1LX5HX8H (X is any amino acid; underlined cysteine and histidine residues coordinate zinc) (12). The zinc fingers are usually found at the C terminus of the protein and bind to the consensus sequence 5’-CNCCC-3’. The N terminus is involved in transcriptional activation and/or repression as well as protein–protein interaction (12). Previous studies show that Krüppel-like proteins typically regulate critical aspects of cellular differentiation and function in diverse cell types (13–16). A role for this gene family in adipocyte biology was recently demonstrated by studies from our laboratory showing that KLF15 is highly expressed in adipose tissue and can induce GLUT4 expression (17). Upon further investigation, we found that KLF2/LKLF was also highly expressed in white and brown adipose tissue in mice. However, in contrast to KLF15, KLF2/LKLF is expressed in preadipocytes but not in mature adipocytes. In this report we provide evidence that KLF2 can function as a negative regulator of adipogenesis via inhibition of PPARγ.

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**To whom correspondence should be addressed: Cardiovascular Division, Brigham and Women’s Hospital, Thorsen Building Room 1123, 20 Shattuck St., Boston, MA 02115. Tel.: 617-278-0142; Fax: 617-732-5132; E-mail: mjain@rics.bwh.harvard.edu.

1 The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; KLF, Krüppel-like Factor; LKLF, lung KLF; PPAR, peroxisome proliferator-activated receptor; ADD1/SREBP1c, adipocyte determination and differentiation factor-1/sterol regulatory element-binding protein-1; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; EV, empty virus; RT, reverse transcriptase; Luc, luciferase.

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MATERIALS AND METHODS

Cell Culture—Adipocyte differentiation using 3T3L1 cells was performed as described previously (18). Cells were cultured in 10% FBS/DMEM until 3 days post-confluence (day 0). Medium was changed to 10% FBS/DMEM supplemented with 0.5 mM MIX (3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1.7 μM insulin). After 48 h (day 2), medium was changed to 10% FBS/DMEM supplemented with 0.25 μM insulin used at day 0. The media of the cultured cells were changed every 48 h during the experiment. Insulin was removed from the maintenance medium on day 4.

For retroviral studies, KLF2 cDNA was cloned into the retroviral vector of green fluorescent protein (a gift of K. Murphy), and retrovirus was generated as described previously (19). For infection of target cells, vector of green fluorescent protein (a gift of K. Murphy), and retrovirus maintenance medium on day 4.

Every 48 h during the experiment. Insulin was removed from the main- stops were performed on plates overexpressing empty virus (EV), KLF2, and KLF15 as described previously (20).

RT-PCR was performed as described previously (20, 21). The primer sequences used for KLF2 were sense 5′-CACGCGAGCAGCCAACA-3′ and antisense 5′-GGGCAAGTCGCACTGGAAT-3′ (Invitrogen). The primer sequences for PPARγ were sense 5′-CCACGACGATCTCCACAATG-3′ and antisense 5′-TGCTTCAAGTCAAGATTACAA-3′ (Invitrogen). The primer sequences for GLUT4 were sense 5′-CGGAGGCACCCCAAGAAA-3′ and antisense 5′-GGGTTACGGGTTACGACGGAG-3′ (Invitrogen). For Sp1, the primer sequences were sense 5′-GGTCAAGCAGATCCGCCCTC-3′ and antisense 5′-GAAGAGGGCAGTGTCACGCT-3′ (Invitrogen) (21).

For the transfection experiment, 3T3-L1 cells were plated in 6-well dishes at a density of 1 × 10^5 cells/well, and transient transfections were performed the following day using FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according to manufacturer’s recommendations. The total amount of DNA used for the transfection assay per well was always held constant at 1 μg. The KLF2 expression plasmid was kindly provided by J. Leiden (Abbott Laboratories, Chicago, IL). Luciferase and β-galactosidase assays were carried out as described previously (16). Promoter activity of each construct was expressed as the ratio of luciferase/β-galactosidase activity. All transfections were performed in triplicate from three independent experiments.

Gel Mobility Shift Assays—Gel mobility shift assays using the GST-KLF2 fusion protein were performed as described previously (22). Modifications of this procedure included a reduction in the amount of PMSF to 200 μg/ml. The radioactive wild-type probe was ~10,000 cpm, and each reaction tube contained 1 μl of rabbit preimmune serum containing dextran (for enhanced binding). Competition studies were performed using 10×, 100×, and 1000× wild-type and mutant competitor. The sequence for the mutant competitor is the same as that used for the mutated primers above. For supershift studies, the anti-KLF2 antibody was kindly provided by J. Leiden (Abbott Laboratories).

