The Novel Gene \textit{fad158}, Having a Transmembrane Domain and Leucine-rich Repeat, Stimulates Adipocyte Differentiation*

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Adipose tissue is a unique organ that plays a key role in energy homeostasis by regulating the balance between energy storage and release according to nutritional status (1). It is now recognized that adipose tissue is a major secretory organ that secretes numerous kinds of cytokines like tumor necrosis factor-\(\alpha\), leptin, plasminogen activator inhibitor-1, adiponectin, and resistin. These factors are known to influence insulin sensitivity, food intake, arteriosclerosis, and several common diseases (2–4). A superfluous accumulation of adipose tissue leads to obesity. Obesity is an important risk factor for diseases like type 2 diabetes mellitus, hypertension, hyperlipidemia, and cardiac infarction (1, 5, 6). The link between obesity and these diseases is thought to come from a loss of secretion of cytokines from adipose tissue.

Obesity is the result of an expansion of individual adipocytes and increase in the number of adipocytes. The generation of new adipocytes results from differentiation mediated by several transcription factors identified as master regulators for adipogenesis. Peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)), \textsuperscript{1} one of these master regulators, was first identified as a protein binding to the enhancer region of adipocyte fatty acid-binding protein 2 (aP2) (7, 8). The ectopic expression of PPAR\(\gamma\) stimulates adipogenesis in non-adipogenic fibroblast cells such as NIH-3T3 (9). The CCAAT/enhancer-binding protein (C/EBP) family is also known to be master regulators of adipocyte differentiation. The ectopic expression of C/EBP\(\alpha\) also enhanced the ability of non-adipogenic cells to terminally differentiate into adipocytes (10).

Before the expression of PPAR\(\gamma\) and C/EBP\(\alpha\), the expression of C/EBP\(\beta\) and C/EBP\(\delta\) is seen from the mid-phase of adipocyte differentiation. These two transcription factors in turn activate the expression of both PPAR\(\gamma\) and C/EBP\(\alpha\) (11, 12). Sterol regulatory element-binding protein-1 (SREBP-1), also named adipocyte determination and differentiation factor 1 (ADD1), is another factor that regulates adipogenesis (13). SREBP-1 directly stimulates the expression of PPAR\(\gamma\) and regulates PPAR\(\gamma\) activity by producing the endogenous ligand of PPAR\(\gamma\) (14). Thus, the events in the mid and late phases of adipocyte differentiation are relatively well characterized. However, the molecular mechanisms underlying the very beginning of adipogenesis remain unknown.

We have previously clarified the initial step of adipocyte differentiation. Using mouse 3T3-L1 fibroblastic cells, a model of adipocyte differentiation, we have isolated the genes whose expression is induced in the early stage of the differentiation process. By the PCR-subtraction cloning method, we isolated 102 genes, including the genes for transcription factors and signaling proteins (15–17). Interestingly, 46 of the genes isolated seem to be new, having no significant similarity with the genes listed in the GenBank\textsuperscript{TM}/EMBL/DDJB databases.

The fragments isolated by the PCR-subtraction method are only 300–500 bp long. Therefore, the first step in this pursuit is to isolate the full-length CDNA and identify the open reading frame (ORF) of the unknown genes. Here we report the isolation and characterization of a novel gene, \textit{fad158} (factor for adipocyte differentiation 158). This gene contains the trans-

\textsuperscript{1} The abbreviations used are: PPAR\(\gamma\), peroxisome proliferator-activated receptor \(\gamma\); \textit{fad}, factor for adipocyte differentiation; C/EBP, CCAAT/enhancer-binding protein; Dex, dexamethasone; GFP, green fluorescent protein; EGFP, enhanced GFP; ER, endoplasmic reticulum; FBS, fetal bovine serum; IBMX, 3-isobutyl-1-methylxantine; IPTG, isopropyl-\(\beta\)-D-galactopyranosidase; LRRC, leucine-rich-repeat-containing ORF, open reading frame; RACE, rapid amplification of cDNA ends; RGS2, regulator of G protein signaling 2; RT, reverse transcription; SREBP-1, sterol regulatory element-binding protein 1; LRR, leucine-rich repeat; NIS, nuclear receptor subfamily 1, group A, member 2; ORF, open reading frame; PPAR, peroxisome proliferator-activated receptor; SREBP, sterol regulatory element-binding protein; TCR, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered protein; WT, wild type; TM, transmembrane region.

