Adipose Tissue Expression of the Lipid Droplet–Associating Proteins S3-12 and Perilipin Is Controlled by Peroxisome Proliferator–Activated Receptor-γ

Knut Tomas Dalen,[1] Kristina Schoonjans,[2] Stine M. Ulven,[1] Mina Susanne Weedon-Fekjaer,[1] Trine Gjesti Bentzen,[1] Hana Koutnikova,[2] Johan Auwerx,[2] and Hilde I. Nebb[1]

In a systematic search for peroxisome proliferator–activated receptor-γ (PPAR-γ) target genes, we identified S3-12 and perilipin as novel direct PPAR-γ target genes. Together with adipophilin and tail-interacting protein of 47 kDa, these genes are lipid droplet–associating proteins with distinct expression pattern but overlapping expression in adipose tissue. The expression of S3-12 and perilipin is tightly correlated to the expression and activation of PPAR-γ in adipocytes, and promoter characterization revealed that the S3-12 and the perilipin promoters contain three and one evolutionarily conserved PPAR response elements, respectively. We furthermore demonstrate that the expression of S3-12 and perilipin is reduced in obese compared with lean Zucker rats, whereas the expression of adipophilin is increased. Others have shown that perilipin is an essential factor in the hormonal regulation of lipolysis of stored triglycerides within adipose tissue. The direct regulation of perilipin and S3-12 by PPAR-γ therefore is likely to be an important mediator of the in vivo effects of prolonged treatment with PPAR-γ activators: insulin sensitization, fatty acid trapping in adipose tissue, reduced basal adipose lipolysis, and weight gain. *Diabetes* 53:1243–1252, 2004

From the [1]Institute for Nutrition Research, University of Oslo, Oslo, Norway; and [2]Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS, INSERM, Illkirch, France.

Address correspondence and reprint requests to Hilde Irene Nebb, Institute for Nutrition Research, University of Oslo, P.O. Box 1046 Blindern, N-0316 Oslo, Norway. E-mail: h.i.nebb@basalmed.uio.no.

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ADRP, adipose differentiation-related protein; aFABP, adipocyte fatty acid–binding protein; BLAST, basic local alignment search tool; CE, cholesterol ester; EMSA, electrophoretic mobility-shift assay; FA, fatty acid; LDAP, lipid droplet–associating protein; MMLV-RT, Moloney murine leukemia virus reverse transcriptase; PPAR, peroxisome proliferator–activated receptor; PPRE, PPAR response element; RAP, RNA arbitrarily primed; RXR, retinoid X receptor; TAG, triacylglycerol; TIP-47, tail-interacting protein of 47 kDa; TZD, thiazolidinedione; WAT, white adipose tissue.

Today's western lifestyle, which involves a high-calorie diet and a lack of exercise, has led to an epidemic of obesity, which often is associated with type 2 diabetes, hypertension, hyperlipidemia, and cardiovascular disease. Increased energy intake results in an imbalance between fat synthesis and degradation, leading to an increase in circulating fatty acids (FAs) and accumulation of lipids in white adipose tissue (WAT). Whereas most tissues store triacylglycerol (TAG), cholesterol esters (CEs), or lipids in relatively small (<1 μm diameter) droplets that can be used as an energy source or for membrane biogenesis, WAT stores most of the body's TAG reservoir in droplets that can exceed 50 μm in diameter (1). Although the interior of those lipid droplets consists largely of neutral lipids, a number of proteins associate with the droplet surface. These include P_{200}, caveolins, vimentin (1,2), mouse adipose differentiation-related protein (ADRP)/human adipophilin (hereafter referred to as adipophilin) (3), perilipin (4,5), S3-12 (6), and tail-interacting protein of 47 kDa (TIP-47) (7).

Perilipin, adipophilin, and TIP-47 exhibit high sequence identity within an NH_2-terminal motif termed PAT-1 (after perilipin, adipophilin, and TIP-47) and a more distally located PAT-2 domain (8,9). A fourth protein, S3-12, has been described along with these PAT family members. S3-12 contains a repeated 33–amino acid motif also found in adipophilin (10), and it shares protein sequence identity to both adipophilin and TIP-47 in the COOH terminus, but not to perilipin (8).

At present, the lipid droplet–associating properties have been thoroughly studied only for adipophilin and perilipin (11,12). Adipophilin associates with smaller neutral lipid storage droplets located within most tissues (3), whereas perilipin is located on the surface of larger TAG droplets in mature adipocytes (13) and on CE droplets in steroidogenic cells (14). Adipophilin is ubiquitously expressed (3) with increasing mRNA expression during adipocyte differentiation (15), in contrast to perilipin, which is expressed in a more restricted pattern confined to WAT and steroidogenic cells (4,14). Perilipin is the major protein kinase A substrate in adipocytes (4), and protein kinase A activation induces phosphorylation of perilipin, ultimately resulting in increased lipolysis, whereas insulin stimulation

acts contrarily by facilitating dephosphorylation of the perilipin protein core (16–18). In concordance with this regulation of lipolysis, mice deficient in perilipin (Plin$^{-/-}$) are resistant to genetic and diet-induced obesity (19) and show enhanced basal lipolytic rate (20). TIP-47 is thought to act as a cargo selection device for trafficking of mannose-6-phosphate receptors from late endosomes to Golgi (21) in addition to its association with lipid droplets (7,9). S3-12 was originally cloned as a surface/membrane-associated protein in adipocytes (10), but later observations suggest that S3-12 coats nascent lipid droplets in adipocytes (6).

Thiazolidinediones (TZDs), a novel class of synthetic antidiabetic drugs, are thought to mediate their effects as high-affinity ligands for PPAR-γ (22). Together with PPAR-α and PPAR-β/δ, PPAR-γ belongs to a subfamily of nuclear receptors that heterodimerize with retinoid X receptors (RXRs) and regulate transcription by binding to specific PPAR response elements (PPREs) in the promoter region of target genes (23,24). PPAR-γ is highly enriched in WAT and coordinates the "thrifty response" by regulating the expression of several adipocyte-expressed genes that control energy storage (23,25). Despite the fact that a number of PPAR-γ target genes have been characterized (26–29), the insulin-sensitizing properties of these drugs are not fully understood. To explore further the function of PPAR-γ in adipose tissue biology, we searched for PPAR-γ targets using RNA arbitrarily primed (RAP)-PCR technology (30) and identified S3-12 as a proven PPAR-γ target gene. Previous observations suggest that the expression of the related adipophilin and perilipin genes are regulated by PPARs (31,32). In this article, we demonstrate that S3-12 and perilipin are directly regulated by PPAR-γ and that the expression of S3-12, perilipin, and adipophilin is altered in a model of obesity.