RESULTS

Expression of KLF2 in Adipose Tissue—We recently reported that a member of the KLF family termed KLF15 is expressed in mature adipocytes and regulates GLUT4 (17). In the course of these studies, we assessed the expression of several other KLFs in adipose tissue. KLF1 (erythroid KLF) and KLF4 (Gut KLF) mRNA were barely detected in mouse adipose tissue (Fig. 1A) (data not shown). However, as shown in Fig. 1A, KLF2 mRNA was strongly expressed in both white and brown adipose tissues. Given the availability of cell lines that faithfully recapitulate adipogenesis in vitro, we assessed the expression of KLF2 during 3T3-L1 differentiation (23). Stimulation of these cells with an empirically derived prodifferentiation mixture leads to a characteristic pattern of induction for various transcription factors and target genes involved in adipogenesis and lipogenesis. We observed that KLF2 mRNA was expressed in 3T3-L1 preadipocytes and markedly diminished upon adipocyte differentiation (Fig 1B). As expected, other factors such as PPARγ, members of the C/EBP family, and adipocyte fatty acid-binding protein were induced with 3T3-L1 differentiation. The expression pattern of KLF2 was most similar to two other transcription factors that have been shown to inhibit adipogenesis, termed GATA3 and 2 (Fig 1B) (data not shown) (24). To verify this expression pattern in primary cells, we assessed the expression of KLF2 in human preadipocytes and adipocytes by RT-PCR (21). Similar to the KLF2 expression pattern in 3T3-L1 cells, KLF2 is highly expressed in human preadipocytes and is markedly reduced in mature adipocytes (Fig 1C). Consistent with previous studies, GLUT4 and PPARγ expression are induced with differentiation, whereas Sp1 levels are minimally affected (Fig 1C).

Overexpression of KLF2 Inhibits Adipogenesis in Vitro—The reduced expression of KLF2 mRNA expression during 3T3-L1 differentiation suggested a potentially inhibitory role in adipogenesis. To test this possibility directly, we retrovirally overexpressed KLF2 and EV as a control in 3T3-L1 cells and induced differentiation. In parallel, we also overexpressed KLF15, which has been shown to promote 3T3-L1 differentiation. As shown in Fig. 2A, compared with EV-infected cells, KLF2-overexpressing cells showed a decrease in intracellular lipid accumulation as evidenced by reduced Oil-Red-O staining (Fig 2A). In contrast, KLF15-infected cells exhibited increased lipid accumulation.

To determine the mechanism by which KLF2 may inhibit 3T3-L1 differentiation, we considered several possibilities. In theory, inhibition of adipocyte differentiation may occur via inhibition of factors that promote differentiation or induction of factors that inhibit adipogenesis. With respect to the latter, we did not observe any effect of KLF2 overexpression on known negative regulators of adipogenesis such as GATA3 (24), β-catenin (25), Smad proteins (26), C/EBP-homologous protein (27), or pret-1 (25) (data not shown). Furthermore, overexpres-
assess the former possibility, we tested whether KLF2 may factors that have been demonstrated to positively regulate second possibility, we did observe a marked inhibition of two ysis using the indicated probes.

Total RNA was harvested at the indicated number of days post-induction of differentiation and subjected to Northern anal-

Constitutive expression of KLF2 inhibits adipocyte differ-

Fig. 2. Constitutive expression of KLF2 inhibits adipocyte differ-

A, effect of KLF2 overexpression on lipid accumulation in 3T3-L1 cells. 3T3-L1 cells were retrovirally infected with EV, KLF2, and KLF15 and subjected to Oil-Red-O staining to assess for lipid accumulation at day 4 differentiation. B, effect of KLF2 overexpression on adipocyte gene expression. 3T3-L1 cells were retrovirally infected with EV and KLF2 and differentiated as described under “Materials and Methods.” Total RNA was harvested at the indicated number of time points, and Northern analysis performed using the indicated probes. C, effect of KLF2 and KLF15 on PPARγ expression. 3T3-L1 cells were retrovirally infected with EV, KLF2, and KLF 15 and differentiated. Cells were harvested at the indicated time points, and Northern analysis performed using the indicated probes.

Fig. 3. KLF2 represses PPARγ2 promoter activity. A, effect of KLF2 on PPARγ2 promoter activity. 3T3-L1 cells were transfected with PPARγ2 promoter (−250-bp Luc) and either KLF2 or ZnF expression construct (left panel). 3T3-L1 cells were transfected with PPARγ2 pro-
moter and C/EBPα and C/EBPβ expression constructs (right panel). Luciferase and β-galactosidase assays were performed as described under “Materials and Methods.” The GST-KLF2 fusion protein was incubated with a 32P-labeled wild-type oligomer extending from base pairs −82 to −93 of the PPARγ2 promoter. A single dominant DNA-protein complex was seen (arrow). Competition and supershift studies (arrowhead) were performed to verify specificity. C, effect of mutation of the CACCC element. Electrophoretic mobility shift assays were performed as described under “Materials and Methods.” The GST-KLF2 fusion protein was incubated with a 32P-labeled wild-type oligomer extending from base pairs −82 to −93 of the PPARγ2 promoter. A single dominant DNA-protein complex was supershifted in the presence of an anti-KLF2 antibody (Fig. 3B, arrowhead) but not IgG. To verify that this site was important in KLF2-mediated repression, we mutated the KLF binding site within the context of the −250-bp Luc-PPARγ2 promoter. As shown in Fig. 3C, mutation of this site significantly attenuated the inhibitory effect of KLF2, suggesting that this site is important for KLF2-mediated inhibition of the PPARγ2 promoter.

