(lipid) homeostasis (1–3). PPARα regulates energy combustion by serving as a key regulator of transcriptional pathways controlling fatty acid oxidation, whereas PPARγ functions as an important regulator of adipocyte differentiation and lipid storage (2, 3). PPARγ is present in two major isoforms, PPARγ1 and PPARγ2, resulting from alternate promoter usage (4). PPARγ2 contains additional 30 amino acids at the N-terminal end relative to PPARγ1, with PPARγ2 expression limited exclusively to adipose tissue where it plays a key role in adipogenesis (5). Recent studies have established that forced expression of PPARγ2 or PPARγ1 can stimulate the differentiation of fibroblasts to adipocytes, and in the process transcriptional pathways essential for the expression of adipocyte specific genes are activated resulting in lipid accumulation (6). Overexpression of PPARγ1 in mouse liver has also been shown to be sufficient for the induction of adipogenic transformation of hepatocytes with adipose tissue-specific gene expression and lipid accumulation (7). In addition to PPARγ, CCAAT/enhancer binding family of transcription factors C/EBPα, β, and δ, and a direct fibroblasts to differentiate into adipocytes (2). The C/EBP-directed adipocyte conversion is mediated through downstream regulator PPARγ (2).

Transcriptional activity of PPARγ and of other nuclear receptors is regulated by the binding of specific ligands and by the recruitment of nuclear receptor coactivators or coregulators (8, 9). The binding of ligands to nuclear receptors influences the recruitment of initial complex of coactivator proteins such as members of p160/SRC-1 family, and CREB-binding protein (CBP), which exhibit histone acetyltransferase activity necessary for remodeling chromatin (8, 9). Docking of other coactivators, either sequentially or combinatorially, manifests as a second multiprotein complex, variously referred to as TRAP-DRIP-ARC mediator complex, which facilitates interaction with RNA polymerase II complexes of the basal transcription machinery (10–12). This second complex, devoid of histone acetyltransferase activity, is anchored by PPARγ-binding protein (PBP), which was initially cloned as PPARγ-interacting protein (PRIP/ASC2/p300)-interacting protein (PRIP) (13). PPRγ also binds to PPARγ-interacting protein (PRIP/ASC2/ RAP250/NRC/TRBP) (14–18), which is also capable of interacting with several other nuclear receptors and with CBP/p300 and TRAP130 of the PBP-anchored mediator complex. In addition, PRIP-interacting protein with RNA methyltransferase activity, designated PIMT (19), forms a complex with CBP/p300, and PBP (20). Thus, the interactions of PRIP with CBP and TRAP130, and of PIMT with PRIP, CBP, and PBP raise the possibility that two major multiprotein cofactor complexes anchored by CBP/p300 and PBP, respectively, merge into one megacomplex on DNA template (21).

Evidence obtained from gene knock-out studies has established that both PBP and PRIP null mutations lead to embryonic lethality, implying that these coactivators influence the physiological functions of many nuclear receptors and possibly other transcription factors (22–26). Recent studies have also established that PPARγ- binding protein (PRIP, PPARγ-interacting protein) with methyltransferase activity; RXR, retinoid X receptor; CBP, cAMP response element-binding protein-binding protein; TRAP, thyroid hormone receptor-associated protein(s); ARC, activator-recruited cofactor; DRIP, vitamin D receptor-interacting protein(s); PIMT, PRIP-interacting cofactor complex; ChIP, chromatin immunoprecipitation; MEFs, mouse embryonic fibroblasts; RT, reverse transcriptase.
lished the critical role for PBP/TRAP220 in PPARγ-stimulated adipogenesis in that PBP−/− mouse embryonic fibroblasts (MEFs) were found refractory to adipocyte differentiation (27). Since PRIP is also a PPARγ coactivator and the disruption of the PRIP gene resulted in lethal phenotype, it appeared necessary to investigate the role of this coactivator in PPARγ-stimulated adipogenesis. Here we show that PRIP−/− MEFs fail to exhibit PPARγ-stimulated adipogenesis even in the presence of PBP, suggesting the need for the presence of both PBP and PRIP for PPARγ-directed adipogenesis.

