Regulation of adipocyte differentiation of 3T3-L1 cells by PDZRN3

Takeshi Honda,* Aiko Ishii,* and Makoto Inui

Department of Pharmacology, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi, Japan

Submitted 24 October 2012; accepted in final form 2 April 2013

PDZRN3, a member of the PDZRN (or LNX) family of proteins, is essential for the differentiation of mesenchymal stem cells into myotubes, but it plays an inhibitory role in the differentiation of these cells into osteoblasts. Given that mesenchymal stem cells also differentiate into adipocytes, we examined the possible role of PDZRN3 in adipogenesis in mouse 3T3-L1 preadipocytes. The expression of PDZRN3 decreased at both the mRNA and protein levels during adipogenic differentiation. RNAi-mediated depletion of PDZRN3 enhanced the differentiation of 3T3-L1 cells into adipocytes as assessed on the basis of lipid accumulation. The upregulation of aP2 and CCAAT/enhancer-binding protein (C/EBP)β during adipogenic differentiation was also enhanced in the PDZRN3-depleted cells, as was the induction of peroxisome proliferator-activated receptor-γ (PPARγ), an upstream regulator of aP2 and C/EBPβ, at both the mRNA and protein levels. Among transcription factors that control the expression of PPARγ, we found that STAT5b, but not STAT5a, was upregulated in PDZRN3-depleted cells at both mRNA and protein levels. Tyrosine phosphorylation of STAT5b, but not that of STAT5a, was also enhanced at an early stage of differentiation by PDZRN3 depletion. In addition, the expression of C/EBPβ during the induction of differentiation was enhanced at the mRNA and protein levels in PDZRN3-depleted cells. Our results thus suggest that PDZRN3 negatively regulates adipogenesis in 3T3-L1 cells through downregulation of STAT5b and C/EBPβ and consequent suppression of PPARγ expression.

MATERIALS AND METHODS

Materials. Recombinant human insulin, 3-isobutyl-1-methylxanthine, dexamethasone, and Oil Red O were obtained from Sigma-Aldrich (St. Louis, MO), and protease and phosphatase inhibitor cocktails were from Roche (Branchburg, NJ). Polyclonal antibodies to PDZRN3 were generated in rabbits and purified as described previously (11). Rabbit polyclonal antibodies to C/EBPβ (sc-61), to STAT5a (sc-1081), and to JunB (sc-73); mouse monoclonal antibodies to C/EBPβ (sc-7692), to PPARγ (sc-7233), and to STAT5b (sc-1656); and goat polyclonal antibodies to aP2 (sc8661) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies to C/EBPβ (no. 2318) and to STAT1 (no. 9172) as well as rabbit monoclonal antibodies to c-Fos (no. 2250) and to c-Jun (no. 9165) were from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal antibodies to Krüppel-like factor 5 (KLF5; ab24331) were from Abcam (Cambridge, MA). Horseradish peroxidase-conjugated mouse monoclonal antibodies to phosphorylated tyrosine (PY-20) were obtained from BD Biosciences (San Jose, CA), and mouse monoclonal antibodies to β-tubulin were from Sigma-Aldrich.

