Coactivator PRIP, the Peroxisome Proliferator-activated Receptor-interacting Protein, Is a Modulator of Placental, Cardiac, Hepatic, and Embryonic Development*

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Nuclear receptor coactivator PRIP (peroxisome proliferator-activated receptor (PPARγ)-interacting protein) and PRIP-interacting protein with methyltransferase activity, designated PIMT, appear to serve as linkers between cAMP response element-binding protein (CREB)-anchored and PBP (PPARγ-binding protein)-anchored coactivator complexes involved in the transcriptional activity of nuclear receptors. To assess the biological significance of PRIP, we disrupted the PRIP gene in mice by homologous recombination. Mice nullizygous for PRIP died between embryonic day 11.5 and 12.5 (postcoitum) due in most part to defects in the development of placenta, heart, liver, nervous system, and retardation of embryonic growth. Transient transfection assays using fibroblasts isolated from PRIP−/− embryos revealed a significant decrease in the capacity for ligand-dependent transcriptional activation of retinoid X receptor α and to a lesser extent on PPARγ transcriptional activity. These observations indicate that PRIP like PBP, CBP, and p300 is an essential and nonredundant coactivator.

Our understanding of the mechanisms underlying transcriptional activation by nuclear receptors has been advanced by the identification of nuclear receptor coactivators or coregulators that appear to influence embryonic development, cell proliferation, and differentiation (1). These include p160/SRC-11 (steroid receptor coactivator-1) family with three members (SRC-1, TIF/GRIPI/SRC-2, and pCIP/AIB1/ACTR/RAC3/TRAM1/SRC3) (2–6). CREB-binding protein (CBP) (7), adenovirus E1A-binding protein p300 (8), peroxisome proliferator-activated receptor-γ (PPARγ)-binding protein (PBP) (9), PPAR-interacting protein (PRIP/ASC-2/RAP250/TRBP/NRC) (10–14) and PPARγ coactivator-1 (PGC-1) (15), among others. Nuclear receptor coactivators contain one or more conserved LXXLL (where L is leucine and X any amino acid) signature motif, which has been found to be necessary and sufficient for ligand-dependent interactions with the activation function-2 domain present in the C-terminal hormone-binding region of the nuclear receptors (1, 6). It is generally held that coactivators play a central role in mediating nuclear receptor transcriptional activity by functioning as at least two large multiprotein complexes formed either sequentially or combinatorially (1). The first complex anchored by CBP/p300 and containing p160 cofactors/SRC-1 cofactors exhibits histone acetyltransferase activity necessary for remodeling chromatin (1, 4, 7, 16), while the second multiprotein complex, variously referred to as TRAP/DRIP/ARC mediator complex, which is anchored by PBP (17–19), facilitates interaction with RNA polymerase II complexes of the basal transcription machinery (1). Deletion of CBP/p300 and PBP genes in the mouse results in embryonic lethality around E11.5 days, indicating that disruption of these pivotal anchoring coactivators affects the integrity of the cofactor complexes, thus altering the function of many nuclear receptors and most likely of other transcription factors (20–24).

Of interest is that the recently identified coactivator designated PRIP/ASC2/RAP250/NRC/TRBP has also been shown to interact with several nuclear receptors and with CBP/p300 and TRAP130 of the TRAP/DRIP/ARC complex (10–14). Thus, PRIP appears to serve as a bridge between the first complex anchored by CBP/p300 and the downstream TRAP/DRIP/ARC mediator complex anchored by PBP. Furthermore, the recently isolated PRIP-interacting protein with RNA methyltransferase activity, designated PIMT (25), forms a complex with CBP, p300, and PBP (26), further attesting to the possibility that two major multiprotein cofactor complexes anchored by CBP/p300 and PBP, respectively, merge into one megacomplex on DNA template (28). Since PRIP and PRIP-binding protein PIMT appear to link the two cofactor complexes under in vitro conditions, we have found it necessary to explore the biological function of PRIP by generating mice with PRIP null phenotype. We now demonstrate that PRIP is critical for the embryonic development, since disruption of the PRIP gene in the mouse leads to embryonic lethality around E11.5 to E12.5 days, implying that PRIP (like CBP/p300 and PBP) is also critical for embryonic development and survival.

