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Prep1 Deficiency Induces Protection from Diabetes and Increased Insulin Sensitivity through a p160-Mediated Mechanism

Francesco Oriente,1 Luis Cesar Fernandez Diaz,3 Claudia Miele,1 Salvatore Iovino,1 Silvia Mori,3 Victor Manuel Diaz,4 Giancarlo Troncone,2 Angela Cassese,1 Pietro Formisano,1 Francesco Blasi,3,4 and Francesco Beguinot1*


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We have examined glucose homeostasis in mice hypomorphic for the homeotic transcription factor gene Prep1. Prep1-hypomorphic (Prep1<sup>i</sup>) mice exhibit an absolute reduction in circulating insulin levels but normal glucose tolerance. In addition, these mice exhibit protection from streptozotocin-induced diabetes and enhanced insulin sensitivity with improved glucose uptake and insulin-dependent glucose disposal by skeletal muscle. This muscle phenotype does not depend on reduced expression of the known Prep1 transcription partner, Pbx1. Instead, in Prep1<sup>i</sup> muscle, we find normal Pbx1 but reduced levels of the recently identified novel Prep1 interactor p160. Consistent with this reduction, we find a muscle-selective increase in mRNA and protein levels of PGC-1α, accompanied by enhanced expression of the GLUT4 transporter, responsible for insulin-stimulated glucose uptake in muscle. Indeed, using L6 skeletal muscle cells, we induced the opposite effects by overexpressing Prep1 or p160, but not Pbx1. In vivo skeletal muscle delivery of p160 cDNA in Prep1<sup>i</sup> mice also reverses the molecular phenotype. Finally, we show that Prep1 controls the stability of the p160 protein. We conclude that Prep1 controls insulin sensitivity through the p160-GLUT4 pathway.

Prep1 is an homeodomain transcription factor belonging to the MEINOX subfamily of the TALE (three amino acid loop extension) proteins (32). Prep1 forms DNA-independent dimeric complexes with all isoforms of the Pbx homeodomain transcription factor, enhancing target specificity and regulatory function (4, 6, 24, 36, 39).

Prep1 is an important gene in development: its downregulation in zebrafish and its deletion in mice induces embryonic lethality (9, L. C. Fernandez-Diaz and F. Blasi, unpublished data). Hypomorphic mice expressing about 2% of Prep1 mRNA (Prep1<sup>i</sup>) have a leaky embryo-lethal phenotype with major anomalies in hematopoiesis, oculogenesis, and angiogenesis (15). About 25% of the homozygous Prep1<sup>i</sup> embryos survive, and the mice are born and live a normal-length life. However, they show defects in T-cell development (38).

In all these systems, the reduction or the absence of Prep1 is accompanied by a reduction of its Pbx partners (9, 15, 38). The reduction in the mouse appears to be cell type and isoform specific (15). Direct studies have shown that the reduction of Pbx expression is due largely to a posttranscriptional mechanism (15, 38), in particular to a decrease in the protein half-life. In fact, dimerization with Prep1 protects Pbx from proteosomal degradation (26).

Previous studies also demonstrated that the generation of Pbx-Prep heterodimers is necessary to enable nuclear localization of Prep1 and to prevent nuclear export of Pbx (5, 20). Indeed, the balance of Prep and Pbx has been shown to be functionally important both during embryogenesis and in adult life (9, 15, 26, 46).

We recently discovered that p160 Myb-binding protein (p160) (44) is a new direct Prep1-interacting protein that competes with Pbx1 for Prep1 binding (11). Thus, Prep1 functions may depend on not only its interaction with Pbx but also that with p160. The role of the Prep1-p160 interaction, however, is still unknown. Interestingly, p160 is a repressor of the regulator of glucose and energy metabolism, PPAR-gamma coactivator-1α (PGC-1α) (13).