* This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, by the Japan Society for the Promotion of Science, and by ONO Medical Research Foundation, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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membrane regions and leucine-rich repeat motifs. Its expression was elevated during the adipogenesis of mouse 3T3-L1 cells. When fad158 expression was knocked down, the ability of 3T3-L1 cells to differentiate declined. Moreover, cells with the ectopic expression of fad158 underwent adipocyte differentiation in the presence of a ligand of PPARγ. Taken together, these results strongly indicate that fad158 is closely related to adipocyte differentiation.

EXPERIMENTAL PROCEDURES
RNA Isolation and Northern Blot Analyses—Total RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's instructions. For Northern blot analyses, 15 μg of total RNA was electrophoresed on a 1% agarose gel containing 2% formaldehyde and then transferred to a Hybond-N+ nylon membrane (Amersham Biosciences). For Northern blot analysis of human tissues, a Multiple Tissue Northern (MTN™) blot containing ~2 μg of poly(A)+ RNA per lane was purchased from Clontech (BD Biosciences Clontech, Palo Alto, CA). Hybridizations were performed under stringent conditions in Express Hyb (BD Biosciences Clontech), according to the manufacturer's directions. Each probe was labeled with [α-32P]dCTP using a BcaBEST labeling kit (Takara Biomedicals, Kusatsu, Japan). The ORF region in each gene was used as a DNA probe, except that the 3′-untranslated region (3′-UTR) of Gα12/13c (GenBank® accession number NM_002314) was used for detection of C/EBPα mRNA in NIH-3T3 cells; use of the ORF region of C/EBPα as a probe resulted in nonspecific bands of high intensity with total RNA from NIH-3T3 cells but not from 3T3-L1 cells. Therefore, several regions were checked, and an appropriate probe was found for C/EBPα, which gave no nonspecific bands.

Fractionation of Fat Cells—The fat cells were prepared as described previously (18). In brief, epididymal fat pads were isolated from male C57Bl/6J mice aged 6 weeks, washed with sterile PBS, minced, and washed with Krebs-Ringer bicarbonate buffer (pH 7.4). Then, the minced tissue was digested with 1.5 mg/ml collagenase type II (Sigma-Aldrich) in Krebs-Ringer bicarbonate containing 4% bovine serum albumin at 37 °C for 1 h on a shaking platform. The undigested tissue was removed with a 5 μm nylon mesh. The digestion fraction was centrifuged at 500 × g for 5 min. Cloning of Full-length cDNA of Mouse fad158—Because fad158 cDNA was isolated as a small 390-bp fragment, the 5′-rapid amplification of cDNA ends (5′-RACE) and 3′-RACE were used for cloning the full-length cDNA. 5′-RACE was performed using a Marathon cDNA amplification kit (BD Biosciences Clontech) following the instructions of the manufacturer. Total RNA was prepared from 3T3-L1 cells 3 h after induction. mRNA was isolated from total RNA using oligotex-dT30 (Daiichi Pure Chemicals, Tokyo, Japan) according to the manufacturer's instructions, the first strand of cDNA was synthesized with oligo-dT primer and avian myeloblastosis virus reverse transcriptase. The second strand was synthesized using a second-strand enzyme mixture containing RNase H, Escherichia coli DNA polymerase I, and E. coli DNA ligase. The resultant double-stranded cDNA was ligated to a Marathon cDNA adaptor with T4 DNA ligase. The PCR for 5′-RACE was performed using the forward primer, AP-1 (5′-CCATCTTAATACGACTCACTATAGGGC-3′), and a fad158-specific reverse primer (5′- GGAGAGAGGCGCGTGTGGTCCAC-3′). The PCR for 3′-RACE was performed with the forward primer, AP-1 (5′-CCATCTTAATACGACTCACTATAGGGC-3′), and a fad158-specific reverse primer, 5′-TGTCGCACCTACGCCTGCCTCGG-3′. The fragments obtained from 5′-RACE and 3′-RACE were subcloned into the T-added EcoRV site of pBluescript KS+ and analyzed by DNA sequencing as described below.