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‡The abbreviations used are: SRC-1, steroid receptor coactivator-1; PPAR, peroxisome proliferator-activated receptor; PRIP, PPAR-interacting protein; PBP, PPAR-binding protein; PIMT, PRIP-interacting protein with methyltransferase activity; RXR, retinoid X receptor; PPIRE, peroxisome proliferator response element(s); RAR, retinoic acid receptor; RARE, retinoic acid response element; CREB, CAMP response element-binding protein; CBP, CREB-binding protein; TRAP, thyroid hormone receptor-associated protein(s); DRIP, vitamin D3 receptor-interacting proteins(s); ARC, activator-recruited cofactor; PRC, PPARα-interacting cofactor complex; ES cells, embryonic stem cells; RT, reverse transcriptase; PCNA, proliferating cell nuclear antigen; MEF, mouse embryonic fibroblast.
**Embryonic Lethality of PRIP Null Mutation**

**MATERIALS AND METHODS**

Construction of Targeting Vector—The genomic DNA fragment containing the full-length PRIP gene was isolated from a mouse 129/Sv F1 bacteriophage library (Genome Systems, St. Louis, MO), using polymerase chain reaction with primers 5′-CAATGACGGCTGGTCTCTG-3′ and 5′-GCTCCTGCACATCACGCTA-3′ designed from mouse PRIP cDNA sequence (10). A Cre-LoxP system was employed to delete exon 7 from the PRIP gene. For this purpose, we constructed a triple-LoxP targeting vector to generate a floxed mouse PRIP-targeted locus.

Generation of PRIP Null Mice—The targeting vector was linearized and electroporated into HM1 embryonic stem (ES) cells (28). Transfected ES cells were selected in the medium with G418 (200 μg/ml) and electroporated into HM1 embryonic stem (ES) cells (28). Transfected ES cells were selected in the medium with G418 (200 μg/ml), and the surviving colonies were screened for homologous recombination by PCR with primers P1 5′-CTTACAGCGTCGAGAAATC-3′ and P2 5′-TAATGGTATGGAACGCCGC-3′. ES cells with the appropriate PRIP floxed targeted locus were further confirmed by Southern blot analysis (Fig. 1B), and the euploid selected ES cells were used for injection into 3.5-day-old blastocysts derived from C57/B6 mouse by the Northwestern University Targeted Mutagenesis Facility to generate chimera mice. Chimeric male mice were bred with wild type C57/B6 females to produce heterozygous offspring, which were then crossed with ELII-cre transgenic mice (29) to delete the DNA fragment between LoxP1 and LoxP3. ELII-cre-mediated recombination occurs early in development (2–8 cells), and mice carrying the allele with deletion were crossed with wild type C57/B6 to achieve germ line transmission. The heterozygous mice with expected deletion were interbred to generate homozygous mutants.

**Genotyping of Mice and Embryos—**DNA was isolated from the tail tips of mice and from the yolk sac of embryos by phenol/chloroform extraction by PCR. The mice carrying the recombination between LoxP1 and LoxP3 on one allele were identified by PCR with primers P1/P2 and P3 (5′-GGCCGCCGATCACTGATA-3′/P4 (5′-TCTCTCTCTCGAGCCGC-3′). Presence of P1/P2 product, while lacking P3/P4 amplification, indicated the deletion of PRIP gene on one allele. The homozygosity for the deletion was detected by the absence of exon 7 as ascertained by PCR with primers P5 5′-ACGGGGCAACAAATAATGATG-3′ and primer P4 (see above) (Fig. 1C).

RT-PCR and Western Blots—For RT-PCR, total RNA was extracted from embryos with TRIzol reagent (Invitrogen). Primers 5′-CTTGAGCGTCGAGAAATC-3′ and 5′-CGAACATGCGTCACTGAGCC-3′ were used to amplify genomic DNA between exons 6 and exons 8 from the PRIP homozygous mutant. To detect PRIP protein in embryos, whole cell lysates were prepared from the embryos by homogenization and probed with anti-mouse PRIP antiserum. The signal was detected by chemiluminescence (ECL detection system).

