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The full-length isoform of the mouse pleckstrin homology domain-interacting protein (PHIP) is required for postnatal growth

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ABSTRACT

PHIP was isolated as an insulin receptor substrate 1 (IRS-1) interacting protein. To date, the physiological roles of PHIP remain unknown. Here we show that mice lacking PHIP1, the full-length isoform of PHIP, are born at normal size but suffer a 40% growth deficit by weaning. PHIP1 mutant mice develop hypoglycemia and have an average lifespan of 4–5 weeks. PHIP1-deficient mouse embryonic fibroblasts (MEFs) grow markedly slower than wild-type MEFs, but exhibit normal AKT phosphorylation and an increased cell proliferation in response to IGF-1 treatment. Together these results suggest that PHIP1 regulates postnatal growth in an IGF-1/AKT pathway-independent manner.

1. Introduction

Insulin and insulin-like growth factor 1 (IGF-1) are key regulators of cell metabolism, proliferation, differentiation and survival [1]. Their potent metabolic and mitogenic effects are initiated by receptor-mediated tyrosine phosphorylation of a family of four insulin receptor substrates (IRS-1 to IRS-4) [2–5]. These proteins, which differ in tissue distribution, subcellular localization and developmental expression, subsequently act as docking sites for the recruitment and activation of a complex network of intracellular Src homology 2-containing signaling molecules [6,7]. Available data from knockout mouse models indicate that IRS-1 and IRS-2, as the principal substrates for insulin and insulin-like growth factor (IGF-1) receptor tyrosine kinases, play a central role in eliciting the pleitropic effects of insulin and IGF-1 [8].

The amino terminus of the IRS proteins contains a highly conserved pleckstrin homology (PH) domain that is critical for insulin/IGF-1 receptor and IRS interactions [9,10]. PHIP (PH-interacting protein) was originally isolated as a 100-kDa (902 amino acids) protein through yeast two-hybrid screening. Biochemical studies revealed that PHIP is a cytoplasmic protein that selectively interacts with the PH domain of IRS-1 [11]. Subsequent functional analysis in human fibroblast cells showed that PHIP is required for insulin receptor-mediated mitogenic and metabolic signal transduction [12]. More recently, a novel 206-kDa (1821 a.a.) isoform of PHIP (PHIP1) containing a WD-40 domain and two bromodomains has been isolated from mouse pancreatic islets [13]. In contrast to PHIP, PHIP1 was shown to be exclusively localized in the nuclear compartment of pancreatic β cells. Based on evidence from overexpression and RNA interference studies in pancreatic β-cell lines, it has been suggested that PHIP1 is involved in control of β-cell proliferation and survival in an IGF-1-dependent and independent manner.

The physiological roles of the PHIP proteins remain unknown. By microinjection of a gene-trapped mouse embryonic stem (ES) cell clone into C57BL/6J blastocyts, we have recently generated mice carrying a gene-trapped *Phip* allele. We report here that mice homozygous for the gene-trap mutation in *Phip* (*Phip*$^{-/-}$) suffer from a severe postnatal growth deficit. The average lifespan of viable mutant mice is 4–5 weeks. These mice are slightly glucose intolerant and develop hypoglycemia. Mouse embryonic fibroblasts (MEFs) from *Phip*$^{-/-}$ mice grow significantly slower than wild-type MEFs, but remain responsive to IGF-1 mitogenic signals. Our data suggest that PHIP1 controls postnatal body growth by functioning independently of the IGF-1/AKT pathway.

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2. Materials and methods

2.1. Mice

Phip gene-trap mice were generated by micro-injection of mouse embryonic stem cell line RRJ571 (Baygenomics) into C57BL/6J blastocysts. The resulting chimeric male founders were crossed to C57BL/6J females to generate F1 heterozygous ($Phip^{+/-}$) mice. $Phip^{+/-}$ mice were then intercrossed to generate F2 homozygous $Phip^{-/-}$ mice or embryos. Genotyping of all animals or embryos was done by PCR using primers F4, R1 and R5 (Supplementary Table 1). All animal experiments were performed in accordance with the Cornell Animal Care and Use Guidelines.

2.2. Physiological studies

Plasma insulin concentrations were measured using a Rat Insulin RIA Kit (Millipore). Glucose tolerance and insulin tolerance tests (GTT and ITT) were performed essentially as described [14]. Briefly, for glucose tolerance test, mice were fasted for 6–8 h and then injected intraperitoneally (IP) with 2 g per kg body weight of D-glucose. Glucose measurements were taken at 0, 5, 15, 30, 60 and 120 min post-injection using an Ascensia Elite XL glucometer (Bayer). Blood was collected from the tail vein at each time point during the glucose tolerance test and plasma insulin levels were determined by ELISA (Crystal Chem). For insulin tolerance test, mice were fasted for 6 h and then injected IP with 0.75 U per kg body weight of regular human insulin (Eli Lilly, IN) dissolved in phosphate-buffered saline (PBS). Glucose measurements were taken at 0, 5, 15, 30, 60 and 120 min post-injection.