Cloning of Full-length cDNA of Human fad158—The reverse transcription coupled-PCR (RT-PCR) was conducted with ReverTra Drama (TOYOBO Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. Total RNA was isolated from HeLa cells as described above. cDNA was synthesized with a random primer and ReverTra Ace (TOYOBO). The 5′ region of the human cDNA of fad158 was amplified using KOD plus (TOYOBO) with a human fad158-specific forward primer, 5′-ACAAGCCATGAGCAGCCA-3′, and reverse primer, 5′-AGAGCTCTGCTGACCAA-3′. In the same way, the 3′ region of the human cDNA of fad158 was amplified with a human fad158-specific forward primer, 5′-GATACACGCGACCATGGC-3′, and reverse primer, 5′-GACTGAAACCGCTTCACC-3′.

DNA Sequencing and Data Base Analyses—The sequence was determined with the automated sequencer DSQ 1000 (Shimadzu Corp., Kyoto, Japan) and an ABI PRISM 310 (Applied Biosystems, Foster City, CA). The human ortholog of fad158 was predicted using the human genome data base. Searches for the human ortholog in human genome databases were performed using BLAST programs accessed via the National Center for Biotechnology Information (NCBI) homepage.

Cell Culture and Differentiation—Mouse 3T3-L1 (ATCC CL173) preadipocyte cells were maintained in Dulbecco's modified Eagle's medium containing 10% calf serum. For the differentiation experiment, the medium was replaced with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 10 μg/ml insulin, 1.5 mm 3-isobutyl-1-methylxantine (IBMX), and 1 μm dexamethasone (Dex) at 2 days post-confluence. Mouse NIH-3T3 (clone 5611, JCRB 00615) fibroblastic cells were maintained in Dulbecco's modified Eagle's medium containing 10% calf serum. fad158-overexpressing NIH-3T3 cells were induced to differentiate by the same medium as the 3T3-L1 cells at 2 days post-confluence with or without addition of 0.5 μl BRL49653 (Hoechst). HeLa cells were maintained in minimal essential medium containing 10% FBS.

Subcellular Localization of FAD158—The pEGFP-fad158 chimeric protein-expressing plasmid pGFP-WT was constructed by subcloning the coding region (amino acids 1–803) into the 3′-end of pEGFP-C1 (BD Biosciences Clontech) and transferred into NIH-3T3 cells. pEGFP-C1 was constructed by subcloning the N-terminal transmembrane region (amino acids 1–404) into the 3′-end in-frame. pGFP-LRR was constructed by subcloning the C-terminal leucine-rich repeat motifs (amino acids 406–803) into the 3′-end in-frame. The same regions were also subcloned into the 5′-end of pEGFP-N1 (BD Biosciences Clontech) and the 3′-end of pCMV-Myc (BD Biosciences Clontech) for generation of WT-GFP (full-length fad158-EGFP), TM-GFP (N-terminal transmembrane region-EGFP), LRR-GFP (C-terminal leucine-rich repeat-EGFP), Mys-TGFP (Myc-full-length fad158), Myc-TM (Myc-N-terminal transmembrane region), and Myc-LRR (Myc-C-terminal leucine-rich repeat), respectively.

3T3-L1 cells were plated on cell dishes (SUMITOMO BAKELITE Co., Ltd., Tokyo, Japan) before transfection. The cells were transfected using FuGENE™ 6 transfection reagent (Roche Applied Science), fixed in 3% paraformaldehyde, 0.1 m M CaCl2, and 0.1 m M MgCl2 for 20 min in room temperature. After the blocking of the aldehyde group with 50 μl NH4Cl in PBS for 5 min at room temperature, each cell disk was incubated with primary antibody, rabbit polyclonal calnexin antibody (H-70) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:50 dilution in PBS), and mouse monoclonal c-Myc antibody (BD Biosciences Clontech; 1:100 dilution in PBS) for 1 h at room temperature. After 5 washes with fresh PBS, the secondary antibody, anti-rabbit TRITC, and anti-mouse fluorescein isothiocyanate (Sigma-Aldrich) were reacted for 30 min at room temperature. After five more washes with fresh PBS, EGF, TRITC, and fluorescein isothiocyanate, signals were detected by fluorescence microscopy.