Histological Analysis and Immunohistochemistry—Age-matched embryos were fixed in paraformaldehyde or 10% of buffered formalin, embedded in paraffin, serially sectioned at 5-μm thickness in sagittal or transverse planes, and stained with hematoxylin and eosin. Immunohistochemical staining for the localization of proliferating cell nuclear antigen (PCNA) was performed using a standard avidin-biotin-peroxidase complex protocol as described previously (24). Giemsa stain was done using the standard protocol.

Isolation of Fibroblasts from Embryos and Transfection of Primary Fibroblasts—Mouse embryonic fibroblasts (MEF) were isolated from E11.5 embryos and cultured in Dulbecco's modified Eagle's medium with 10% of fetal bovine serum as described (22). For transfection, 2 × 10⁶ of MEFs were plated in six-well plates for overnight culture. The transfections were carried out with LipofectAMINE-2000 Plus reagent according to manufacturer's instruction (Invitrogen). Plasmids pCMV-PPARγ, pPRES-TK-LUC, pCMV-RXR, pXRX-TK-LUC, pCMV-RAR, and pRAR-TK-LUC were used as described previously (10, 25, 26). β-Galactosidase assay and luciferase vector pCMVβ was used as a cotransfectant, which served as control for transfection efficiency. Cell extracts were prepared 36 h after transfection and were assayed for luciferase and β-galactosidase activities.

**RESULTS**

**Disruption of PRIP Gene in Mice—**We constructed a conditional knockout allele of PRIP by using the Cre/loxP recombination system according to the strategy in Fig. 1A. To generate conventional PRIP knock-out mice (in which the gene is permanently inactivated at the germ cell stage), it was necessary to induce recombination between loxP sites. To achieve heterozygosity, PRIP-targeted mice were bred with homozygous Cre transgenic mouse line, EII-cre (29). The EII-cre mice carry the Cre transgene under the control of the adenosuvirus ELII promoter and express Cre recombinase only in early mouse embryos (2–8 cell stage), and it induces the recombination between the two loxP sites with the same orientation (29). We detected the expected, all types of recombinants among the offspring (29). The recombination between loxP1 and loxP3 resulted in the deletion of PRIP exon 7 and a reading frame shift to generate a stop codon right after the fusion between exon 6 and exon 8. The chimeras were crossed to wild type mice to produce heterozygous mice carrying one recombinant PRIP allele, and the homozygous mice were obtained from heterozygous mating. By sequencing the RT-PCR products, exon 7 was not found in mRNA transcribed from the recombinant PRIP allele, and the reading frameshift was introduced by the deletion leading to a premature stop codon (data not shown). As the result, only 488 amino acids at the N terminus containing no LXXLL motif can possibly be translated from the mRNA, but no PRIP protein was detected by Western blot analysis (data not shown).

**Embryonic Lethality and Growth Retardation of PRIP Null Mice—**Among 26 new-born pups, and 54 mice that were 3 weeks old generated from intercrosses between heterozygous PRIP mutant mice, no homozygous mutants were detected. Genotyping the embryos at different stages of gestation showed that no PRIP null embryos survived beyond E13.5 (13.5 days postcoitum). However, heart beating was observed among the majority of viable PRIP null embryos recovered between E11.5 and E12.5 and few were moribund or dead. These observations indicate lethality occurred in a relatively narrow window of time as no viable PRIP null embryos were seen at E13.5. The viable PRIP1/−/− embryos recovered at E11.5 and E12.5 exhibited clear evidence of growth retardation compared with the wild type and heterozygous littermates. PRIP1/−/− embryos appeared strikingly different at the gross level at E12.5, they were pale and smaller in size than their PRIP1+/− and PRIP1+/+ littermates (Fig. 2, A and B). Normally, extraembryonic mesoderm of the yolk sac gives rise to blood and endothelial cells, which form blood islands. The extraembryonic membrane covering PRIP null embryos contained fewer vessels in contrast to wild type yolk sac with its well developed blood vessel network (Fig. 2B). In addition, superficial vasculature was less obvious in
PRIP null mutants (Fig. 2D). While the liver of wild type embryos was easily visualized by its rich vasculature through the skin, only a pale primitive liver bud was discerned in PRIP−/− embryos (Fig. 2D). Failure of palatal shelf to fuse, abnormal finder separations, and developmental abnormalities in brain were detected in PRIP−/− mutants (not illustrated).