2.3. Morphological studies

Hindlimb skeletal muscle, liver and lung were dissected from wild-type and mutant mice at 3 weeks of age, fixed in 4% paraformaldehyde (PFA) in PBS at 4 °C overnight and then processed for paraffin embedding. 5-μm sections were mounted on glass slides and stained with hematoxylin and eosin. All images were acquired using an Axiovert 40 microscope (Zeiss) equipped with an AxioCam camera. Hepatocyte and skeletal muscle fiber sizes were measured using the AxioVision software (Version 4.1).

2.4. RNA isolation and quantitative RT-PCR

RNA was isolated using the TRIZOL RNA Isolation Kit (Invitrogen). For quantitative RT-PCR analysis, total RNA was treated with DNase I for 10 min and purified using the RNAqueous-Micro Kit (Ambion). cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen). Quantitative PCR (qPCR) was performed using Power SYBR Green PCR Master Mix on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). All qPCR primers (Supplementary Table 1) were designed using the PrimerSelect program of Lasergene 7.1 Sequence Analysis Software (DNAStar). Quantification of expression and normalization were done essentially as described [15].

2.5. Cell proliferation assays

Wild-type and $Phip^{-/-}$ MEF cells were isolated and cultured as described [16]. For IGF-1 treatment, MEF cells were plated at a density of 2.4×10^4 cells per 60-mm dish in complete medium. After 12 h of culture, the cells were serum-starved overnight and then 100 ng/ml of human IGF-1 (National Hormone and Peptide Program, UCLA) was added into the serum-free medium. Mock treatment was carried out by adding an equal volume of PBS into the culture medium. Cells were counted daily for 6 consecutive days.

[^{3}H] Thymidine incorporation assay was performed essentially as described [17]. Briefly, wild-type and $Phip^{-/-}$ MEF cells were plated in triplicates in 48-well plates at a density of 7.5×10^3 cell/well and grown in complete medium overnight. MEFs were serum-starved for 24 h and subsequently cultured in basal medium with 25, 50, 100 and 200 ng/ml of recombinant human IGF-1 for 18 h in the presence of [Methyl-^{3}H] thymidine at a final concentration of 1 μCi/well (MP Biochemicals, CA). MEFs were then washed 3 times with cold PBS. DNA was precipitated with 500 μl of cold 10% trichloroacetic acid and solubilized by the addition of 100 μl of 0.3 N NaOH. The amount of [^{3}H] thymidine incorporated into DNA was measured by liquid scintillation counting and normalized to the amount of total cellular protein.

2.6. AKT phosphorylation and Western blot analysis

$Phip^{+/+}$ and $Phip^{-/-}$ MEFs were seeded at 5×10^6 cells per 100-cm dish and cultured for 16 h in complete medium. The MEFs were serum-starved overnight and treated with 100 ng/ml IGF-1 for 0, 5, 10 and 30 min. Total proteins were extracted using cell extraction buffer containing 0.05 M Tris–HCl at pH 8.0, 0.15 M NaCl, 5.0 mM EDTA, 1% NP-40 and a protease inhibitor cocktail (MBL) at 4 °C. After centrifugation, soluble protein in the extract was quantified using bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Proteins were fractionated by SDS-10% PAGE, transferred to nitrocellulose membranes and probed with primary antibodies recognizing total AKT and AKT phosphorylated on serine 473 (Cell Signaling). Primary antibody binding was detected by using horseradish peroxidase coupled to goat anti-rabbit immunoglobulin G (IgG) or donkey anti-goat IgG (1:10,000). The antibody complex was visualized by incubation with the Lumi-Light Western blotting substrate (Roche-Mannhein, Mannhein, Germany).

2.7. Statistical analysis

Differences between compared groups were evaluated by performing two-tailed Student's *t*-test and $P < 0.05$ is considered significant.

3. Results

3.1. Generation and molecular characterization of Phip gene-trap mutant ($Phip^{-/-}$) mice

The *Phip* gene-trap mice were generated by microinjection of a mouse embryonic stem (ES) cell clone, RRJ571, into C57/B6 blastocysts. This clone contains an exon-trap cassette in intron 4 of the *Phip* gene. To identify the insertion site for the gene-trap, we carried out PCR-based chromosome walking using a series of forward and reverse primers specific to intron 4. The trap is located approximately 1516-bp downstream of exon 4 (Fig. 1A).

The gene-trap cassette in *Phip* contains a strong splicing acceptor site and a β-galactosidase-neomycin (βgeo) fusion gene. This cassette is expected to block splicing between exon 4 and 5, resulting in a fusion protein containing the 63 amino acids of the N-terminus of PHIP1 and (βgeo (Fig. 1B). To assess this, we performed semi- and quantitative RT-PCR analyses of liver RNAs from wild-type ($Phip^{+/+}$), heterozygous ($Phip^{+/-}$) and homozygous ($Phip^{-/-}$) mice using *Phip* and βgeo specific primers (Fig. 1C, arrows). As expected, a wild-type amplicon was detected in $Phip^{+/+}$and $Phip^{+/-}$ mice, but not in $Phip^{-/-}$ mice; in contrast, a Phip-βgeo fusion amplicon was detected in $Phip^{+/-}$ and $Phip^{-/-}$ mice, but not in