Cell culture. Mouse 3T3-L1 preadipocytes were obtained from American Type Culture Collection (Manassas, VA) and were maintained under 5% CO₂ at 37°C in growth medium consisting of DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). For induction of differentiation into adipocytes, the cells were cultured for 2 days after achieving confluence and were then exposed to growth medium containing MDI (0.5 mM 3-isobutyl-1-
Fig. 1. Expression of PDZRN3 during 3-isobutyl-1-methylxanthine, dexamethasone, and insulin (MDI)-induced differentiation of 3T3-L1 cells into adipocytes. A–C: 3T3-L1 cells were exposed (or not) to MDI to induce differentiation into adipocytes and were stained with Oil Red O at 5 days after induction (A). Stained area was measured by image analysis and expressed relative to the value for cells not exposed to MDI (B). Triglyceride content of the cells was also measured at 7 days after induction (C). Data in B and C are means ± SE from 10 and nine independent experiments, respectively. ***P < 0.001. D: cell lysates prepared at the indicated times after the induction of adipogenesis were subjected to immunoblot analysis with antibodies to PDZRN3, to peroxisome proliferator-activated receptor-γ (PPARγ), to CCAAT/enhancer-binding protein (C/EBP)β, to aP2, and to α-tubulin (loading control). E: intensity of the PDZRN3 band in immunoblots similar to that in D was measured by densitometry, normalized by that of the corresponding α-tubulin band, and expressed as a percentage of the value for time 0. Data are means ± SE from 5 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs. time 0. F: total RNA prepared from cells at the indicated times after the induction of adipogenesis was subjected to RT and real-time PCR analysis of PDZRN3 mRNA. Data are expressed as a percentage of the value for time 0 and are means ± SE from 3–6 independent experiments. **P < 0.01, ***P < 0.001 vs. time 0.

Fig. 2. Effect of RNAi-mediated depletion of PDZRN3 on the differentiation of 3T3-L1 cells into adipocytes. A: 3T3-L1 cells infected (or not) with adenoviral vectors encoding either short hairpin (sh)RNAs specific for mouse PDZRN3 mRNA or a scrambled control shRNA were constructed as described previously (8). 3T3-L1 cells were seeded at a density of 5 × 10⁴ cells per well in 24-well plates, cultured in growth medium for 12 h, and infected with adenoviruses at a multiplicity of infection of 10 for 24 h, after which the medium was replaced with growth medium. An adenoviral vector encoding a form of mouse PDZRN3 mRNA that is resistant to the PDZRN3 shRNA was constructed as described previously (8).

Oil Red O staining and quantification of triglyceride content. 3T3-L1 cells were fixed with 4% formaldehyde for 15 min at room temperature, washed with PBS, and incubated for 15 min at room temperature with 0.3% Oil Red O in isopropanol. The stained cells were washed with 60% isopropanol and then photographed. In triglyceride measurement, cells were washed with PBS, and then lysed with PBS containing 0.5% Triton X-100. After incubation at 80°C for 5 min, the lysates were centrifuged, and the amounts of triglyceride in
the supernatants were measured according to the manufacturer’s instruction using a WAKO LabAssay Triglyceride Kit (Wako Pure Chemical, Osaka, Japan). Triglyceride content is expressed as milligrams of triglyceride per milligrams of protein.

**Immunoprecipitation and immunoblot analysis.** 3T3-L1 cells were washed twice with a solution containing 150 mM NaCl and 20 mM Tris-HCl (pH 7.5) and then lysed in a solution containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 20 mM Na2VO4, 20 mM NaF, 5 mM EDTA, 0.3% Triton X-100, phosphatase inhibitor cocktail, and protease inhibitor cocktail. The lysates were analyzed for protein content with the use of a DC Protein Assay Kit (Bio-Rad, Hercules, CA), and equal amounts of total lysate protein were subjected to SDS-PAGE. The separated proteins were transferred to a PVDF membrane (Millipore, Billerica, MA), nonspecific sites of which were blocked before its consecutive exposure to primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL). Immune complexes were detected with the use of a Chemi-Lumi One detection kit (Nacalai Tesque, Kyoto, Japan) and Amersham Hyperfilm ECL (GE Healthcare/Amersham Biosciences). The beads were washed with lysis buffer additional presence of protein G-Sepharose beads (GE Healthcare/Amersham Biosciences). The beads were washed with lysis buffer five times and then suspended in SDS sample buffer for immunoblot analysis with PY-20 antibodies.