The importance of Pbx and Hox protein interaction in glucose homeostasis, pancreatic cell proliferation, and pancreas development has been studied. Transgenic mice expressing Pbx interaction-defective Pdx1 genes were used to investigate the requirements for Pdx1-Pbx complexes in pancreatic morphogenesis, islet cell differentiation, and glucose homeostasis (12). In these studies, Pdx1-Pbx complexes were dispensable for glucose homeostasis and differentiation of stem cells into ductal, endocrine, and acinar lineages; however, they were essential for expansion of these cell populations during development. Further studies of transheterozygous Pbx1<sup>+/−</sup> Pdx1<sup>+/−</sup> mice revealed in vivo genetic interactions between Pbx1 and Pdx1 that are essential for postnatal pancreatic function (22). Indeed, these mice developed age-dependent overt diabetes mellitus, while Pbx1<sup>+/−</sup> and Pdx1<sup>+/−</sup> mice showed only reduced glucose tolerance. Mutations of Pdx1 protein cause diabetes in both mice and humans (1, 18, 27, 43), suggesting that Pbx1 perturbation may also determine susceptibility to diabetes.

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tes. Whether the perturbation of other MEINOX transcription factors, like Prep1, affects the development and function of the endocrine pancreas and glucose tolerance is presently unknown.

It is not known whether Prep1 has any role in glucose and energy metabolism. Because of the ability of Prep1 to interact with both Pbx1 and p160, we have studied glucose homeostasis in Prep1I−/− mice. We show that adult Prep1−/− animals exhibit enhanced sensitivity to insulin action and are protected from developing streptozotocin-induced diabetes. The increased sensitivity to insulin in these mice is due, at least in part, to a novel Pbx1-independent and p160-dependent mechanism. We demonstrate, for the first time, a role for Prep1 in glucose homeostasis mediated by the newly identified interactor p160.

MATERIALS AND METHODS

Materials. Media, sera, antibodies for cell culture, and the Lipofectamine reagent were from Invitrogen (Grand Island, NY). The anti-Prep1 polyclonal antibody and pBOS-Prep1 vector were described previously (3). pSG5-Pbx1, PSG5-Prep1hyp, and pRUFneo-p160 vectors were described previously (11). PGC-1α and GLUT4 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The p160 antibody was from Zymed Laboratories (San Francisco, CA). Protein electrophoresis reagents were purchased from Bio-Rad (Richmond, VA), and Western blotting and enhanced chemiluminescence (ECL) reagents were from Amersham Biosciences (Arlington Heights, IL). All other chemicals were from Sigma (St. Louis, MO).

Generation and characterization of Prep1-hypomorphic mice. Prep1 targeted mice were generated by gene trapping by Lexikon Genetics, Inc. (The Woodlands, TX) and have been described (15, 38). In the experiments reported in this paper, heterozygous mice were backcrossed with wild-type C57BL/6 mice for four generations. All animal handling conformed to the regulations of the Ethics Committee on Animal Use of H. S. Raffaele (IACUC permission number 207). Southern blot analysis of EcoRI-digested total DNA from tail biopsy specimens or yolk sacs was accomplished through a 132-bp double-stranded Prep1 cDNA probe prepared from full-length Prep-1 cDNA with the forward primer 5′-ATG ATGCCAGACAGACGCTAAGTATA-3′ and the reverse primer 5′-GGGG TCTGAGACTCGATGGGAGGACTC-3′. The PCR genotyping strategy employed the oligonucleotides Prep-R1 and LTR2 (sequences provided below), which amplify a 230-bp fragment in the disrupted allele, while the Prep-F1–Prep-R1 pair amplifies a 300-bp fragment of the wild-type allele. Sequences of oligonucleotides are as follows: Prep-F1, 5′-CCAGGGCAATGAAGAC CGTCTGGA-3′; Prep-R1 5′-GGGGATGCTCAACATTTAAGAAGAC CGTCTGGA-3′; LTR2, 5′-CAAATGCTGCTTACTAAGCTAGCTTGCC-3′.

Pancreatic islet sections were stained with hematoxylin and eosin. For morphometry, pancreases were obtained from 3-month-old control and hypomorphic mice fasted overnight and then injected with glucose (2 g/kg body weight) intraperitoneally. Venous blood was subsequently collected by tail clipping at 0, 15, 30, 45, 60, 90, and 120 min after injection to determine blood glucose levels. To assess ex vivo insulin secretion, islets were isolated from 6-month-old mice by collagenase digestion and subsequent centrifugation on a Histopaque (Sigma-Aldrich) gradient (23). A total of 20 islets were manually selected and preincubated in Dulbecco’s modified Eagle’s medium (Life Technologies) at 37°C in a 5% CO₂ atmosphere for 24 h. Islets were further incubated in Krebs-Ringer buffer (120 mmol/liter NaCl, 1.2 mmol/liter MgSO₄, 5 mmol/liter KCl, 10 mmol/liter NaHCO₃, 1.3 mmol/liter CaCl₂, and 1.2 mmol/liter KH₂PO₄) for 30 min and then stimulated at 37°C with various concentrations of glucose for 1 h. Islets were subsequently collected by centrifugation, and supernatants were assayed for insulin content by RIA.