RESEARCH DESIGN AND METHODS

Materials. RNasin and restriction enzymes were purchased from Promega (Madison, WI). Cell culture reagents, oligonucleotides, and chemicals were obtained from Sigma (St. Louis, MO); and Moloney murine leukemia virus reverse transcriptase (MMLV-RT) was obtained from Invitrogen Life technologies (Carlsbad, CA).

Culturing and transfection of cells. 3T3-L1 and SGBS cells were cultured and differentiated into adipocytes as described (33,34). COS-1 cells (American Type Culture Collection, Fairfax, VA) were transiently transfected in six-well dishes (Corning, Corning, NY) with reporter (5 μg) and cotransfected with pSV-β-galactosidase (3 μg) and RXR-α and PPAR-γ expression vectors (1 μg each) with calcium phosphate precipitation (34,35). Relative luciferase activity was normalized against β-galactosidase activity (control = 1).

3T3-L1 cells were transfected with reporter (1 μg) and pRL-SV40 (0.1 μg, as internal control) and expression vectors (0.2 μg of each) with Lipofectamine Plus (Promega). Cells were harvested in 200 μl of lysis buffer, and luciferase activities were measured with the Dual Luciferase assay kit (Promega). All transfections are representative of at least three individual experiments performed in triplicate. Error bars represent SD.

Preparation and analysis of RNA and electrophoretic mobility-shift assays. Total RNA was extracted with Trizol reagent (Invitrogen). RNA (10 or 20 μg) was separated on a 1% agarose formaldehyde/MOPS gel and blotted and hybridized as described (34). 36B4 (acidic ribosomal phosphoprotein PO) mRNA was used as a loading control (28,36). Partial or full-length cDNAs were generated by RT-PCR from mouse (3T3-L1) or human (SGBS) total RNA using the ImProm-II Reverse Transcription System (Promega), amplified with PfuTurbo and cloned into pPCR-Script vector (Stratagene, La Jolla, CA). Primer sequences are listed in supplemental Table 1 (online appendix available at http://diabetes.diabetesjournals.org). Digested purified fragments were used for labeling.

Proteins were synthesized from pSG5-hPPAR-γ (37), pCMX-mRXR-α, and pCMX expression vectors (34) using a TNT T7-coupled in vitro transcription/translation system (Promega). Oligonucleotide probes were labeled using T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP (Amersham Bioscience, Buckinghamshire, U.K.). Electrophoretic mobility-shift assay (EMSA) was performed as described (34). EMSAs and Northern blots were visualized by Phosphor Imaging and analyzed with ImageQuant software (Amersham).

RAP-PCR analysis. Total RNA isolated from four culturing flasks was pooled and used for mRNA isolation with oligo(dT)-cellulose columns (Amersham, #27-9258-02) to obtain pure mRNA. RAP-PCR screening (Stratagene; #200440) was performed with the following primer pair combinations (left primers were used alone in cDNA synthesis step): A1-A2, A1-A3, A2-A3, A2-A4, A3-A4, A3-A1, A4-A1, A4-A2, C1-C2, C1-C3, C2-C3, C2-C4, C3-C4, C3-C5, C4-C1, C4-C2, C5-C2, and C5-C3. The differentially expressed bands were cloned and sequenced with ABI-377 DNA sequencer (Applied Biosystems, Foster City, CA).

Promoter and protein sequence identification and comparison. Full-length cDNAs (supplemental Fig. 1) were used to search nonredundant and high-throughput genomic sequence databases by the basic local alignment search tool (BLAST) (38) to identify promoter sequences. Human and mouse promoters were compared by Blast2 alignment (39).

The mouse S3-12 protein sequence (10) was used to search nonredundant peptide sequence databases with protein-protein BLAST (Matrix: BLOSUM-62 or PAM30). S3-12 and PAT proteins (accessions: perilipin, #AAN77870; adipophilin, #NP_031434; and TIP-47, #BAB28291) were compared with Blast2 alignment and clustalW (EMBOSS package).

Cloning and mutagenesis of promoters. Full-length promoters were amplified with PfuTurbo (Stratagene) with mouse (Clontech, #6650-1) or human (Clontech, #6550-1) genomic DNA as template (34). Primers are listed in supplemental Table 1. Deletion constructs for the S3-12 and perilipin promoters were made by restriction digestion combined with PCR with nested primers. Site-directed mutagenesis of PPREs was performed with PCR (34).

Animal experiment. Male C57BL/6J mice (B&K Universal, Sollentuna, Sweden; 10 weeks, 30 g) were killed by cervical dislocation to obtain skeletal muscle and adipose tissue samples. Lean and fatty (*fa/fa*) Zucker rats (Iffa Credo, Les Oncins, France; 6 weeks) were maintained in a temperature-controlled (25°C) facility with 12-h/12-h fixed light-dark cycles and free access to water. The food intake of lean rats that had free access to food was monitored and served as a basis to determine the amount of food given to the food-restricted group. This last group received 15% less food than the ad libitum–fed rats. The experiment was approved by the local ethical committee and performed according to French and European Union ethical standards. Rats were killed by decapitation, using a guillotine after an overnight fast. Tissue samples were dissected, quickly frozen in liquid nitrogen, and stored at −80°C until RNA extraction.

RESULTS

Identification of differentially expressed transcripts by RAP-PCR. In a systematic search for novel PPAR-γ target genes, we used RAP-PCR technology (30) to compare the mRNA expression pattern in samples from differentiated 3T3-L1 cells that were treated with a synthetic PPAR-γ agonist (darglitazone; 1 μmol/l) for 24 h against control cells (vehicle; DMSO). Fifty-nine transcripts were differentially expressed; 31 were decreased, whereas 28 were increased by darglitazone. All transcripts were cloned, sequenced, and identified by BLAST search (results not shown). The validity of this approach was proved by the presence of the well-established PPAR-γ target gene aP2/aFABP (adipocyte fatty acid–binding protein) (28) among the darglitazone-induced transcripts. One of the fragments differentially amplified with the A4-A1 primer pair (Fig. 1*A*, +7; compare *lanes 1* and *2*) corresponded to the mouse S3-12 gene product. We verified the induction of S3-12 by Northern analysis, where a 2.1-fold increase in S3-12 mRNA was found upon darglitazone treatment (Fig. 1*B* and *C*). As positive controls, the PPAR-γ target genes aFABP and liver X receptor-α (35,40) were induced 1.9-fold and 1.5-fold, respectively.