**RT-PCR analysis.** Total RNA was prepared from 3T3-L1 cells with the use of an SV total RNA isolation system (Promega), and portions of the RNA were reverse transcribed with the use of a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). The resulting reaction mix was subjected to real-time PCR analysis with a FastStart Universal SYBR Green Master Kit (Roche) and the Applied Biosystems StepOne Plus real-time PCR system (Life Technologies, Carlsbad, CA). Relative expression of the genes of interest was estimated with the ΔΔCt method and with G3PDH as a reference gene. The sequences of PCR primers (sense and antisense, respectively) were 5′-CTGACT-CGCTCCGCGCCTG-3′ and 5′-CGGGATGCTCTTCGAGCCAAGG-3′ for mouse PDZRN3; 5′-ATAAAGTCCTTCCCGCTGACCAAAGCC-3′ and 5′-GGGTCTTCCACTGAGAATAATGACAGC-3′ for mouse PPARγ; 5′-ATTACACTCTGACTTGCA-3′ and 5′-GTCGAACTCGCCCATCTTGG-3′ for mouse STAT5a; 5′-TCCCTGTGAGCCCGCAAC-3′ and 5′-GGTGAGGGTCGTGTCATGACT-3′ for mouse STAT5b; 5′-CAAGCTGAGCAAGAGTACA-3′ and 5′-GCCCTCAGCCGCTG-3′ for mouse STAT5a; 5′-CAAGCTGAGCAAGAGTACA-3′ and 5′-GCCCTCAGCCGCTG-3′ for mouse STAT5b. The resulting reaction mix was subjected to real-time PCR analysis with a FastStart Universal SYBR Green Master Kit (Roche) and the Applied Biosystems StepOne Plus real-time PCR system (Life Technologies, Carlsbad, CA). Relative expression of the genes of interest was estimated with the ΔΔCt method and with G3PDH as a reference gene. The sequences of PCR primers (sense and antisense, respectively) were 5′-CTGACT-CGCTCCGCGCCTG-3′ and 5′-CGGGATGCTCTTCGAGCCAAGG-3′ for mouse PDZRN3; 5′-ATAAAGTCCTTCCCGCTGACCAAAGCC-3′ and 5′-GGGTCTTCCACTGAGAATAATGACAGC-3′ for mouse PPARγ; 5′-ATTACACTCTGACTTGCA-3′ and 5′-GTCGAACTCGCCCATCTTGG-3′ for mouse STAT5a; 5′-TCCCTGTGAGCCCGCAAC-3′ and 5′-GGTGAGGGTCGTGTCATGACT-3′ for mouse STAT5b; 5′-CAAGCTGAGCAAGAGTACA-3′ and 5′-

**Fig. 3.** Effects of PDZRN3 depletion on the expression of aP2, C/EBPα, PPARγ, and C/EBPβ in 3T3-L1 cells during adipogenesis. A: 3T3-L1 cells infected with adenoviral vectors for PDZRN3 (KD-1 or -2) or control (Scramb) shRNAs were induced to differentiate into adipocytes by culture in growth medium containing MDI. Cell lysates prepared at the indicated times were subjected to immunoblot analysis with antibodies to the indicated proteins. B–D: normalized abundance of aP2 (B), C/EBPα (C), and PPARγ (D) at 48 h after the addition of MDI in immunobLOTS similar to that in A was determined by densitometry. Data are expressed relative to the value for cells expressing the control shRNA and are means ± SE from 8 to 10 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs. the value for Scramb. E: total RNA isolated from cells treated as in A was subjected to RT and real-time PCR analysis of PPARγ mRNA. Data are expressed relative to the value for noninfected cells at time 0 and are means ± SE from 4–5 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.
AGCTGCTCCACCTTTCTCTG-3' for mouse C/EBPβ; and 5'-CTC-CCACTTTCCACTTCG-3' and 5'-CATACCAGAAATGAGC- TTGACAA-3' for mouse G3PDH.

Statistical analysis. Data are presented as means ± SE and were analyzed by one-way ANOVA followed by post hoc comparison among groups or by two-tailed unpaired Student’s t-test between two groups. A P < 0.05 was considered statistically significant.