For low-dose streptozotocin treatment, mice fasted for 6 h prior to intraperitoneal (i.p.) injection with streptozotocin (40 mg/kg body weight) dissolved in sterile citrate buffer or with the vehicle, citrate buffer (0.05 M sodium citrate, pH 4.5). Streptozotocin was administered for five consecutive days, and glycemia and body weight were measured during the next 4 weeks. Some mice were sacrificed during the streptozotocin treatment to evaluate the morphological alteration of the pancreas.

In vivo glucose utilization. An intravenous flash injection of 1 μCi of the nonmetabolizable glucose analog 2-[1-¹³C]deoxy-d-glucose (2-DG) (Amersham Pharmacia Biotech) and an i.p. injection of insulin (0.75 mU·g of body weight⁻¹) were administered to random-fed mice. The specific blood 2-DG clearance was determined with 25-µl blood samples (tail vein) obtained 1, 10, 20, and 30 min after injection using the Somogyi procedure (42). Tibialis skeletal muscle tissue samples were removed 30 min after injection. The glucose utilization index was determined by measuring the accumulation of radiolabeled compounds (14). The amount of 2-DG–6-phosphate per milligram of protein was divided by the integral of the concentration ratio of 2-DG to unlabeled glucose measured. Glucose utilization is expressed as picomoles per milligram of protein per minute.

p160 gene delivery in skeletal muscle of Prep1-hypomorphic mice. We adopted an established technique (19, 40) to inject mouse tibialis muscles with 100 μg of pRUFneo-p160 naked DNA (encoding a Flag-tagged p160 cDNA), using 1-ml syringes and 27-gauge needles. After 48 h, mice were sacrificed and muscles were processed for RNA and/or protein extraction as described in the appropriate section.

FIG. 1. Insulin and glucagon levels in Prep1-hypomorphic mice. For determination of circulating insulin (A) and glucagon (B) levels, control and hypomorphic mice fasted overnight and were then injected with glucose (3 g/kg body wt) intraperitoneally. Plasma insulin concentrations were quantitated at the indicated times by radioimmunoassay. Glucagon levels were assessed in the fasting state. Data are means plus standard deviations for 12 mice/group. Asterisks indicate statistically significant differences (∗, P < 0.05; **, P < 0.01).
Cell culture procedures and transfection. L6 skeletal muscle cells were cultured at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 2% l-glutamine, 10,000 U/ml penicillin, and 10,000 μg/ml streptomycin. Transient transfection of Prep1, Prep1HR, P160, and Pbx1 plasmids or small interfering RNA oligonucleotides was performed by the Lipofectamine method according to the manufacturer’s instructions. For these studies, 60 to 80% confluent cells were washed twice with Optimem (Invitrogen) and incubated for 8 h with 5 μg of plasmid construct or with 12 μg of oligonucleotides and 45 to 60 μl of Lipofectamine reagent. The medium was then replaced with DMEM with 10% fetal calf serum, and cells were further incubated for 15 h before being assayed.

2-DG uptake by the L6 cells was measured as previously reported (7). Briefly, confluent cells were incubated in DMEM supplemented with 0.25% albumin for 20 min at 37°C. The medium was aspirated, and cells were further incubated for 30 min in glucose-free HEPES buffer (5 mmol/liter KCl, 120 mmol/liter NaCl, 1.2 mmol/liter MgSO₄, 10 mmol/liter NaHCO₃, 1.2 mmol/liter KHPO₄, and 20 mmol/liter HEPES [pH 7.8], 2% albumin). The cells were incubated with 100 nmol/liter insulin and incubated for 18 h at 37°C. The medium was aspirated, and cells were further incubated for 15 h before being assayed.