Establishment of Antisense fad158 mRNA-Expressing Transformers—3T3-L1 cells that stably express the antisense mRNA of fad158 under the regulation of isopropyl-1-thio-β-D-galactopyranoside (IPTG) were established using the LacSwitch II inducible mammalian expression system (Stratagene, La Jolla, CA) according to the manufacturer's directions. The fragment covering from −5 to +355 was amplified by PCR with primers containing XhoI and KpnI sites. After digestion with restricted enzymes, the fragment was subcloned into pOPRSV/MCS in an antisense orientation. pOPRSV containing antisense fad158 or empty pOPRSV was co-transfected into 3T3-L1 cells with pCMV-Lac1, which expresses the lac repressor, using LipofectAMINE (Invitrogen). The stable transformants were selected with neomycin (G418) and hygromycin. Single drug-resistant colonies were isolated, stored individually, and used for the adipocyte differentiation analyses.

Establishment of FAD158-expressing Transformants—NIH-3T3 cells that stably express fad158 were established using a Retro-X system (BD Biosciences Clontech) according to the manufacturer's instructions, except that pDONAI (Takara) was used as a retroviral vector. The full-length cDNA of fad158 was subcloned into the vector pDONAI. pDONAI-fad158 or pDONAI empty vector was introduced into PT67 using calcium phosphate co-precipitation techniques as described by Chen and Okayama (19). The virus transiently expressed in medium containing 10% fetal bovine serum (FBS), 10 μg/ml insulin, 1.5 mm 3-isobutyl-1-methylxantine (IBMX), and 1 μm dexamethasone (Dex) at 2 days post-confluence. Mouse NIH-3T3 (clone 5611, JCRB 00615) fibroblastic cells were maintained in Dulbecco's modified Eagle's medium containing 10% calf serum. fad158-overexpressing NIH-3T3 cells were induced to differentiate by the same medium as the 3T3-L1 cells at 2 days post-confluence with or without addition of 0.5 μl BRL49653. HeLa cells were maintained in minimal essential medium containing 10% FBS.

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RESULTS

Expression of fad158 during the Early Stage of Adipocyte Differentiation—We originally isolated fad158 as one of many unknown genes, the expression of which was elevated at 3 h after induction (15). To investigate the role of fad158 during adipocyte differentiation, we first determined the expression level of fad158 by Northern blot analysis (Fig. 1). To monitor changes in fad158 expression levels during adipocyte differentiation, 3T3-L1 cells were stimulated with inducers, and total RNA was prepared at various points. Although the expression of fad158 was rarely detectable before induction (0 h), it was quickly elevated after induction, reached a maximum at 6 h, and decreased to 24 h. This result indicates that fad158 is transiently expressed in the early stage of adipocyte differentiation.

To clarify the agents sufficient for the expression of fad158, the expression level of fad158 was determined when only one inducer (FBS, insulin, Dex, or IBMX) was added to the medium. The result showed that none of these agents induced the inducer (FBS, insulin, Dex, or IBMX) was added to the medium. The result showed that none of these agents induced the expression of known genes, the expression of which was elevated at 3 h after induction. Indeed, the length of fad158 was 390 bp. Therefore, we attempted to isolate a full-length cDNA of fad158 using 5′-RACE and 3′-RACE methods (Fig. 3). 5′-RACE was performed using cDNA prepared from 3T3-L1 cells 3 h after induction, and a 764-bp cDNA fragment containing a first methionine at 236 bp was isolated. A 2111-bp cDNA fragment containing a stop codon was isolated by 3′-RACE. By combining these two cDNA fragments, fad158 was found to consist of 2851 bp with an ORF of 803 amino acids (GenBank™ accession number AB081509). Because BLAST searches of databases identified no significant matches against proteins of known function, fad158 seemed to be a novel gene.

Genomic Distribution of Mouse fad158—The mouse genome data base was made public by The Mouse Genome Sequencing Consortium (20, 21). Using this data base, a BLAST search for mouse fad158 was performed. The result indicated that mouse fad158 existed at locus 5E5 of mouse chromosome 5 and consisted of four exons divided by three introns (Fig. 4A). Sequences of the exon/intron junctions in the data base revealed that the GT/AG rule was preserved in all cases.