S3-12 was recently demonstrated to associate on the surface of lipid droplets (6). PAT members with similar

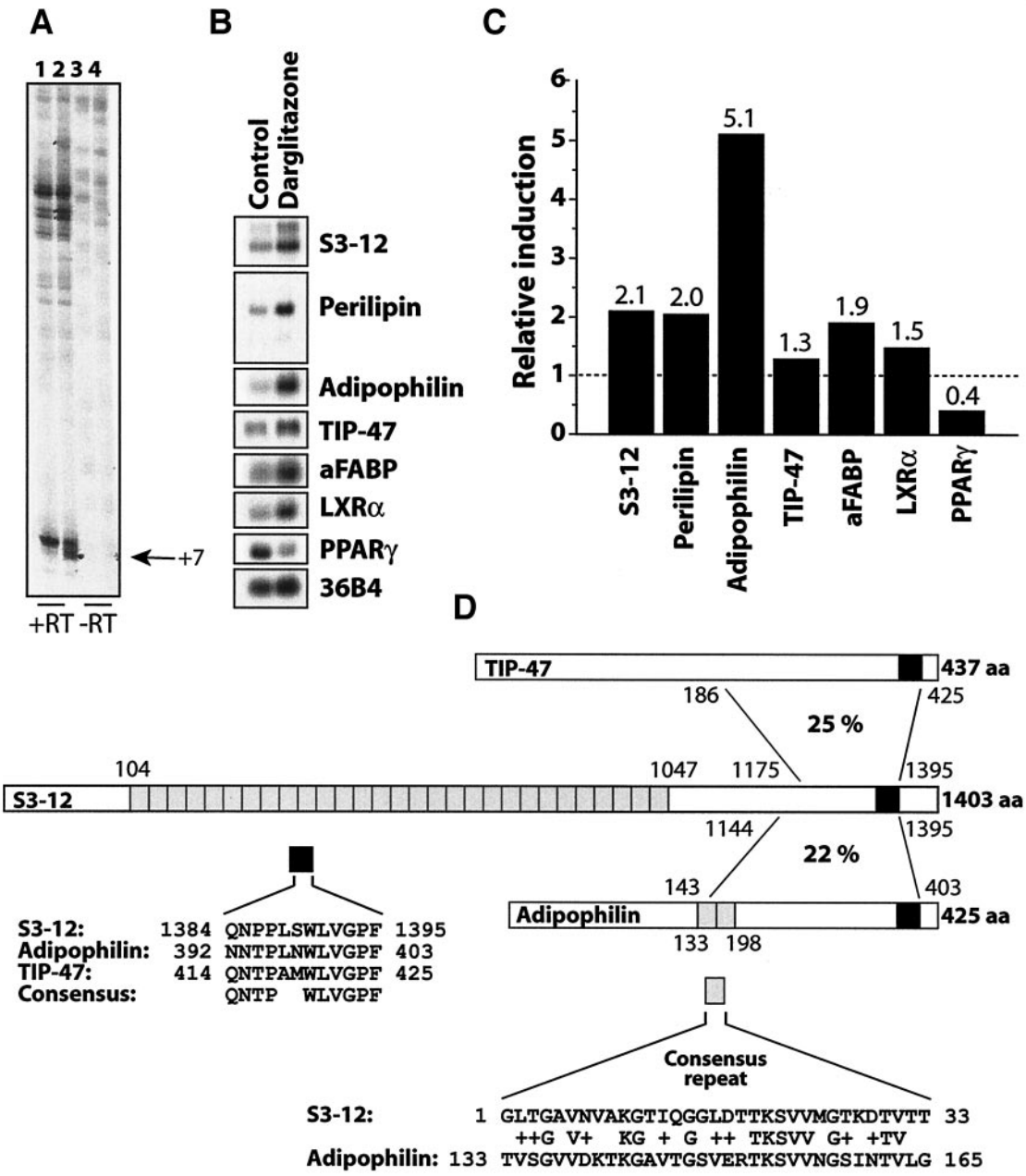

FIG. 1. Identification of S3-12 by RAP-PCR analysis and protein sequence relationship to PAT proteins. *A*: A section of the RAP-PCR gel (A4-A1 primer pair) showing induction of a transcript (+7) originating from mouse S3-12. Samples were incubated with MMLV-RT (+RT) or without MMLV-RT (−RT) as a control for proper cDNA synthesis. *B*: Verification of upregulation after darglitazone treatment in mRNA samples (5 μg/lane) used for RAP-PCR screening. *C*: Relative fold increase by darglitazone treatment. *D*: Protein sequence identity between mouse S3-12, adipophilin, and TIP-47. The previously reported 33–amino acid repeat found in S3-12 and adipophilin (light gray box) is not present in TIP-47. A 12–amino acid region is highly conserved among all three proteins (■).

cellular location, i.e., adipophilin and perilipin, have previously been reported to be induced by PPAR activators (31,32), suggesting a role for PPAR-γ in the regulation of these genes. Hence, we determined the effects of darglitazone on the expression of PAT genes. Perilipin and adipophilin mRNAs were induced 2.0- and 5.1-fold, respectively, whereas TIP-47 remained unchanged (1.3-fold; Fig. 1*B* and *C*).

S3-12 is a peripheral member of the PAT family. S3-12 shares sequence identity to PAT proteins (8,9), and to determine eventually sequence relationships with other proteins, we searched with mouse S3-12 against available protein databases. As reported previously, S3-12 showed protein sequence identity with both TIP-47 and adipophilin in the COOH terminus (1,8) (Fig. 1*D*; relative number of conserved amino acids), but the 33–amino acid motif (light gray) repeated twice in adipophilin and 29 times in S3-12 (10) seems not to be present in TIP-47. Close to the COOH terminus, we discovered a novel, unique 12–amino acid motif (■), only found in these proteins. No evident sequence identity to the conserved PAT1 domain in PAT proteins, perilipin, or other proteins assembled in public databases was found.