RESULTS

Downregulation of PDZRN3 during adipogenesis in 3T3-L1 cells. Exposure of 3T3-L1 preadipocytes to MDI induced their differentiation into adipocytes, as revealed by their accumulation of lipid (Fig. 1, A–C). Immunoblot analysis showed that the abundance of PPARγ and C/EBPα was increased 2 days after the induction of differentiation and that the expression of αP2 increased markedly thereafter (Fig. 1D). Whereas the abundance of PDZRN3 protein appeared to be transiently increased at 1 day after induction of differentiation, this effect was not statistically significant (Fig. 1E). Rather, the amounts of both PDZRN3 protein (Fig. 1, D and E) and mRNA (Fig. 1F) decreased during adipogenesis.

Promotion of adipogenesis by depletion of PDZRN3. To examine the possible role of PDZRN3 in adipogenesis, we determined the effect of its RNAi-mediated depletion on the differentiation of 3T3-L1 cells. Knockdown of PDZRN3 by infection of cells with adenoviral vectors encoding two different shRNAs (KD-1 and -2) specific for PDZRN3 mRNA (Fig. 2A) was associated with a marked increase in the extent of Oil Red O staining at 5 days (Fig. 2B) and a significant increase in triglyceride content at 7 days (Fig. 2C) after the induction of adipogenesis, compared with those apparent in cells infected with an adenovirus encoding a scrambled version of KD-1. Depletion of PDZRN3 thus resulted in enhancement of adipogenesis. Analysis of the cells at an earlier stage of differentiation revealed that the abundance of C/EBPα and αP2 was greater in the PDZRN3-depleted cells than in the control cells (Fig. 3, A–C). The expression of PPARγ, an upstream regulator of C/EBPα and αP2, was also increased at both the mRNA and protein levels as a result of PDZRN3 depletion (Fig. 3, A, D, and E). Restoration of PDZRN3 expression in PDZRN3-depleted cells by infection with an adenovirus encoding a RNAi-resistant form of the protein resulted in normalization of the amounts of PPARγ and αP2 (Fig. 4, A and B). Such effects were not apparent on infection of PDZRN3-depleted cells with an adenovirus encoding β-galactosidase (LacZ). Furthermore, overexpression of PDZRN3 in 3T3-L1 cells not subjected to RNAi had no effect either on the expression of PPARγ or αP2 during the induction of adipogenesis (Fig. 4C) or on the extent of Oil Red O staining at 5 days (Fig. 4D) or triglyceride content at 7 days (Fig. 4E) after the induction of adipogenesis.

Increased expression of STAT5b and C/EBPβ in PDZRN3-depleted cells. The upregulation of PPARγ during early adipogenic differentiation is mediated by the action of various transcription factors including AP-1, C/EBP, KLF, and STAT proteins. The expression of C/EBPβ was induced earlier than that of PPARγ during the differentiation of 3T3-L1 cells into adipocytes (Fig. 3A). At 6 h after the addition of MDI, the abundance of C/EBPβ was significantly higher in PDZRN3-depleted cells than in cells expressing the control shRNA (Figs. 3A and 5A). The amount of C/EBPβ mRNA at 2 h after the addition of MDI, at which time the level peaks in 3T3-L1 cells (5), was also higher in the PDZRN3-depleted cells than in the control cells (Fig. 5B). The expression of C/EBPα, KLF5, and the AP-1 components c-Fos, JunB, and c-Jun was induced at 2 h after the addition of MDI, consistent with previous observations (4, 20), but the amounts of these proteins did not differ significantly between the PDZRN3-depleted and control cells (Fig. 5, C–G).
The expression of STAT5b, but not that of STAT1 or STAT5a, was markedly increased in PDZRN3-depleted 3T3-L1 cells, compared with that in cells expressing the control shRNA, before the induction of adipocyte differentiation (Fig. 6A). Similarly, the amount of STAT5b mRNA, but not that of STAT5a mRNA, was increased in the PDZRN3-depleted cells (Fig. 6B). Consistent with previous observations (16), the expression of STAT5a and STAT5b in control 3T3-L1 cells was found to be upregulated at a late stage of adipocyte differentiation (Fig. 6C). In PDZRN3-depleted cells, however, such upregulation of STAT5b was not observed, presumably because of its increased expression level before the addition of MDI (Fig. 6C). The amounts of the tyrosine-phosphorylated (activated) forms of STAT5a and STAT5b increased gradually after exposure of control cells to MDI before declining at 5 h (Fig. 6D). The extent of this transient activation of STAT5b, but not that of STAT5a, was significantly increased in PDZRN3-depleted cells (Fig. 6, D and E).