2-DG uptake by the L6 cells was measured as previously reported (7). Briefly, confluent cells were incubated in DMEM supplemented with 0.25% albumin for 18 h at 37°C. The medium was aspirated, and cells were further incubated for 30 min in glucose-free HEPES buffer (5 mmol/liter KCl, 120 mmol/liter NaCl, 1.2 mmol/liter MgSO₄, 10 mmol/liter NaHCO₃, 1.2 mmol/liter KHPO₄, and 20 mmol/liter HEPES [pH 7.8], 2% albumin). The cells were incubated with 100 nmol/liter insulin for 30 min, supplemented during the final 10 min with 0.2 mmol/liter [14C]2-DG. Cells were then solubilized, and 2-DG uptake was quantitated by liquid scintillation counting.

Western blot analysis and immunoprecipitation procedures. Tissue samples were homogenized in a Polytron (Brinkman Instruments) in 20 ml T-PER reagent per gram of tissue according to manufacture (Pierce). After centrifugation at 10,000 rpm for 5 min, supernatant was collected. Cells were solubilized in lysis buffer (50 mmol/liter HEPES [pH 7.5], 150 mmol/liter NaCl, 10 mmol/liter EDTA, 10 mmol/liter Na₂P₂O₇, 2 mmol/liter Na₃VO₄, 100 mmol/liter NaF, 10% glycerol, 1% Triton X-100, 1 mmol/liter phenylmethylsulfonyl fluoride, 10 mg/ml aprotonin) for 1 h at 4°C, and lysates were centrifuged at 5,000 × g for 20 min. Total or immunoprecipitated homogenates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.45-μm Immobilon-P membranes. Upon incubation with primary and secondary antibodies, immunoreactive bands were detected by ECL according to the manufacturer’s instructions (30).

Real-time reverse transcription (RT)-PCR analysis. Total cellular RNA was isolated from tibialis muscle and L6 cells by using an RNaseasy kit (Qiagen Sciences), according to manufacturer’s instructions. One microgram of tissue or cell RNA was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen). PCR products were analyzed using Sybr green mix (Invitrogen). Reactions were performed using Platinum Sybr green qPCR Super-UDG using an iCycler IQ multi-color real-time PCR detection system (Bio-Rad). All reactions were performed in triplicate, and β-actin was used as an internal standard. Primer sequences used were as follows: GLUT4 R, 5′-AATGATGCGCAATGAGAAAGG-3′; GLUT4 F, 5′-CAAAATTTTGGAGCATCGAG-3′; Prep1-F, 5′-GAACCTGTGCTTCGCTGCAAG-3′; Prep1-R, 5′-AGCAAGGACTGACTACAG-3′; P160 F, 5′-AGCACAAAGAATGACTACAG-3′; P160 R, 5′-GCCATGCTGTTGACATCCTCTC-3′; PGO-1α F, 5′-AGGGTCATCGTTTGTGGTCAG-3′; PGO-1α R, 5′-GCCGCTTACCCGAACGCTAG-3′; Pbx1 R, 5′-CTATGACTCTGCCGTCCGTTCCGTG-3′; Pbx1 R, 5′-GATGCCCTGCGGACTGTACATCTGACTG-3′; β-actin F, 5′-GCGTGACATCAAAGAAGAG-3′; and β-actin R, 5′-ACTGTTCTGGCATAGAGAAG-3′.

Statistical procedures. Data were analyzed with Statview software (Abacus Concepts) by one-factor analysis of variance. P values of less than 0.05 were considered statistically significant. The total area under the curve for glucose response during the insulin tolerance test was calculated by the trapezoidal method (45).

RESULTS

Prep1-hypomorphic mice are protected from streptozotocin-induced hyperglycemia. To address the role of Prep1 in glucose metabolism, we compared wild-type, Prep1H/H, and Prep1i/i mice. Six groups (22/group) of male and female Prep1-hypomorphic and control mice were analyzed at 3 and 12 months of age. Following determinations of basal glycemas, mice were injected intraperitoneally with glucose (2 mg·kg⁻¹), followed by further blood glucose level determinations at the indicated times as described in Materials and Methods. Data are means ± standard deviations for each group of animals. Areas under the curves showed no significant difference between groups.

FIG. 2. Glucose tolerance tests in Prep1-hypomorphic mice. Six groups (22/group) of male and female Prep1-hypomorphic and control mice were analyzed at 3 and 12 months of age. Following determinations of basal glycemas, mice were injected intraperitoneally with glucose (2 mg·kg⁻¹), followed by further blood glucose level determinations at the indicated times as described in Materials and Methods. Data are means ± standard deviations for each group of animals. Areas under the curves showed no significant difference between groups.