MATERIALS AND METHODS

Construction of Retrovirus Vector—The retroviral vector expressing PPARγ1 was constructed by inserting mouse PPARγ1 cDNA isolated from PCMV-PPARγ into pMSCVneo and the construct was verified by sequencing. Phoenix ecotropic retrovirus packaging cells and PT67 cells (Clontech) were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. To infect MEFs with PPARγ retrovirus, 1 × 105 cells were grown for 24 h and changed to the medium collected from 10-cm plate of virus producing PT67 cells. Cells were incubated with retrovirus for 24 h and then cultured in fresh medium. Forty-eight hours after infection, cells were selected with 200 μg/ml G418 for 8 days. Induction of adipogenesis was carried out as described (5). Briefly, after cells grew into confluence, they were treated with culture medium containing 0.5 mM 3-isobutyl-1-methyl-xantine (Sigma), 1 μM dexamethasone (Sigma), 5 μg/ml insulin (Sigma), and 0.5 μM rosiglitazone or Me2SO for 48 h. Cells were then changed to the medium containing 5 μg/ml insulin (Sigma) and 0.5 μM rosiglitazone or Me2SO. The induction lasted for 8 days with the medium being replaced every 2 days. The fat droplets in cells that exhibited adipogenesis were revealed by Oil Red O staining.

Immortalization of Wild-type and PRIP−/− Embryonic Fibroblasts—Wild type and PRIP−/− primary MEFs were isolated from E11.5 littermate embryos as described (24). Self-immortalization of the MEFs was achieved by re-plating the cells every 3 days at a density of 2.6 × 106 continuously for more than 5 months. PBP−/− MEFs were obtained as described previously (22).

Cell Culture, Retrovirus Infection of MEFs, and Induction of Adipogenesis—MEF cell lines were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. To infect MEFs with PPARγ retrovirus, 1 × 105 cells were grown for 24 h and changed to the medium collected from 10-cm plate of virus producing PT67 cells. Cells were incubated with retrovirus for 24 h and then cultured in fresh medium. Forty-eight hours after infection, cells were selected with 200 μg/ml G418 for 8 days. Induction of adipogenesis was carried out as described (5). Briefly, after cells grew into confluence, they were treated with culture medium containing 0.5 mM 3-isobutyl-1-methyl-xantine (Sigma), 1 μM dexamethasone (Sigma), 5 μg/ml insulin (Sigma), and 0.5 μM rosiglitazone or Me2SO for 48 h. Cells were then changed to the medium containing 5 μg/ml insulin (Sigma) and 0.5 μM rosiglitazone or Me2SO. The induction lasted for 8 days with the medium being replaced every 2 days. The fat droplets in cells that exhibited adipogenesis were revealed by Oil Red O staining.

Northern Analysis and RT-PCR—Total RNA was extracted from cultured cells using TRIzol (Invitrogen) according to the manufacturer's instructions. For Northern blotting, 20 μg of total RNA was used for each sample. To detect PBP mRNA in wild type and PRIP−/− MEFs, RT-PCR was carried out with primers 5'-TGTATCCGGCTCTTTCCAAATCC-3' and 5'-AGTGATGGATGATTCCAGG-3' (4). To detect PRIP mRNA in wild type and PBP−/− MEFs, RT-PCR was performed with primers 5'-TTTTATGGATGACGGCGGC-3' and 5'-CATCATATGCGCGCCG-3' (15). Total RNA (1 μg) was used for each sample for RT-PCR analysis using One-Step RT-PCR kit (Invitrogen). Primers for PPARγ, PPARα, PPARδ, PPARβ and COX2 were designed on the basis of the published sequences. PCR products were separated on 2% agarose gels and visualized under UV light.

Immunoprecipitation and Immunoblot Analysis—ChIP assay, cells were cross-linked with 1% formaldehyde at room temperature for 10 min and processed for the isolation of nuclei. Immunoprecipitation (ChIP) assay, cells were crosslinked with 1% formaldehyde and then sonicated on ice to shear chromosomal DNA (28). After centrifugation, the supernatant was diluted 10-fold with dilution buffer (16.7 mM Tris-Cl, pH 8.1, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl) and with preimmune serum-coupled protein A beads and salmon sperm DNA. Immunoprecipitation was performed by incubating the preclared lysate with specific antibody at 4°C for 12 h. Immune complexes were pulled down by protein A-agarose beads and washed sequentially for 10 min each with washing buffer I (0.1% SDS, 1% Triton-X100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.1, 150 mM NaCl), buffer II (1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.1, 500 mM NaCl), and buffer III (0.25 mM LiCl, 1% Nonidet P-40, 1% deoxycholate, 10 mM Tris-Cl, pH 8.1). DNA in the immune complexes was extracted and used as the template in PCR reaction.