**DISCUSSION**

We have shown that PDZRN3 inhibits adipogenesis in 3T3-L1 cells by suppressing the master regulator of this process, PPARγ, which is itself under the control of several transcription factors (4, 20). One of these factors, STAT5, undergoes tyrosine phosphorylation and translocates to the nucleus early during adipogenesis, and it drives differentiation by inducing the expression of PPARγ (1, 6, 13). Given that the expression of STAT5, in particular that of STAT5b, was found to be upregulated and that the MDI-induced phosphorylation of STAT5b was enhanced in PDZRN3-depleted cells, PDZRN3 likely regulates the expression of PPARγ through STAT5b. In addition, the abundance of C/EBPβ, a transcription factor that also plays a key role in the induction of PPARγ expression (2, 21, 22), was greater in PDZRN3-depleted cells than in control cells after the addition of MDI. These observations thus suggest that PDZRN3 controls adipogenesis in 3T3-L1 cells by regulating the expression of both STAT5b and C/EBPβ.

Mesenchymal stem cells are capable of differentiating into myotubes, osteoblasts, and adipocytes. We previously showed that PDZRN3 is upregulated during myogenic and osteoblastic differentiation of C2C12 cells (8, 11). The expression of PDZRN3 increases early (<12 h) after the induction of osteoblastic differentiation, whereas the protein is upregulated later (>48 h) after the induction of myogenic differentiation. In contrast, PDZRN3 was found to be downregulated during adipogenesis in 3T3-L1 cells. PDZRN3 has an inhibitory role in both osteoblastic and adipogenic differentiation, whereas it is essential for myogenic differentiation (8, 11). Upregulation of PDZRN3 during osteoblastic differentiation is likely important for negative feedback control of the differentiation process, whereas downregulation of PDZRN3 appears to facilitate adiogenic differentiation. PDZRN3 thus responds to different stimuli and plays different roles in three different differentiation pathways. The performance of multiple functions by PDZRN3 might be facilitated by its interactions with different binding partners mediated through its PDZ domains or COOH-terminal PDZ binding motif.

We also previously showed that the depletion of PDZRN3 increased LR6 (low-density lipoprotein receptor-related pro-
tein 6) expression in C2C12 cells, resulting in the promotion of osteoblastic differentiation through enhancement of Wnt signaling (8). Upregulation of LRP6 in response to depletion of PDZRN3 was not apparent in 3T3-L1 cells, however (data not shown). Furthermore, neither Wnt3a nor the Wnt signaling inhibitor Dkk had a substantial effect on the promotion of adipogenesis by depletion of PDZRN3 in these cells (data not shown). LRP6 thus does not appear to contribute to the regulation of adipogenesis by PDZRN3 in 3T3-L1 cells, even though Wnt signaling has previously been shown to inhibit the differentiation of preadipocytes into adipocytes (14).