As with the mouse genome data base, we used the human genome data base to predict the full-length human fad158 ORF and to analyze the genomic distribution of human fad158 (22). To isolate the full-length ORF of human fad158, we first predicted the ORF region of human fad158 based on the search result. By splicing out the introns and combining the exons, the ORF of human fad158 was predicted. To isolate human fad158 including the entire ORF, we constructed primer sets as indicated under “Experimental Procedures” and performed RT-PCR using a template prepared from total RNA extracted from HeLa cells. From sequence analyses of the resultant fragments, the human fad158 cDNA was concluded to be 2575 bp long, with a 2409-bp ORF encoding 803 amino acids (GenBank™ accession number AB081509). A BLAST search of the human genome data base revealed the existence of a human homologue of fad158 on chromosome 1 at locus 1P22 that consisted of three exons divided by two introns. Comparing the genomic structure of mouse and human fad158, it was found that almost 95% of both ORFs was included in the last exons. In both genomes, the first methionine exists in the exon in front of the last exon.

The Deduced Protein Primary Structure of fad158—The deduced 803-amino-acid sequence of the mouse and human fad158 ORF included four transmembrane domains and eight leucine-rich repeat motifs at the C-terminal end. Interestingly, the positions of these two domains were well conserved in mouse and human fad158 (Fig. 4B). The leucine-rich repeat were digested by RsaI for non-bias cloning (15).
sequences of mouse fad158 are aligned in Fig. 4C. Comparing these sequences with the leucine-rich repeat consensus sequences, it was found that the consensus residues were well conserved. Thus, it is clear that these proteins contain leucine-rich repeat motifs. The same result was obtained by the multiple alignments of human fad158 leucine-rich repeat motifs (the nucleotide and amino acid sequences that are not presented here are available in GenBank under accession numbers AB081508 and AB081509).

Expression of fad158 in Various Tissues—To determine the tissue distribution of human fad158 mRNA, we next performed Northern blot analysis. As shown in Fig. 5A, two transcripts were obtained in most adult tissues. Whereas moderate levels of expression were observed in heart, lung, and peripheral blood leukocytes, a high level of expression was observed in skeletal muscle.

The adipose tissue isolated from mouse was fractionated into a stromal vascular fraction and adipocytes. Then the expression of fad158 in both fractions was analyzed by Northern blotting. As shown in Fig. 5B, the expression of fad158 was very weak in both fractions. However, the expression in the stromal vascular cells was slightly stronger than that in the adipocytes, indicating that the expression of fad158 is dominant in the preadipocytes (Fig. 5B).

Subcellular Localization of FAD158—To further characterize fad158, the subcellular localization of FAD158 was determined by transient transfection of an EGFP-fad158 expression vector into 3T3-L1 cells. The signals were detected by fluorescence microscopy. The wild type FAD158 localized in the cytoplasm (GFP-WT in Fig. 6A). The truncated mutants, which only contain the four N-terminal transmembrane regions of FAD158, were also distributed in the cytoplasm (GFP-TM in Fig. 6A). Interestingly, another truncated mutant that does not contain the N-terminal transmembrane regions was distributed throughout the cell including the nucleus as detected in the control (GFP-LRR in Fig. 6A).

Because these three GFP-fad158 chimeric constructs have GFP at the N-terminal site, we next constructed fad158-GFP chimeric plasmids in which GFP stays in the C terminus and also made plasmids having a smaller Myc tag. As shown in Figs. 6, B and C, the same distribution pattern of GFP or Myc was obtained as that of GFP at the N-terminal site. Because the subcellular distribution of truncated mutants containing the four N-terminal transmembrane regions of FAD158 was unchanged compared that of the wild type, it seems that FAD158 distributes to the membrane structure in the cytoplasmic region.

Therefore, we next conducted immunofluorescent staining using antibody against calnexin, which is a marker of the endoplasmic reticulum (ER) (23, 24, 25). The distribution of wild type FAD158 (WT-GFP) and the truncated mutant containing the four transmembrane regions (TM-GFP) overlapped with the staining pattern of calnexin. In contrast, the truncated mutant without the four transmembrane regions (LRR-GFP) did not co-localize with calnexin (Fig. 6B). When myc was used as a smaller tag, the same result was obtained (Fig. 6C). These results indicated that FAD158 is anchored to the ER through its four N-terminal transmembrane regions.