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RESULTS

Prep1-hypomorphic mice are protected from streptozotocin-induced hyperglycemia. To address the role of Prep1 in glucose metabolism, we compared wild-type, Prep1H/H, and Prep1i/i lit-
termate mice. We observed a gene dosage-dependent reduction in plasma insulin levels both under fasting conditions and upon glucose loading (Fig. 1A). As with insulin, circulating glucagon levels were also depressed in the Prep1-hypomorphic mice (Fig. 1B).

In spite of the absolute reduction in the insulin levels, i.p. glucose loading indicated preserved glucose tolerance in both Prep1+− and Prep1i−/i mice, with no gender-related difference (Fig. 2). In addition, glucose tolerance remained normal in 12-month-old hypomorphic mice, suggesting protection of these animals from developing glucose tolerance abnormalities. To analyze this hypothesis further, we targeted β-cell function in the Prep1-hypomorphic mice with low-dosage (40 mg/kg) streptozotocin administration. As shown in Fig. 3A, this treatment reduced fasting plasma insulin levels by almost 40% in both Prep1+/− and wild-type littermates. By day 12 of streptozotocin treatment, the wild-type mice developed overt hyperglycemia, accompanied by a 12% reduction in body weight (P < 0.05; Fig. 3B and C). In contrast, the Prep1+/− mice showed twofold-smaller changes in plasma glucose levels with no significant weight loss. These data show that, despite their absolute hypoinsulinemia, Prep1+/− mice are protected from streptozotocin-induced diabetes. Similar experiments could not be performed on homozygous Prep1+/+ mice because of their frailness.

Prep1 gene hypomorphism causes enhanced insulin sensitivity in glucose disposal. TUNEL (terminal deoxynucleotidyl-
transferase-mediated dUTP-biotin nick end labeling) assays revealed no difference in streptozotocin-induced β-cell apoptosis in hypomorphic and control mice (data not shown). We hypothesized therefore that the protection from development of hyperglycemia observed in the Prep1-hypomorphic mice could result from improved insulin sensitivity of glucose disposal. Indeed, upon i.p. insulin injection, the Prep1 and Pbx1 heterozygous and Prep1 homozygous mice developed more sustained and prolonged hypoglycemia than control mice (glucose area-under-the-curve difference between hypomorphic and control mice was significant at the P < 0.001 level), with a Prep1 gene dosage effect (Fig. 4A). Importantly, in vivo insulin-mediated 2-DG uptake by the skeletal muscle was almost 2.5- and 3.5-fold improved, respectively, in the Prep1 and in the Prep1 mice (Fig. 4B). These results suggested a molecular mechanism operating in skeletal muscle and compensating for the absolute hypoinsulinemia caused by Prep1 deficiency.

Skeletal muscle from Prep1-hypomorphic mice shows altered expression of GLUT4, PGC-1α, and p160. To address the mechanisms responsible for increased insulin-mediated glucose disposal in Prep1-hypomorphic mice, we investigated the expression of several proteins playing an important role in insulin sensitivity and glucose disposal. Protein levels measured by immunoblotting of insulin receptor (IR), IR substrate 1 (IRS-1), IRS-2, Akt, PKB, PKC-δ, ERK1/2, and Pbx1 were not significantly different in Prep1/+, Prep1−/−, and control mice (Fig. 5A). Interestingly, however, GLUT4 expression was increased by 75 and 25%, respectively, in skeletal muscle of Prep1/+, Prep1−/−, and control mice (Fig. 5A). Similar to GLUT4, the mRNA and protein expression of PGC-1α (29) was also upregulated in the muscle of Prep1-hypomorphic mice. The protein (though not the mRNA) levels of the PGC-1α repressor and Prep1-binding Pbx competitor p160 were reduced by 30 and 50%, respectively, in the Prep1/− and the Prep1 mice (Fig. 5A and B). At variance from muscle, liver expression of p160 as well as of PGC-1 exhibited no difference in Prep1-hypomorphic and in control mice, indicating tissue selectivity in Prep1 action (data not shown).