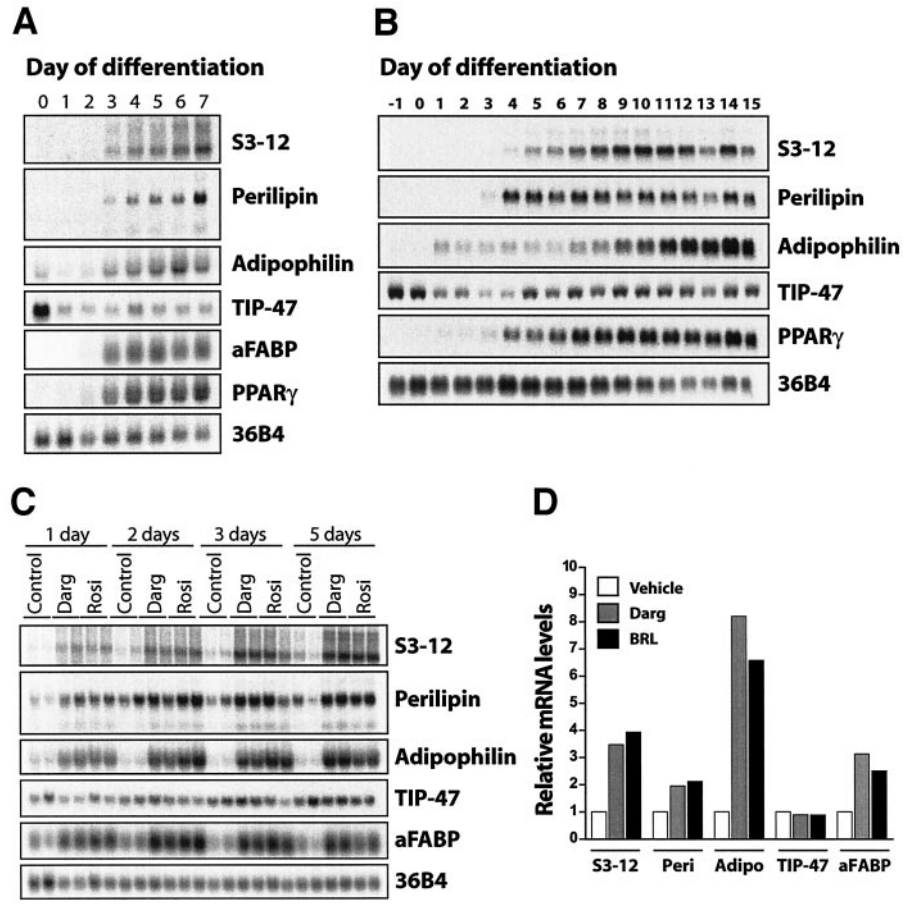

FIG. 2. Expression of S3-12 and PAT proteins during adipocyte differentiation and after PPAR-γ activation. Expression (20 μg total RNA/lane) during differentiation of mouse 3T3-L1 (*A*) and human SGBS cells (*B*). Differentiation was initiated 1 day after confluence (day 0). *C*: Expression in differentiated 3T3-L1 cells after treatment with darglitazone (Darg; 1 μmol/l) or rosiglitazone (Rosi; 10 μmol/l). *D*: Relative expression of S3-12, perilipin (Peri), adipophilin (Adipo), TIP-47, and aFABP after 5 days of stimulation.

Expression of S3-12 and perilipin in adipocytes correlates to the expression and activation of PPAR-γ. The mRNA expression of S3-12 (10), perilipin (4), and adipophilin (3,15) is induced during mouse adipocyte differentiation; hence, we investigated whether the induction of these genes was associated with transcription of PPAR-γ. The expression of S3-12, perilipin, and aFABP mRNAs was robustly induced between days 2 and 3, matching the appearance of the PPAR-γ transcript (Fig. 2*A*). Increased adipophilin mRNA was also correlated with the appearance of PPAR-γ; however, adipophilin was also already present in confluent cells (day 0), with declining expression levels at days 1 and 2. The expression of TIP-47 decreased from day 0 with a subsequent lower steady-state expression.

To determine whether the same induction pattern applies to humans, we examined the mRNA expression pattern during differentiation of SGBS cells, a human adipocyte cell strain (33). A low expression level of the PPAR-γ transcript was detected already at day −1, but a robust induction of PPAR-γ, S3-12, and perilipin was observed from days 3 to 4 (Fig. 2*B*). Adipophilin was strongly induced at day 1, with no further induction at day 4, suggesting that adipophilin is differently regulated than S3-12 and perilipin in humans. Again, TIP-47 expression decreased from day 0 and remained relative unchanged during differentiation, suggesting that TIP-47 is unaffected or even repressed by adipocyte differentiation.

To evaluate changes in the expression levels after long-term PPAR-γ activation, we differentiated 3T3-L1 fibroblasts for 13 days and consecutively stimulated them for 1, 2, 3, or 5 days with two different PPAR-γ activators: darglitazone (1 μmol/l) or rosiglitazone (10 μmol/l). Both TZDs induced the expression of S3-12, perilipin, adipophilin, and aFABP, whereas the expression of TIP-47 was unaffected (Fig. 2*C* and *D*). The expression increased more with long-term (5 days) compared with short-term (1 day) stimulation. In differentiated SGBS cells, the expression of S3-12 and perilipin was increased after 1 and 2 days of stimulation with the PPAR-γ ligand darglitazone, whereas the expression of both adipophilin and TIP-47 was relatively unaffected (results not shown).

The S3-12 and perilipin promoters respond to PPAR-γ activation. To determine potential PPREs in the S3-12 and perilipin promoters, we mapped and analyzed the 5′ upstream regulatory promoter sequences of both genes. Alignment of the mouse and human S3-12 promoters revealed two highly conserved regions (74 and 69% sequence identity), both containing promising PPREs (supplemental Fig. 1). Similarly, the human and mouse perilipin promoters contained particularly one highly conserved region (78% sequence identity) with a promising PPRE.

We thereafter cloned the mouse S3-12 (−2,070 to 638) and the human perilipin (−3,918 to 576) promoters into the pGL3-basic Luciferase reporter vector and subsequently transfected these constructs transiently into COS-1 cells. The S3-12 and perilipin reporters were induced up to 300- and 20-fold, respectively, when cotransfected with RXR-α and PPAR-γ expression vectors in the