Antisense Experiment on fad158 in 3T3-L1 Cells—We next examined the effect of fad158 during adipocyte differentiation. We attempted an antisense experiment to knock down the expression of fad158 during the adipocyte differentiation of mouse 3T3-L1 cells. For this purpose, a lac switch mammalian expression system was used. By co-transfecting pOPRSV1-fad158 antisense and pCMVLac1 into 3T3-L1 cells and then selecting with G418 and hygromycin, we isolated stable transfectants expressing antisense fad158 mRNA. As a control, the empty pOPRSV1 vector and pCMVLac1 were co-transfected, and transfectants were selected and isolated in the same manner.

Twelve hours before the treatment of these stable transfectants with inducers, 5 mM IPTG was added to the medium to induce the expression of antisense fad158 mRNA to knock down the endogenous expression of fad158. We have analyzed the expression level of fad158 in IPTG-treated or untreated fad158 antisense-expressing cells by Northern blotting. The reduction in expression was slight, but the same results were obtained in the two independent clones (different clone of stable transfectant). Because the reduction was slight, we performed RT-PCR. Because RT-PCR is a semi-quantitative method, we confirmed the reproducibility, and the result showed that the expression level of fad158 was decreased, whereas that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was unchanged by the addition of IPTG (Fig. 7A).

Using these stable transfectants the effect of antisense fad158 was determined. The control cells and antisense fad158-harboring cells were grown to confluence. At 2 days post-confluence, the cells were treated with inducers with or without IPTG. After 8 days, the cells were fixed and stained with oil red O to discern the oil droplets. In control cells the addition of IPTG had no effect on the adipogenesis, and the cells differentiated with an accumulation of oil droplets (data not shown). The antisense fad158-harboring cells not treated with IPTG also differentiated well. On the other hand, when IPTG was added to antisense fad158-harboring cells, the storage of oil droplets was reduced (Fig. 7B). To confirm this result we used two other independent clones and performed the differentiation experiments again. The whole plate of one clone stained with oil red O is shown in Fig. 7C. The quantitation of amounts of triacylglycerol is also shown. These results showed a reduction in the accumulation of fat pads and triacylglycerol, indicating that fad158 expression is necessary for adipocyte differentiation.

The expression levels of several marker genes were also analyzed by Northern blotting. During the differentiation into adipocytes, the expression level of PPARY, a master regulator for adipogenesis, declined, and the expression of its target gene aP2 was also reduced. The expression level of another master regulator, C/EBPα, also declined, and slight reductions in the expression of C/EBPβ and C/EBPδ were observed (Fig. 7D).

Overexpression of fad158 in NIH-3T3—As described above, the expression of mouse fad158 is rapidly induced at the early stages in the differentiation of 3T3-L1 cells into adipocytes and was restricted to conditions for the differentiation. Moreover, with the knocking down of the expression of fad158, the ability to differentiate was reduced. To further characterize the func-
tion of this gene during adipogenesis, we next established stable cell lines that overexpress fad158 in NIH-3T3 cells using a retroviral system. Fig. 8A shows the ectopic expression of fad158 in the stable transformants. When the fad158 gene was used as a probe to detect fad158 expression, a 6.4-kilobase mRNA band was obtained from fad158-expressing cells as an exogenous transcript.

Using these stable transformants, we next tested whether the cells could differentiate into mature adipocytes. It is known that NIH-3T3 cells do not differentiate when exposed to inducers that stimulate the differentiation of preadipocyte 3T3-L1 cells. Indeed, when the control stable transformant was treated with inducers at 2 days post-confluence, it did not reveal any morphological changes (data not shown). Then, we tested the stable transformants of fad158-overexpressing cells. However, the same result was obtained, and no differentiation was observed (data not shown). Next, we performed the same experiment in the presence of BR469653, a ligand for PPARγ. Quite interestingly, on the addition of this ligand, fad158-overexpressing NIH-3T3 cells differentiated into mature adipocytes, and the accumulated oil droplets were stained with oil red O. In contrast, the control cells retained the fibroblastic morphology even after 1 week of treatment with the inducers and ligand, and oil droplets were not detected (Fig. 8B).