Immunoprecipitation and Immunoblot Analysis—Cells were infected with His-tagged adenovirus PIMT as described previously (21). Cells were collected 48 h after virus infection and lysed in lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 0.5 mM PMSF). Immunoprecipitation with anti-His tag and immunoblotting were carried out as described previously (20).

RESULTS

PPARγ1-induced Adipogenesis in PRIP−/− MEFs—PPARγ plays an essential role in the transcriptional regulation of adipocyte-specific genes during adipogenesis (2, 5). Introduction of PPARγ into either primary or immortalized MEFs stimulates adipogenesis (5, 27). Both PPARγ isoforms, PPARγ1 and PPARγ2, have been shown to have the similar adipogenic capacity (28). To test the requirement of PRIP in PPARγ-mediated adipogenesis, immortalized MEF cell lines were established from E11.5 PRIP−/− embryos and their wild type littermates. PRIP−/− mouse embryos die between E11.5 and E12.5 (postcoitum) due in most part to defects in the development of placenta, heart, liver, nervous system, and retardation of embryonic growth (24–26). Wild type and PRIP−/− MEF cell lines were infected with retroviruses expressing PPARγ1 and then induced with differentiation medium containing PPARγ1 ligand rosiglitazone to maximize cell differentiation or vehicle (dimethyl sulfoxide) alone. Wild type MEFs expressing PPARγ1 and treated with rosiglitazone exhibited adipocyte conversion (Fig. 1, A and C). In contrast, retroviral expression of PPARγ1 failed to induce adipogenesis in PRIP−/− MEFs even in the presence of rosiglitazone (Fig. 1, B and D). Consistent with the morphological changes, mRNA and protein levels of adipocyte specific gene aP2 were markedly induced in wild type MEFs exhibiting adipogenesis, whereas aP2 mRNA and protein were not detectable in PRIP−/− MEFs that failed to reveal adipogenesis (Fig. 2, A and C). On Northern blotting, PPARγ1 mRNA level in PRIP−/− MEFs were at a higher level than that noted in the wild type MEFs showing the differentiated morphology (Fig. 2A). These results demonstrated that PPARγ1 was unable to stimulate adipogenesis in the absence of PRIP. Primary PRIP−/− MEFs also showed defects in PPARγ1-stimulated adipogenesis (data not shown).
PRIP Is Required for PPARγ-mediated Adipogenesis

Expression of PBP in PRIP−/− Cells—Recently, PPARγ-coactivator PBP, the anchor protein for TRAP-DRIP-ARC-PRIC complex (13, 14), has been shown to be essential for adipogenesis (27). Since PBP−/− MEFs have been shown to be defective in PPARγ2-stimulated adipogenesis, it was important to ascertain the relative levels of PBP mRNA in wild type and PBP−/− MEFs (Fig. 2B). PBP mRNA levels, as assessed by RT-PCR, were essentially similar in wild type and PBP−/− MEFs. There was also no difference in the level of PRIP mRNA expression in PBP−/− MEFs (Fig. 2B). These results indicate that the defects in PPARγ1-induced adipogenesis caused by the absence of PRIP and PBP are independent of each other.

Recruitment of PBP, CBP, and PIMT to aP2 Gene Promoter in PRIP−/− MEFs during Adipogenesis—Transcriptional activation by PPARγ requires recruitment of nuclear receptor coactivator complexes to remodel chromosome structure and facilitate transcriptional initiation (8, 9). To investigate the impact of the absence of PRIP on the ability of PPARγ to recruit coactivator complexes, ChIP assay was utilized to examine the association of aP2 gene promoter with coactivators in wild type and PRIP−/− MEFs that received adipogenesis treatment (Fig. 3). Using antibodies against PPARγ, PRIP, CBP, PIMT, or PBP, protein-DNA immune complexes were pulled down from cell lysates, and the DNA fragments in the complexes analyzed by PCR with primers (5′-AAATTCAGAAGATAAACA-CATTATT-3′ and 5′-ATGCCCTGACCATGTGA-3′) spanning the PPARγ-responsive element (AGGTCAAATGTGT) in the aP2 gene promoter region (29). The presence of endogenous PPARγ was detected on the aP2 promoter in wild type and PRIP−/− cells, and this association was prominent in the presence of ligand. PPARγ occupancy was increased by expressing exogenous PPARγ and addition of PPARγ ligand. Recruitment of CBP, and PBP to the aP2 promoter by PPARγ, was not visibly altered in PRIP−/− MEFs in response to retroviral expression of PPARγ1, whereas the recruitment of PIMT to the aP2 promoter in response to exogenous PPARγ1 was less robust in PRIP−/− cells (Fig. 3), suggesting that PRIP is needed for PIMT recruitment and for the formation of a large multi-protein transcriptional complex (20, 21).