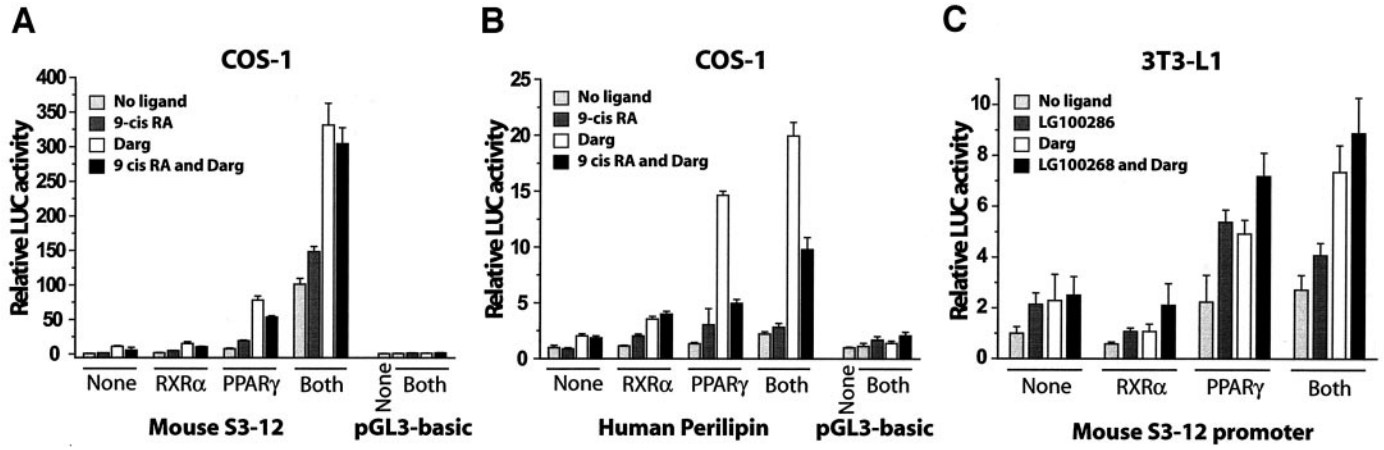

FIG. 3. The S3-12 and perilipin promoters respond to PPAR-γ activation. Transfection of full-length reporters into COS-1 cells. *A*: Mouse S3-12. *B*: Human perilipin. Medium was supplemented with vehicle (DMSO; light gray), 9-*cis*-retinoic acid (9-cis-RA; 1 μmol/l; dark gray), darglitazone (Darg; 1 μmol/l; white), or both ligands (■). *C*: S3-12 reporter transfected into differentiated 3T3-L1 cells. After 3 h, cells were incubated in medium containing vehicle (light gray), LG100268 (1 μmol/l; dark gray), darglitazone (1 μmol/l); white), or both ligands (■) for 48 h.

presence of appropriate ligands (Fig. 3*A* and *B*). In adipocytes, the S3-12 reporter showed a maximal ninefold induction by cotransfection of expression vectors in the presence of the rexinoid LG100268 (1 μmol/l) and darglitazone (1 μmol/l; Fig. 3*C*). The lower maximal induction in this cell system is probably due to the presence of endogenously expressed receptors and ligands.

The S3-12 and perilipin promoters contain binding sites for the RXR-α/PPAR-γ heterodimer. To map the most PPAR-γ–responsive region, we transfected deletion constructs of the S3-12 reporter (Fig. 4*A*) into COS-1 cells and found the −2,070 to −380 upstream region to contribute strongest to the PPAR-γ–mediated induction of the reporter (Fig. 4*B*). We then point-mutated the candidate PPREs, either alone or in combinations (Fig. 4*C*), and transfected these constructs into COS-1 cells. Single mutations of PPREs reduced the reporter activity modesty, whereas mutations of all three PPREs reduced reporter activity more than sixfold (Fig. 4*D*). The triple-mutated promoter construct still retained a weak response to PPAR-γ, suggesting that the promoter contains an eventual additional PPRE. Similar transfection studies of the human perilipin promoter also indicated the presence of a functional PPRE (Fig. 4*E* and *F*).

To demonstrate in vitro binding of PPAR-γ to the identified PPREs, we performed EMSAs with double-stranded oligonucleotides containing the PPREs of the S3-12 and perilipin promoters (supplemental Fig. 2; oligo sequences). A specific protein/DNA complex was observed only in the presence of in vitro translated RXR-α and PPAR-γ proteins (Fig. 4*G*). These complexes were specific, because the binding was competed by excess unlabeled wild-type but not by unlabeled mutated oligonucleotides.

Expression of S3-12, perilipin, and adipophilin correlates to the expression of PPARs. To evaluate the tissue distribution of S3-12 and PAT proteins in relation to the expression of PPARs, we probed human (Fig. 5*A*) and mouse (Fig. 5*B*) multiple tissue blots. A relatively strong S3-12 signal was obtained in skeletal muscle, both in human and in mouse, in disagreement with the weaker expression reported previously (10). To verify this unexpectedly higher muscle expression, we isolated RNA from skeletal muscle and two different WAT depots from two independent mice and compared the relative S3-12 expression in these samples. Again, a significant skeletal muscle S3-12 expression was observed (Fig. 5*C*). After prolonged exposure, skeletal muscle expression of perilipin was observed on the two commercial multitissue blots. However, no perilipin signals were detected in skeletal muscle from the muscle/WAT blot, supporting the previously highly restricted adipose tissue expression (4,8,14). This lack of a perilipin signal also eliminates the possibility that fat contamination of the muscle sample could explain the observed S3-12 muscle expression. Further analysis will be needed to determine whether there might be discordance between the S3-12 mRNA and protein levels, as observed for adipophilin in differentiated 3T3-L1 cells (3), or whether factors such as mouse strains, conditions of feeding, or animal activity influence the level of S3-12 expression. Adipophilin mRNA was expressed in all tissues examined, although at different levels, supporting the ubiquitous expression reported previously (3). TIP-47 mRNA was easily detected in all tissues examined, with elevated levels in human placenta and skeletal muscle. It is interesting that the expression of S3-12, perilipin, and adipophilin mRNAs overlapped well with that of RXR-α, PPAR-γ, and PPAR-α.

The expression of S3-12, perilipin, and adipophilin is altered in a model of obesity. Storage of TAG within lipid droplets is a major function of the adipose cell, and alteration in this storage capacity is associated with numerous diseases (23,25). Recent reports have demonstrated that perilipin is an important hormone-regulated "switch" that controls lipolytic rate and, hence, efflux of FAs from adipose tissue (19,20,41). To determine whether changes in the expression of S3-12 and PAT genes could be associated with obesity and insulin resistance, we examined the expression in an animal "diabesity" model.

Three groups of Zucker rats were compared, lean rats receiving 15% less food (lean food restricted) than control lean rats and fatty rats fed ad libitum. After 4 weeks on this food regimen, the total body weight of rats was found to be 220 ± 4 g (lean food–restricted rats), 273 ± 7 g (lean