DISCUSSION

Members of the Krüppel-like family of transcription factors have been shown to play important roles in cellular differenti-

KLF2 was initially identified through a homology screening strategy using the zinc finger domain of KLF1 as a probe (29). Studies targeting KLF2 in mice have shown an essential role in embryonic development as these animals die during mid-gestation because of abnormal blood vessel development (30). In addition, T-cell maturation (15).
well as lung development (31) is impaired. However, despite the importance of KLF2, very few direct targets have been identified (22). In this regard, the observations from this study may bear important implications not only for adipocyte biology but also for the role of KLF2 in other tissues.

In this report, we provide evidence that the Krüppel-like factor KLF2 is a negative regulator of adipogenesis. Consistent with an inhibitory role, KLF2 is expressed in preadipocytes and expression is markedly reduced in mature adipocytes (Fig. 1). In addition, overexpression of KLF2 markedly inhibits lipid accumulation in 3T3-L1 cells (Fig. 2). This ability is specific to KLF2, whereas another member of this family, KLF15, augments lipid accumulation. To understand the basis for the inhibitory effect of KLF2, we considered several possibilities such as (a) inhibition of factors that have been shown to positively regulate adipocyte differentiation or (b) induction of factors identified as negative regulators of adipocyte differentiation. With respect to the latter possibility, we did not observe any effect on the expression of several previously identified negative regulators such as GATA3 (24), β-catenin (25), Smad proteins (26), CHOP (27), or pref-1 (32). With respect to factors that promote adipogenesis, KLF2 did not significantly affect the expression of C/EBPβ and C/EBPδ, two upstream regulators of adipogenesis. In contrast, KLF2 potently inhibits the expression of PPARγ, the central regulator of adipogenesis. Given that PPARγ expression is essential for adipocyte development, it is probable that this inhibition is critical for KLF2-mediated inhibition of adipocyte differentiation. In addition to PPARγ, KLF2 also inhibits expression of both C/EBPα and ADD1/SREBP1c, two factors that play important roles in adipocyte differentiation. A recent study (8) suggests that the principal role of C/EBPα is to induce and maintain PPARγ expression. As such, the inhibition of C/EBPα expression may contribute to the reduced levels of PPARγ seen in KLF2-overexpressing cells, thereby leading to the inhibition of adipocyte differentiation. Furthermore, ADD1/SREBP1c has been shown to augment adipogenesis via direct induction of PPARγ expression as well as through production of an endogenous PPARγ ligand (9–11). The inhibition of ADD1/SREBP1c expression by KLF2 may therefore lead to both a reduction in PPARγ expression and activation. Thus, KLF2 may arrest the adipogenic program through inhibition of PPARγ expression and inhibition of factors that positively regulate PPARγ expression and function.

The mechanism by which KLF2 is able to inhibit PPARγ expression has yet to be fully elucidated. As suggested above, part of the mechanism may be via the inhibition of factors such as C/EBPα and ADD1/SREBP 1c, which positively regulate PPARγ expression and function. However, our data also suggest that KLF2 can inhibit PPARγ2 promoter activity, thus supporting a direct mechanism. As shown in Fig. 3C, KLF2 inhibited PPARγ2 promoter activity by ∼70%. An examination of the PPARγ2 promoter revealed the presence of a single consensus Krüppel binding site. Gel shift studies demonstrate that KLF2 can bind to this site (Fig. 3B), and promoter mutation analyses support a role for this site in mediating the inhibitory effect of KLF2 (Fig. 3C). Importantly, however, the mutation of this site alone was not sufficient to completely abrogate KLF2-mediated inhibition of promoter activity, suggesting that non-DNA binding-dependent mechanisms are probably involved for the full inhibitory effect. It is also noteworthy that the KLF binding sites lie in between the two binding sites identified by Tong et al. (24) as essential for GATA-mediated inhibition of the PPARγ2 promoter activity. The proximity of these two sites raises the possibility of a cooperative interaction between these two factors and is the subject of future investigations.

Our current understanding of the transcriptional basis for adipogenesis highlights the function of several major positive and negative regulatory families such as the C/EBPs, PPARs, and GATA factors. We recently reported (17) a role for the Krüppel-like factor KLF15 in adipocyte biology by virtue of its ability to induce the insulin-sensitive glucose transporter GLUT4. In this report, we provide a novel function for another KLF family member, KLF2, as a negative regulator of adipogenesis. Taken together, these studies support a role for the Krüppel-like factors as potential regulators of adipogenesis. Future studies evaluating these factors in vivo will help elucidate more precisely the role of Krüppel-like factors in adipocyte biology.

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