P160 has been shown to directly interact with Prep1 in various cell lines (11). We also obtained evidence of Prep1-p160 interaction in tibialis muscle extracts of wild-type mice by immunoprecipitating with anti-Prep1 and blotting with p160 antibodies (Fig. 5C). Indeed, reduced coimmunoprecipitation was noted in the Prep1/− extracts, with no interaction in the Prep1/− extracts. These findings raised the possibility that, in the skeletal muscle, Prep1 downregulation results in activation of the glucose transport machinery by decreasing p160 levels. p160 mediates Prep1-dependent GLUT4 downregulation in L6 skeletal muscle cells. We analyzed this hypothesis further by transiently transfecting Prep1 cDNA in differentiating L6 skeletal muscle cells, leading to a ninefold overexpression of Prep1 in these cells (Fig. 6A). Consistent with the findings in muscles of hypomorphic mice, overexpression of the wild-type Prep1 cDNA determined a >70% inhibition of insulin-stimulated 2-DG uptake by these cells (Fig. 6B) and a similar decline in the expression of GLUT4 protein and mRNA (Fig. 6A and C). These effects were accompanied by a threefold decline in PGC-1α protein and mRNA levels (Fig. 6A and D) and opposite changes in the levels of p160 protein (though p160 mRNA remained unchanged) (Fig. 6A and E).

To test for the specificity of the Prep1 effect, we transfected the Prep1HR1 mutant, in which two leucines of the HR1 region of Prep1 are mutated to alanine, which prevents binding to both Pbx1 and p160 (11). Transfection with the Prep1HR1 mutant had no effect either on GLUT4, PGC-1α, p160, and Pbx1 protein levels (Fig. 6A) or on 2-DG uptake (Fig. 6B). However, transfection of p160 cDNA blocked GLUT4 and PGC-1α expression to an extent comparable to that caused by Prep1 (Fig. 6A) and decreased 2-DG uptake (Fig. 6B). Pbx1 levels were low in the L6 cells and did not change upon transfection with Prep1 cDNA but were further decreased by p160 (Fig. 6A). Overexpression of Pbx1, on the other hand, caused a twofold increase in PGC-1α and GLUT4 levels (Fig. 6A), a similar decrease of p160 (Fig. 6A), and an increase in 2-DG uptake (Fig. 6B).

Delivery of p160 in skeletal muscle reverts the Prep1−/− phenotype. Next, we injected 100 μg of p160 cDNA into the tibialis muscles of wild-type, heterozygous, and homozygous litter-mate mice. Consistent with the above findings, ectopic expression of p160 in tibialis muscles of Prep1−/− and Prep1−/− mice by naked DNA delivery markedly reduced PGC-1α and GLUT4 levels, thereby rescuing the molecular phenotype (Fig. 7). The introduction of p160 cDNA into the muscles of wild-type mice also led to a reduction of PGC-1α and GLUT4. The data are therefore consistent with the view that Prep1 downregulates PGC-1α and GLUT4, and thereby the insulin-dependent glucose transport machinery, through p160 and not Pbx1.

Prep1 regulates proteasomal degradation of p160. Prep1 controls the half-life of Pbx at the protein stability level (15, 26). To test whether Prep1 can likewise regulate the stability of p160, we exposed L6 myotubes to the protein synthesis inhibitor cycloheximide and found that this treatment reduced p160 cellular levels in a time-dependent manner (Fig. 8A). Interestingly, the degradation of p160 in the presence of cycloheximide was abolished in the Prep1-overexpressing cells, implying that,