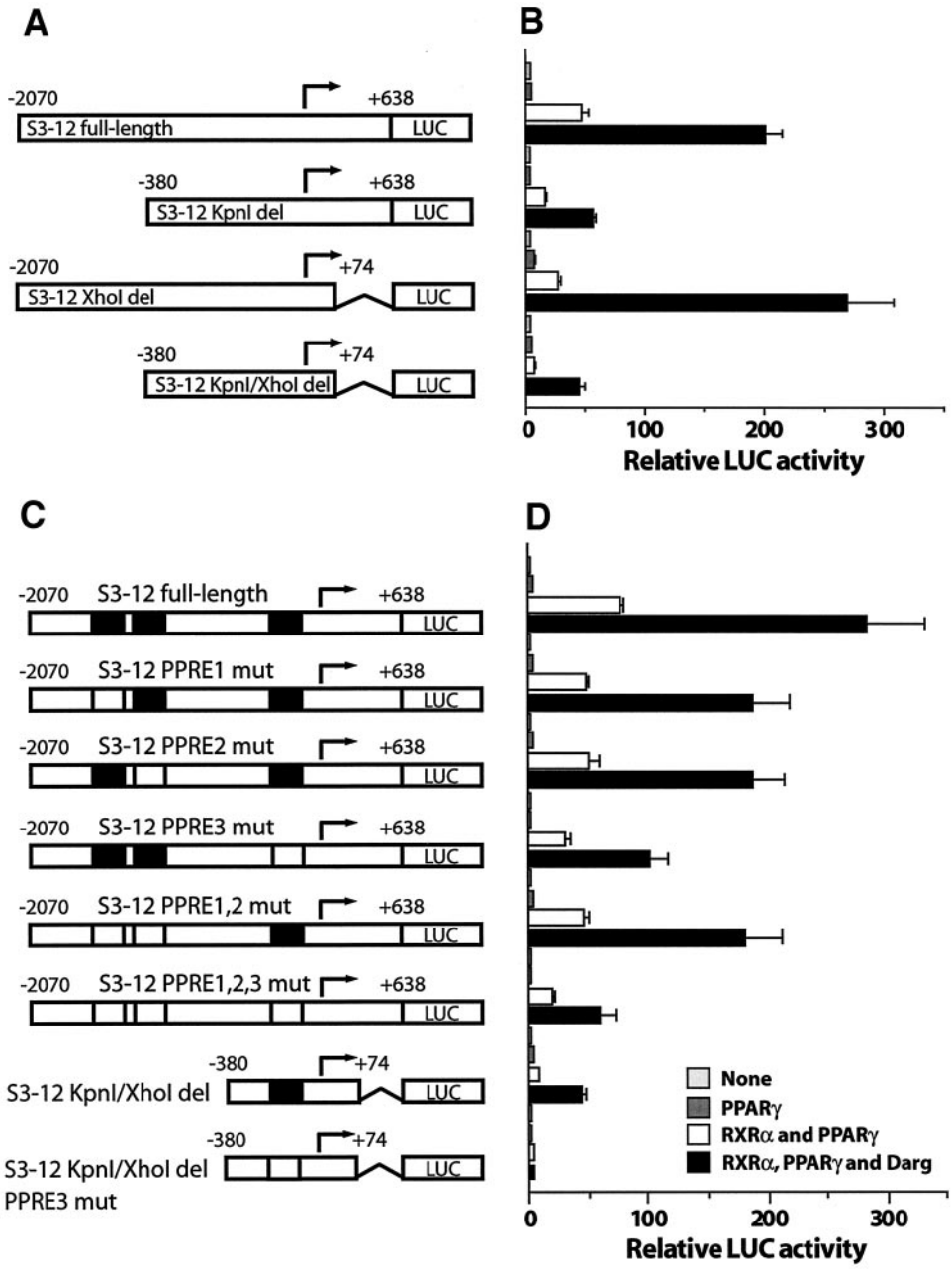

FIG. 4. Mapping of the PPAR-γ responsive region in the S3-12 and perilipin promoters. *A*: A schematic presentation of S3-12 reporter constructs. *B*: Transient transfection into COS-1. Cells were cotransfected with none (light gray), RXR-α (dark gray), or RXR-α and PPAR-γ expression vectors (□ and ■) and incubated in medium containing vehicle (light gray, dark gray, and □) or darglitazone (Darg; 1 μmol/l; ■). *C*: Constructs containing targeted mutations of S3-12 PPREs. *D*: S3-12 constructs transfected into COS-1. *E*: Constructs containing targeted mutations of perilipin PPREs. *F*: Perilipin constructs transfected into COS-1. *G*: Binding of RXR-α/PPAR-γ to PPREs. The competition was performed using unlabeled oligonucleotides as competitors in 10-, 20-, and 50-fold molar excess.

controls) and 328 ± 13 g (fatty rats fed ad libitum) with a corresponding increase in perirenal fat pad weight (Fig. 6*A*). Total RNA was subsequently extracted from perirenal WAT to evaluate basal mRNA expression levels. The expression of S3-12 and perilipin mRNA was higher in lean controls compared with the two other groups, whereas adipophilin was robustly increased in the fatty group (Fig. 6*B*). The expression of the PPAR-γ target gene aFABP was not different among lean and obese groups, in agreement with previous observations (40,42). As expected, the leptin mRNA was reduced in food-restricted animals and highly increased in fatty Zucker rats. The expression of TIP-47 and PPAR-γ (not shown) did not vary among the three groups. Repression of adipose perilipin and S3-12 expression, combined with induced adipophilin expression, therefore is a feature of the obese and partly insulin-deficient Zucker rat model.

DISCUSSION

PPAR-γ is required to maintain the adipocyte phenotype by directly binding to and transactivate response elements (PPREs) in a number of adipocyte-specific genes (23,24). Previously characterized PPAR-γ target genes facilitate uptake (lipoprotein lipase [26], fatty acid transport pro-