Next, we determined the expression profiles of adipocyte marker genes. The expression of PPARγ and SREBP-1, which are known to be master regulators of adipogenesis, gradually increased by day 8 in the fad158-overexpressing cells. Lipoprotein lipase and adipocyte fatty acid-binding protein 2, which are known to be the target genes of PPARγ, were also expressed about 1 week after induction. On the other hand, the expression level of the C/EBP family did not change during the adipogenesis of fad158-overexpressing NIH-3T3 cells. Notably, C/EBPα expression was almost undetectable during the incubation (Fig. 8C). Taken together, these results strongly suggested that the fad158 gene has some functional role in adipogenesis, and these effects seem to be linked with the PPARγ pathway, not the C/EBP pathway.

DISCUSSION

The transcription factor PPARγ is thought to be a master regulator of adipogenesis for several reasons. First, the expression of many adipocyte-specific proteins is regulated by PPARγ, and the promoters of these genes contain a PPAR-responsive element (26, 27). Second, overexpression of PPARγ in fibroblastic non-adipocytes resulted in differentiation into mature adipocytes (9, 26). Finally, embryonic stem cells or embryonic fibroblast cells established from PPARγ−/− mice did not differentiate into mature adipocytes (26, 28, 29).

The C/EBP family is known to be another master regulator of adipocyte differentiation. Embryonic fibroblast cells derived from C/EBPα−/− mice did not differentiate into mature adipocytes (26, 30). C/EBPβ and C/EBPδ have the ability to activate the expression of PPARγ and C/EBPα (26). When C/EBPα was overexpressed in PPARγ−/− embryonic fibroblast cell cultures, it did not restore the role of PPARγ in adipogenesis. These findings strongly suggested that the PPARγ pathway is unified, and C/EBPα induces adipogenesis via this pathway (31). Thus, recent reports explain relatively well the molecular mechanisms for the mid and late phases of adipocyte differentiation. However, the earliest step of adipocyte differentiation is still unclear.

We have previously isolated the genes expressed at the beginning of the differentiation process (15–17). Of these, almost half were unknown genes. In this study, we have cloned a 2851-bp cDNA of mouse fad158. In the Northern blot analyses, two major forms were observed. However, no other isoform of fad158 was observed in RT-PCR experiments using various
primers, which could detect unidentified exons. In this study, \textit{fad158} was isolated as a 2851-bp cDNA, and this cDNA seems to correspond to the smaller band obtained by Northern blotting. It is possible that the larger band is due to the usage of a different promoter. It is also possible that it is derived from the usage of a different poly(A) additional signal and has a very long 3'-untranslated region.

We have predicted the human ortholog sequence using the human genome data base sequenced by The International Human Genome Sequencing Consortium (22) and constructed by the NCBI. Using this information, we cloned the human 2575-bp \textit{fad158} cDNA by RT-PCR. The resultant cDNA sequences were 100% similar to the sequence predicted using the Human Genome Data base. Thus, this is an easy and simple way of cloning the human ortholog when one has the mouse cDNA sequence. The mouse genome data base was also made public by The Mouse Genome Sequencing Consortium (20, 21). This enables us to predict the mouse ortholog using the human cDNA sequence in the same way. Both cDNAs of mouse and human \textit{fad158} contained the entire ORF and encoded 803 amino acids. The amino acid sequences of these two clones are highly conserved with similarity of 97.5%.

A BLAST search in a mouse cDNA data base with the full-length cDNA of mouse \textit{fad158} revealed four genes with about 50% similarity to \textit{fad158}. The two genes found here were isolated by the RIKEN full-length cDNA project and named leucine-rich repeat-containing 5 (\textit{LRRC5}) and \textit{LRRC8}. The third gene was T-cell activation leucine repeat-rich protein (\textit{TA-LRRP}). The last gene was registered in the data base with GenBank\textsuperscript{TM} accession number XM_146206.

We performed RT-PCR to detect the mRNAs of these four genes and found that they actually exist. The deduced protein structure of \textit{LRR5}, \textit{LRR8}, and \textit{TA-LRRP} contained three transmembrane regions and eight leucine-rich repeat motifs at the...
C-terminal end. The deduced amino acid sequence of XM_146206 contained four transmembrane regions and eight leucine-rich repeat motifs at the C-terminal end. Because the expression of fad158 was elevated during the adipogenesis of 3T3-L1 cells, we determined the expression level of these four genes, LRRC5, LRRC8, TA-LRRP, and XM_146206 in the adipogenesis of 3T3-L1 cells. Interestingly, the expression level of these genes did not change during the adipogenesis of 3T3-L1 cells, indicating that only fad158 is related to adipocyte differentiation (data not shown). In a database search of the human genome, four related genes were also identified in humans (data not shown).