Lack of Organized Spongiotrophoblast Layer in PRIP Mutant Placenta—In wild type placenta at E12.5 contains three distinct trophoblast cell structures: the innermost labyrinthine layer, the intermediate spongiotrophoblast layer, and the outermost trophoblast giant cell layer. The labyrinthine zone formed by the fusion of chorion with allantois is composed of extensively branched fetal blood vessels and maternal blood sinuses among strands of diploid trophoblast cells that separate the maternal blood sinus from fetal blood vessels. In PRIP null placenta, no compact layer of spongiotrophoblast cells was observed between labyrinth zone and trophoblast giant cell layer. Instead, islands of spongiotrophoblast-like cells dissociated from trophoblast giant cell layer and migrating into the labyrinth zone were common occurrence in PRIP−/− placenta (Fig. 3B). In wild type placenta blood sinuses are filled with maternal blood cells throughout the labyrinthine layer (Fig. 3, A, C, and F), whereas most of the tortuous vessels in the PRIP−/− placenta were enlarged, ruptured, and generally empty (Fig. 3, B, D, and F). While the choioallantoic fusion appeared to occur in PRIP mutant placenta, chorionic trophoblast cells clustered in labyrinth had multiple nuclei, and these clusters showed insufficient blood vessel branching. These changes are reminiscent of some of the placental defects observed in PBP null mutants (22, 24). However, changes in PRIP−/− placenta appeared less profound when compared with PBP−/− placenta (24). Nucleated fetal erythroblasts in PRIP−/− placenta had irregular shaped nuclei with very little cytoplasm (Fig. 3F). Trophoblast cell proliferation in PRIP null placenta as assessed by PCNA immunostaining was significantly lower than that observed in PRIP+/+ placenta (data not shown).

PRIP−/− Embryos Manifest Cardiac Defects—Inefficient pumping by the heart leading to circulation failure is one of the major causes of embryonic lethality during middle gestation. The development of heart requires coordinated differentiation of several embryonic lineages, including the myocytes of myocardium, the endothelial cells of the endocardium, and the cells of the neural crest that form the outflow tract. At E12.5, the heart of PRIP−/− embryos exhibited defects involving all three lineages (Fig. 4, A and B). In PRIP−/− heart, the epicardium, consisting of a single layer of mesothelial cells lining against the compact layer of myocardium, appeared to separate from underlying myocardium. In some regions of compact layer of PRIP−/−, the adhesion of myocytes was disrupted and red blood cells penetrated through myocardium (arrowheads). C and D, PCNA staining for the proliferating cells in ventricles of E12.5 wild type (C) and PRIP−/− (D) embryos. Fewer dark brown PCNA-positive cells are present in the compact layer of PRIP−/− ventricle.
The inset apoptotic bodies (cardium (Fig. 4, C) of PRIP/H11002 somewhat in that PRIP seemed to influence RXR maximally. The activities of the three nuclear receptors examined here differed dramatically potential, although the contribution of PRIP to the activities of the three nuclear receptors require PRIP to achieve their full transcription appears similar to that noted in PBP null and PPAR

Defective Hepatopoiesis and Hepatic Hematopoiesis in PRIP<sup>−/−</sup> Mutants—Liver of PRIP null mutants appeared considerably smaller in size when compared with their littermates (Fig. 5, A and B). The function of the liver at E12.5 days is to become the major site of hematopoiesis so as to gradually replace yolk sac based hematopoiesis. Histological examination of PRIP<sup>−/−</sup> liver revealed reduction in hepatocyte population and an increase in hepatocyte apoptosis (Fig. 5D). A marked decrease in the number of erythroid progenitors was also evident, and these cells had large nuclei with scant minimally hemoglobinized cytoplasm. Liver exhibited large numbers of megakaryocytes.

Differential Reduction of Transactivation by Nuclear Receptors in PRIP<sup>−/−</sup> Primary Fibroblasts—To assess the impact of loss of PRIP on transcriptional activities of nuclear receptor, we isolated MEFs from PRIP<sup>−/−</sup> and PRIP<sup>+/+</sup> embryos. They were used for assaying the transcriptional activities of PPARγ, RXR, and RAR. In wild type MEFs transfected with an RXR expressing vector and RXR-responsive element-linked reporter, the addition of RXR-ligand 9-cis-retinoic acid induced marked increase (~76-fold) in the transcription (Fig. 6A). In PRIP<sup>−/−</sup> MEFs, the induction of ligand-mediated RXR transcription was markedly reduced (~3-fold). Transcription assays with PPARγ (Fig. 6B) and RARα (data not shown) showed that the influence of PRIP was only modest. These results demonstrated that nuclear receptors require PRIP to achieve their full transcriptional potential, although the contribution of PRIP to the activities of the three nuclear receptors examined here differed somewhat in that PRIP seemed to influence RXR maximally.