Recruitment of PBP, CBP, and PIMT to aP2 Gene Promoter in PRIP−/− MEFs during Adipogenesis—Proroperty of adipocytes. Catalase immunostaining is used as protein loading control.

DISCUSSION

In this study, we have examined the role of nuclear receptor coactivator PRIP in the PPARγ1-directed adipogenesis using wild type and PPRIP−/− MEFs. Our findings provide evidence that PRIP is required for adipocyte differentiation and for the expression of adipocyte specific aP2 gene. The refractoriness of PRIP−/− MEFs to PPARγ-stimulated adipogenesis is similar to that described for PBP/PRIP−/− MEFs (27), suggesting that both PRIP and PBP play important roles in mediating the adipocyte differentiating effects of PPARγ. These two coactivators were isolated using PPARγ as bait in the yeast two-hybrid screen and were identified as coactivators for PPARγ and other nuclear receptors (13–18). PBP has emerged as a central piece of large TRAP-DRIP-ARC-PRIC multiprotein coactivator complex.
PRIP Is Required for PPARγ-mediated Adipogenesis

(10–12, 21), whereas PRIP and PRIP-binding protein PIMT have been found recently to serve as linkers between CBP- and PBP-anchored cofactor complexes (18, 20). The embryonic lethality observed with the disruption of PBP and PRIP genes and the failure of MEFs derived from the PBP and PRIP null mutants to differentiate into adipocytes under PPARγ stimulation suggest that both PBP and PRIP are vital for the successful completion of transcriptional activity of several genes involved in adipogenesis and possibly in the development and differentiation necessary for ontogeny.

The mechanism for the failure of PRIP−/− MEFs to undergo PPARγ1-stimulated adipogenesis may involve inadequate docking or linkage between CBP/p300-anchored coactivators with PBP-anchored TRAP-DRIP-ARC-PRIC multiprotein complex. Since PRIP binds CBP/p300 and TRAP/DRIP130, a component of TRAP complex, the absence of PRIP most likely interferes with linkage of CBP/p300-anchored coactivator complex with PBP-anchored complex, thus curtailing the transcriptional signaling (18, 20). Absence of PRIP might further interfere with these protein-protein interactions because PRIP binding protein PIMT directly binds with both CBP/p300 and PBP (20). Interestingly, ChIP assays revealed reductions in PIMT recruitment to the aP2 promoter in response to exogenous PPARγ1, implying that PRIP is needed to recruit PIMT to the coactivator complex. ChIP assays also revealed reduction in exogenous PPARγ1 recruitment to aP2 promoter in PRIP−/− MEFs (Fig. 3). This may be due to the requirement of PRIP for the stable formation of PPARγ1-RRx heterodimers on the aP2 promoter or reduced amount of RRx in PRIP−/− MEFs in that PRIP−/− MEFs were shown to exhibit marked repression of RRx-mediated transcriptional activity (24). Immunoprecipitation and immunoblotting data reveal that in PRIP−/− MEFs the binding of CBP to PIMT is reduced, and this may also have functional implications.

It is now well established that PPARγ and C/EBPα are critical transcription factors in adipogenesis (1, 2). Genetic analysis of adipogenesis has revealed that PPARγ promotes adipogenesis in C/EBPα-deficient cells, but the converse is not true in that C/EBPα has no ability to promote adipogenesis in the absence of PPARγ (2). The studies of Ge and co-workers (27) with PBP−/− MEFs, and the observations reported here using PRIP−/− MEFs clearly establish the importance of these two coactivators in PPARγ-stimulated adipogenesis. These two coactivators appear to function as downstream effectors of PPARγ, and both are required for the successful completion of the adipogenic program. This assumption is based on the observation that PRIP−/− MEFs used in this study express PBP (Fig. 2B). Likewise PBP−/− MEFs also express PRIP mRNA to the same level as wild type MEFs, and the absence of either one of these coactivators interferes with PPARγ-stimulated adipogenesis. In essence, lack of PBP may result in the disruption of TRAP-DRIP-ARC-PRIC complex formation and absence of PRIP may interfere with the linkage and passage of transcriptional signal through the combined CBP/p300- and PBP-anchored complex. Additional studies are needed to assess the role of PRIP in in vivo adipogenesis using PRIP conditional null mice.

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