The time course of the downregulation of PDZRN3 during adipogenesis coincided with that of aP2 upregulation. Given that PDZRN3 plays an inhibitory role in adipogenesis, an increase in its expression level might be expected to block this process. Overexpression of PDZRN3 in 3T3-L1 cells, however, did not inhibit adipogenesis, as assessed on the basis of the expression of PPARγ and aP2 as well as of the Oil Red O staining and measurement of triglyceride content. Although it remains unclear why overexpression of PDZRN3 did not inhibit adipogenesis in 3T3-L1 cells, it is possible that PDZRN3 antagonizes adipogenesis through its interaction with one or more binding proteins and that the expression levels of these binding proteins in 3T3-L1 cells are lower than or equal to that of endogenous PDZRN3. Under such circumstances, overexpression of PDZRN3 would not be expected to inhibit
adipogenesis, whereas the restoration of PDZRN3 expression in PDZRN3-depleted cells would be expected to and did normalize the differentiation process.

Our results suggest that PDZRN3 is a key determinant of the basal expression level of STAT5b in 3T3-L1 cells. The increased abundance of STAT5b in PDZRN3-depleted 3T3-L1 cells resulted in enhancement of MDI-induced activation of STAT5b. On the other hand, depletion of PDZRN3 had no substantial effect on the basal abundance of STAT5a or on its activation at an early stage of differentiation. STAT5a and STAT5b might be subjected to differential transcriptional control, given that their relative abundance varies among tissues (7). It remains unclear, however, how the basal abundance of STAT5b is regulated in 3T3-L1 cells and how PDZRN3 contributes to such regulation. Knockout mice lacking either STAT5a or STAT5b have a reduced fat pad size (17), and both STAT5a and STAT5b were previously shown to be activated (tyrosine phosphorylated) at an early stage of 3T3-L1 cell differentiation (13). These findings indicate the importance of both proteins in adipogenesis. A functional difference between STAT5a and STAT5b was previously suggested by the finding that the ectopic expression of STAT5b did not induce adipogenesis in nonprecursor fibroblast cell lines, whereas STAT5a or the combination of STAT5a and STAT5b did so (6). Our results now show that the expression of STAT5a and that of STAT5b are regulated differentially by PDZRN3 and that STAT5b plays an important role in the modulation of adipogenesis by PDZRN3.

We found that the MDI-induced activation of STAT5b and expression of C/EBPβ were enhanced in PDZRN3-depleted cells. Both STAT5b and C/EBPβ contribute to the induction of PPARγ expression, which drives adipogenesis (4, 20). Previous studies have shown that the activation of STAT5 is closely linked to growth hormone signaling (10, 15) and that activated STAT5 is downregulated by a proteasome-dependent pathway (19). On the other hand, cAMP regulatory element-binding protein has been shown to participate in the induction of C/EBPβ (23). In addition to the regulation of STAT5b expression, PDZRN3 might thus also contribute to the regulation of C/EBPβ expression by cAMP signaling. It is also possible that the increased abundance of STAT5b in PDZRN3-depleted 3T3-L1 cells enhances transcription of the C/EBPβ gene, given that STAT5 has been found to increase the expression of C/EBPβ and its transactivation activity at the PPARγ gene promoter (10, 18). Further studies are necessary to determine how the expression of STAT5b and C/EBPβ is regulated by PDZRN3.

REFERENCES


GRANTS

This work was supported in part by a Yamaguchi University research project on stress and by Grants-in-Aid for Scientific Research (to M. Inui) and a Grant-in-Aid for Scientific Research (C) (24590332 to T. Honda) from the Japan Society for the Promotion of Science.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: T.H. and A.I. performed experiments; T.H. and A.I. analyzed data; T.H., A.I., and M.I. interpreted results of experiments; T.H. and A.I. prepared figures; M.I. conceived and designed of research; M.I. drafted manuscript; M.I. edited and revised manuscript; M.I. approved final version of manuscript.

Received for publication April 19, 2013. Accepted in revised form September 1, 2013.

AJP-Cell Physiol • doi:10.1152/ajpcell.00343.2012 • www.ajpcell.org