FIG. 5. Expression profile of skeletal muscle from Prep1-hypomorphic mice. (A) Tibialis muscles from Prep1-hypomorphic and control mice were dissected, solubilized, and subjected to Western blotting with IR, IRS1, IRS2, Akt, PKCζ, ERK1/2, GLUT4, PGC-1α, p160, and Pbx1 antibodies. Blots were revealed by ECL and autoradiography, and bands were quantitated by laser densitometry and normalized for actin. Data are means plus standard deviations of duplicate determinations for 12 mice/group. (B) The abundance of RNAs for the indicated proteins was determined by real-time RT-PCR analysis of total RNA isolated from tibialis muscles of the hypomorphic and control mice, using beta-actin as an internal standard. Data are means plus standard deviations for four independent experiments in each of which reactions were performed in triplicate using the pooled total RNAs obtained from six mice/genotype. (C) Protein lysates (250 μg) from tibialis muscles of wild-type and Prep1-hypomorphic mice were precipitated with Prep1 antibody or with nonimmune mouse immunoglobulin G (mlG) followed by blotting with p160 antibody, ECL, and autoradiography. The same lysates were directly blotted with antiactin antibodies for normalization. The autoradiograph shown is representative of four independent experiments.
FIG. 6. Effect of Prep1 overexpression in L6 skeletal muscle cells. (A) L6 myotubes were transiently transfected with the Prep1, Prep1_{HR} mutant, p160, and Pbx1 cDNAs or with the empty vector (CTRL). Cells were solubilized, and lysates were analyzed by SDS-PAGE and subjected to Western blotting with GLUT4, PGC-1α, p160, and Pbx1 antibodies. As a control, filters were rebotted with actin antibodies. Bands were revealed by ECL and autoradiography. The autoradiograph shown is representative of five independent experiments. (B) L6 myotubes transfected with the Prep1, Prep1_{HR} mutant, p160, and Pbx1 cDNAs or with the empty vector (CTRL) were exposed to 100 nM insulin, and 2-DG uptake was assayed as described in Materials and Methods. Data are means plus standard deviations from four independent experiments, each in duplicate. (C, D, and E) Levels of mRNAs encoding GLUT4, PGC-1α, and p160 in cells transfected with the Prep1, Prep1_{HR} mutant, p160, and Pbx1 cDNAs were quantitated by real-time RT-PCR analysis, using beta-actin as an internal standard. Data are means plus standard deviations from four independent experiments.
at least in part, upregulation of p160 by Prep1 (Fig. 6) involves posttranslational mechanisms. To better define these events, we also incubated L6 cells with the proteasome inhibitor MG132. As shown in Fig. 8B, exposure to this agent for 6 h increased p160 intracellular levels by 10-fold. In the Prep1 overexpressing cells, the proteasome inhibitor induced a further 2.5-fold accumulation of the protein. Also, in these same cells, p160-Prep1 coprecipitation was 3-fold more effective than in cells expressing only their physiological complement of Prep1 (Fig. 8C). These data indicate that Prep1 interaction enhances p160 protein stability and explain the mechanism of p160 decrease in Prep1/+/ mice. Thus, we conclude that the Prep1 function on glucose disposal depends on its interaction with p160. This represents the first identified Pbx1-independent function of Prep1.

Effect of Prep1 deficiency on islet development and islet function. As the Prep1 partner Pbx1 is required for pancreas development and adult function (22), and in view of the lower insulin and glucagon plasma level of Prep1/+/ mice, we also tested whether Prep1 deficiency has an effect on endocrine pancreas function. We first compared islet morphology in adult (3-month-old) Prep1/+/, Prep1/−/−, and wild-type littermates. In these animals, pancreas weight correlated with the genotype, being lowest in the homozygous Prep1/−/− mice (Table 1). However, the percentage of the area occupied by islets, including both a and b cells, was normal. Also, whole-islet and b-cell masses were decreased in the Prep1/−/− mice. The heterozygous Prep1/+/ mice displayed an intermediate phenotype (Table 1). This phenotype could be assessed by histological and immunohistochemical examination of pancreatic sections (Fig. 9) showing smaller islets, with normal spatial distribution of a and b cells. The smaller size of the islets is consistent with the reduced plasma levels of insulin and glucagon (see Fig. 1).

Since Prep1 stabilizes Pbx1 (15) and since Pbx1 is important in pancreas development and glucose tolerance (22), we measured
Pbx-1 levels in pancreatic tissue by immunoblotting. As shown in Fig. 10A, Pbx1 levels were severely reduced in extracts of Prep1-hypomorphic pancreas. Again, the extent of this reduction correlated with the genotype. Other Pbx isoforms as well as p160 show a reduction similar to that of Pbx1 (data not shown). Importantly, however, islets isolated from the Prep1-hypomorphic and control littermates mice exhibited comparable insulin secretion responses when exposed to 16.7 mM glucose-containing culture medium.

**TABLE 1. Morphometric analysis of Prep1 hypomorphic pancreas**

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Islet area/pancreatic area</th>
<th>% in islet</th>
<th>Wt or mass (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Beta cells</td>
<td>Alpha cells</td>
</tr>
<tr>
<td>Control</td>
<td>0.54 ± 0.07</td>
<td>77.21 ± 4.52</td>
<td>17.15 ± 2.10</td>
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<tr>
<td>Prep1+/−</td>
<td>0.50 ± 0.04</td>
<td>76.54 ± 3.11</td>
<td>15.75 ± 2.25</td>
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<tr>
<td>Prep1−/−</td>
<td>0.48 ± 0.04*</td>
<td>77.32 ± 2.12</td>
<td>16.33 ± 3.24</td>
</tr>
</tbody>
</table>