**DISCUSSION**

The nuclear hormone receptors comprise a superfamily of transcription factors that regulates coordinated expression of gene networks involved in developmental, physiological, and metabolic processes (1). Notable among this nuclear receptor superfamily is PPAR subfamily comprising of three isoforms, PPARα, PPARγ, and PPARβ/δ, since these receptors have emerged in recent years as a critical player in regulating energy metabolism. In an effort to understand the factors controlling cell and gene specific transcriptional events initiated by nuclear receptors, the ligand-binding domain of nuclear receptors was used in the yeast two-hybrid screen to identify receptor interacting proteins (2–7). During the past 7 years, more than 25 nuclear receptor coactivators have been cloned raising the issue of redundancy, since these coactivators generally appear promiscuous in their coactivation potential.

To fully appreciate the in vivo biological functions of these coactivators, molecular genetic approaches are being increasingly employed. Previous studies have demonstrated that mice lacking SRC-1 or p/CIP/SRC-3 are viable and manifest either partial or full redundancy for certain nuclear receptor actions (32–36). In contrast, deletion of more general coactivators such as CBP/p300 and PBP in mice leads to embryonic lethality implying that these are essential coactivators (20–24). Thus, there appear to be at least two broad classes of coactivators: essential and redundant. Our observations reported here now add coactivator PRIP to the class of essential coactivators because of the embryonic growth retardation, defects in placental, cardiac and hepatic development, and embryonic lethality. Embryonic lethality was noted between E11.5 and E12.5 days with no viable embryos at E13.5. Defects were noted in heart (reduced amount of myocardium and noncompaction), liver (small liver with reduction in hepatocyte population, and hepatocyte apoptosis), and defects in erythropoiesis (reduced hemoglobinization) and placenta (maturation block of trophoblast with vascularization defect). The placental defects, although not as pronounced as those encountered in PBP<sup>−/−</sup> placenta (22, 24),
Nevertheless, most tissues in the embryo and adult have minimal expression of PRIP, except in the heart, where it is highly expressed. PRIP has also been shown to be crucial for embryonic development, as mice with a null mutation in PRIP exhibit cardiac failure and embryonic death. PRIP interacts with the GATA family of transcription factors and thus influences erythrocyte differentiation. PRIP overexpression has been observed in breast cancer, and it has been shown that PRIP has better preference for RXR than for PPAR and RAR. These observations strongly suggest that PRIP is indispensable for embryonic development because PBP null mutation leads to embryonic death around E11.5 of mouse development. PBP null mutation also causes defects in the development of placental vasculature similar to those encountered in PPARα mutants, supporting the requirement of PBP for PPARα function in vivo. PRIP null mutants also exhibited cardiac failure because of noncompaction of the ventricular myocardium and resultant ventricular dilatation. There was also paucity of retinal pigment, excessive systemic angiogenesis, a deficiency in the number of megakaryocytes, and an arrest in erythrocyte differentiation. We showed that PBP interacts with GATA family of transcription factors and thus influences the development of vital organ systems (24). Consistent with this view is the fact that encoding PBP is amplified and overexpressed in breast cancer (39). Like PBP, PRIP is also highly amplified and overexpressed in human breast and colon tumors (11), suggesting that both PBP and PRIP by virtue of their coactivating function may augment cell proliferation and neoplastic progression. Finally, the PRIP null MEFs exhibited marked repression of XIXR-mediated transcriptional activity as compared with PPAR and RAR. These observations strongly suggest that PRIP has better preference for RXR than other nuclear receptors, and some of the abnormalities noted in PRIP null mutants may be due to inhibition of RXR function. Further studies are needed to examine the role of PRIP in various tissues by generating PRIP conditional null mice.

Addendum—Deletion of the AIB3 (ASC-2) gene has been described recently (40).