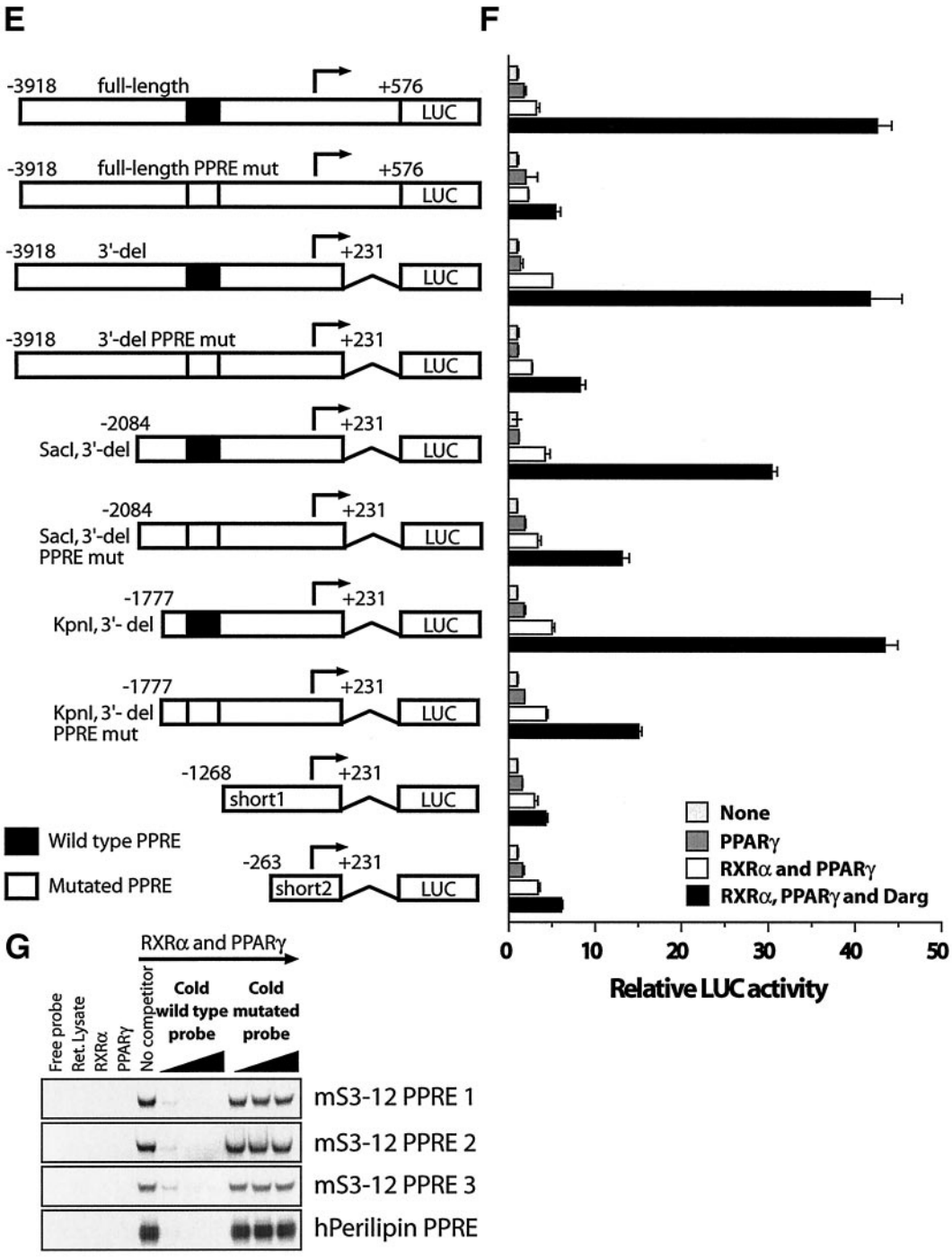

FIG. 4—*Continued.*

tein-1 [27]), cytosolic binding (aFABP [28]), and activation of FAs for TAG synthesis (acyl-CoA synthetase [29]). In this study, several observations convergently demonstrate that S3-12 and perilipin are directly regulated by the RXR-α/PPAR-γ heterodimer. S3-12 and perilipin mRNAs are induced during adipocyte differentiation, matching the timing of PPAR-γ induction, and induced by PPAR-γ activation. These effects are driven by RXR-α/PPAR-γ heterodimerization onto evolutionarily conserved PPREs located in both promoters. Increased expression of perilipin (32) and S3-12 (43,44) has been reported after PPAR-γ activation, supporting our analyses. Thus, our work extends the list of characterized PPAR-γ target genes and establishes a novel role for PPAR-γ in adipose tissue as a direct regulator of genes that are important for lipid droplet formation and maintenance.

It is interesting that regulation of S3-12 and PAT proteins by PPAR-γ is not a general phenomenon, because numerous experiments indicate that TIP-47 is not a PPAR-γ target gene. The first report to experimentally confirm TIP-47 as a component of lipid droplets (7) has also been discussed (45). Although later analysis strongly

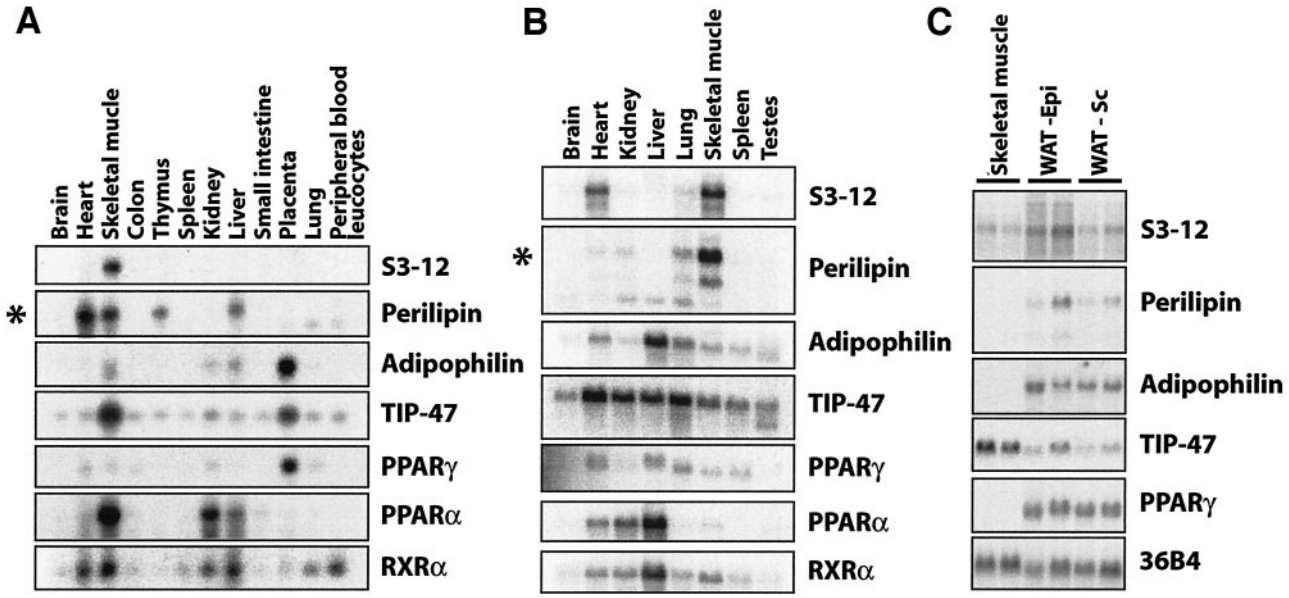

FIG. 5. Tissue distribution of S3-12 and PAT proteins in human and mouse. Expression (2 μg mRNA/lane) in human (*A*) and mouse (*B*). Perilipin mRNA was detected only upon longer exposure (*). *C*: Expression in skeletal muscle and epididymal (EPI) and subcutaneous (SC) WAT. Total RNA (15 μg/lane) isolated from single individuals.

supports lipid droplet association for TIP-47 (9), further analyses are definitely needed to clarify whether lipid droplet association for TIP-47 has any biological meaning or whether trafficking of mannose-6-phosphate receptors from late endosomes to Golgi (21) is the major function for this protein. In contrast, a recent observation suggests that PPARs might be involved in the regulation of adipophilin (31). A DR-1 element in the mouse adipophilin promoter that recruits PPAR-β/δ in macrophages has been described (46). However, analyses in our laboratory have failed to demonstrate that the human version of this DR-1 element is able to recruit RXR-α/PPAR-γ and transactivate the human adipophilin promoter (data not shown). Also, adipophilin expression is altered opposite to S3-12 and perilipin in obese compared with lean rats, suggesting that adipophilin is differently and perhaps more complexly regulated than S3-12 and perilipin.