To determine the subcellular localization, transfection experiments using EGFP-fad158 chimeric plasmid were performed. The results indicated a unique feature of fad158. EGFP-FAD158 was found in cytoplasm, and this distribution is dependent on the four transmembrane regions at the N-terminal end. Furthermore the immunohistochemical experiment with the ER marker calnexin revealed that FAD158 localizes to the ER through the four N-terminal transmembrane regions. This structure was also conserved in human FAD158, suggesting the importance of these regions for anchoring fad158 to the ER. FAD158 also contained a leucine-rich repeat at the C-terminal end. The leucine-rich repeat consensus motif is LXXLXXLXXLXXLXX, the first 11–12 residues of which are highly conserved (32, 33). This motif is found in a number of proteins with diverse functions (32, 33). Insulin and epidermal growth factor receptors are known to contain leucine-rich repeat motifs in domains L1 and L2. L1 and L2 were found to be important for ligand binding (32). The leucine-rich repeat motifs at the C-terminal end of NOD2 function to activate NF-κB and confer susceptibility to Crohn's disease by altering the activation of NF-κB in monocytes (34). We do not have any information on how the leucine-rich repeat motifs of fad158 function. However, it is interesting that fad158, having the leucine-rich repeat motifs, is expressed at the beginning of

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**Fig. 7. Functional analysis of fad158 by reducing its expression in 3T3-L1 cells.** A, the expression level of fad158 was determined by RT-PCR. The expression level of fad158 with or without IPTG treatment is indicated, and relative intensity is also shown. The expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a control. B, adipocyte differentiation experiment in fad158 knockdown 3T3-L1 cells. IPTG-induced antisense fad158-expressing 3T3-L1 stable transfectants or control cells (transfected with the empty vector pOPRSVI/MCS) were treated with inducers, IBMX, Dex, insulin, and FBS with or without IPTG to knock down the expression of fad158. After 8 days, the cells were stained with oil red O to detect oil droplets. C, adipocyte differentiation experiment in fad158 knockdown 3T3-L1 cells. The same experiment as in B was done with another independent cell line. The cells were stained with oil red O, and the amounts of triacylglycerol accumulated were also determined. D, Northern blot analyses of adipocyte marker genes during the differentiation of fad158 knockdown cells. Total RNA was isolated at various time points during adipocyte differentiation of fad158 knockdown 3T3-L1 cells. aP2, adipocyte fatty acid-binding protein.
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The expression of fad158 was determined by Northern blot analysis. The retroviral exogenous gene expression is shown. fad158 mRNA is expressed fused with the neomycin-resistance gene. B. differentiation experiment of fad158-overexpressing NIH-3T3 cells. NIH-3T3 cells stably expressing fad158 or control cells (infected with empty vector) were treated with inducers IBMX, Dex, insulin, and FBS with BRL49653, which is a ligand for PPAR. After 8 days, the cells were stained with oil red O to detect oil droplets. C. Northern blot analyses of adipocyte marker genes during differentiation from fad158-overexpressing NIH-3T3 cells. Total RNA was isolated at various time points of adipocyte differentiation of fad158-overexpressing NIH-3T3 cells. Staining with ethidium bromide (EtBr) for ribosomal RNA is shown as a control. aP2, adipocyte fatty acid-binding protein 2.

Acknowledgments—We thank Drs. B. M. Spiegelman (Dana-Farber Cancer Institute, Harvard Medical School), S. L. McKnight (University of Texas Southwestern Medical Center), and R. Sato (University of Tokyo) for generously providing the plasmids containing cDNAs of PPARγ, C/EBPs, and SREBP-1, respectively. We also thank Glaxo-SmithKline for the gift of BRL49653.

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J. Biol. Chem. 2004, 279:34840-34848.
doi: 10.1074/jbc.M312927200 originally published online June 7, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M312927200

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