Perilipin, adipophilin, and TIP-47 were initially grouped as PAT proteins because of sequence identity within the NH_2-terminal PAT-1 domain (8,9). S3-12 has also been described as peripheral member (6), despite the fact that the NH_2-terminal domain of S3-12 seems to lack a conserved PAT sequence. Because lipid droplet association properties are independent of this domain (11,12), it is arguable whether S3-12 should be classified as a PAT protein, although there is a clear sequence identity between S3-12 and ADRP and TIP-47 in the COOH terminus. Rather, it might be more appropriate to describe S3-12 as a lipid droplet–associating protein (LDAP) until possible specific biological properties for the conserved PAT domain have been clarified. Nevertheless, it seems appropriate to ascribe S3-12 to a subfamily consisting of the above three genes that are all independently demonstrated to accumulate on the surface of lipid droplets. Except for their common lipid droplet–associating properties, these genes vary in their transcriptional regulation and the presence of protein domains conserved among these proteins. At least four unique domains are present in these proteins (PAT1, PAT2, and the 33– and 12–amino acid repeats), but none of the proteins seem to contain all of these domains. These genes are also expressed in distinct tissues but with overlapping expression in WAT. Because the physiological response to FAs and lipids differs among tissues, it is likely that different LDAPs have acquired distinct functions to fine-tune lipid metabolism according to the particular needs of these tissues. To understand the differential regulation and function of these LDAPs therefore definitely will be important to rationalize how nutrition, physical exercise, and pharmacological interventions affect FA metabolism. A transition from using adipophilin to perilipin as the main LDAP occurs during differentiation of 3T3-L1 cells, accompanied with the formation of larger, unilocular lipid droplets (3). This change is important, as perilipin is more suited to control lipolysis by responding to hormonal signals, such as insulin and glucagon (16–18). Recent experiments have nicely demonstrated that the presence of perilipin on the lipid droplet surface is essential to obtain a low basal but a high stimulated lipolytic rate within adipose tissue (20,41). In the obese rat model, we observed a repression of perilipin and S3-12 expression, in combination with a robust increase of adipophilin. Induction of adipophilin in WAT was also a consistent feature in $Plin^{-/-}$ mice (19,20), suggesting that induction of adipophilin compensates for the decrease (in the obese rats) or loss (in $Plin^{-/-}$ mice) of perilipin as coating protein. It is interesting that although $Plin^{-/-}$ mice are resistant to genetic (19) and diet-induced obesity (20), they have elevated plasma levels of leptin, insulin, glucose, and TAG (20) in the range of those seen in obese animal models. These observations indicate that perilipin expression is crucial to control the storage of TAGs within lipid droplets in WAT to avoid undesirable consequences of uncontrolled lipolysis, such as insulin resistance.

The mechanisms underpinning the insulin-sensitizing, antidiabetic, and hypolipidemic actions of TZDs are at present not fully understood. As high-affinity ligands for

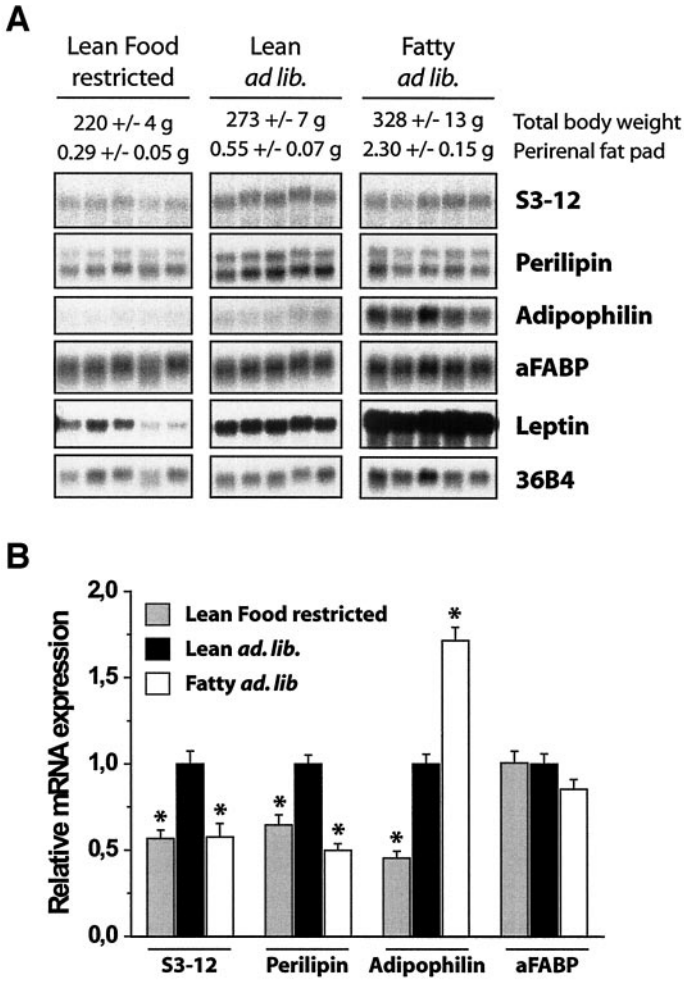

FIG. 6. Basal expression of S3-12 and PAT genes in WAT is dysregulated in an obese animal model. *A*: Expression in food-restricted (lean food restricted) and control lean Zucker rats (lean *ad lib*) and in fatty Zucker rats (fatty *ad lib*). Total RNA (20 μg/lane on the same blot) isolated from perirenal fat pads. *B*: Basal expression in each group. Statistical differences were evaluated with one-way ANOVA analysis (*$P < 0.01$).

PPAR-γ (22), they are thought to mediate their effects through regulation of PPAR-γ target genes within WAT. Increased transcription of these target genes is believed to remove FAs from circulation and promote their storage in WAT (25). LDAPs, such as perilipin and S3-12, are clearly pertinent in this context, because they control both storage and release of FAs within WAT. It is interesting that reduced perilipin protein levels was recently reported in obese compared with lean women, linking the reduced perilipin expression to increased basal lipolysis (47). Reduced expression of PPAR-γ and its target genes is well documented to be associated with obesity and type 2 diabetes (25). The establishment of S3-12 and perilipin as novel direct PPAR-γ target genes therefore is of considerable importance to better understand the physiological effects of PPAR-γ activators. Increased transcription of these LDAPs might indeed explain several of the insulin-sensitizing effects of TZDs: the observed FA trapping in adipose tissue, the reduced basal lipolysis, and the weight gain observed by prolonged stimulation by PPAR-γ activators (48).

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