*Mice were analyzed as described in Materials and Methods. Data are means ± standard deviations for eight Prep1+/− and an equal number of Prep1−/− and wild-type (control) mice. Asterisks indicate statistically significant differences (*, P < 0.05; **, P < 0.01).
We have shown that Prep1-hypomorphic mice have a complex phenotype with at least two metabolically relevant features. One is increased insulin sensitivity in skeletal muscle accompanied by protection from streptozotocin-induced diabetes. The second is pancreatic islet hypoplasia accompanied by absolute hypoinsulinemia.

So far, the Pbx proteins have been considered the unique partners of Prep1 (8, 4, 21, 33, 34, 35). However, it was recently shown that p160 can compete with Pbx1 for binding to Prep1 (11), although the functional in vivo relevance of this interaction is unknown.

Pbx1 has a major role in pancreas development, as Pbx1/H11001/H11002 embryos feature pancreatic hypoplasia as well as defective endocrine and exocrine cell differentiation prior to death at embryonic day 15 to 16 (22, 41). An important role of Pbx1 in pancreatic adult function is also shown by the reduced glucose tolerance phenotype of heterozygous Pbx1/H11001/H11002 mice and by the overt diabetic phenotype of the compound Pbx1/H11001/H11002/Pdx1/H11001/H11002 mice (22). In the present paper, however, we show that the increased insulin sensitivity occurring in the skeletal muscle of Prep1i/H11001 and Prep1i/H11002 mice does not depend on Pbx1. First, Pbx1 mRNA and protein levels were unchanged in skeletal muscle (unlike other organs) (Fig. 5A and B and Fig. 10). Second, studies with L6 cells show that overexpression of Prep1 and Pbx1 have opposite effects. Prep1 reduces the level of PGC-1α and GLUT4 in these cells (Fig. 6). Overexpression of Pbx1, on the other hand, boosts the levels of PGC-1α and GLUT4. Thus, Pbx1 and Prep1 have opposite effects on insulin-dependent glucose disposal by muscle.

At variance with Pbx1, p160 is key to Prep1 action on glucose disposal, consistent with the known function of p160 in regulating PGC-1α and energy metabolism in skeletal muscles.
Indeed, we show that Prep1 increases the levels of p160 in muscle cells (Fig. 6). Overexpression of p160 in the L6 cells and direct delivery of a p160 cDNA in the muscle of Prep1+/+ and Prep1+/ mice mimic the effect of Prep1, decreasing PGC-1α and GLUT4 expression. No change of PGC-1α mRNA or protein was detected in the liver of the Prep1-hypomorphic mice (data not shown), suggesting that the interplays between these molecules are cell type selective. Together, these data are in agreement with several published papers reporting a positive correlation between PGC-1α and GLUT4 in rodent (29) and human (2) muscle cells and in humans in vivo (37) and may account for the enhanced insulin sensitivity in muscles of Prep1-hypomorphic mice (Fig. 6). Previous work by Miura et al. demonstrated GLUT4 downregulation in transgenic mice overexpressing PGC-1α (31). However, as suggested by those authors, a reduction in the lower-molecular-weight PGC-1α form might have caused the negative correlation between PGC-1α and GLUT4 levels in this model.

Cy cloheximide experiments demonstrated that the regulation of the p160 expression by Prep1 was, at least in part, posttranslational. Furthermore, the stabilizing effect of MG132 shows that p160 degradation involves the proteasome. In the presence of excess Prep1, the proteasome inhibitor had a much smaller effect (Fig. 8), indicating that Prep1 interaction stabilizes p160 and induces p160 escape from proteasomal degradation.

The present results highlight a novel regulatory mechanism of insulin sensitivity occurring in the skeletal muscle upstream of the p160/PGC-1α complex and operated by the balance of p160 and Pbx1. When p160 exceeds Pbx1, it binds Prep1, is stabilized, and represses GLUT4 and insulin sensitivity. When Pbx1 is present in excess, the reverse occurs. These regulatory mechanisms must be more complex, however, since one would otherwise expect that a decrease in Pbx1 would directly lead to decreased insulin sensitivity, which is not the case. Indeed, Prep1+/−/ mice display a slightly improved insulin sensitivity accompanied by early impairment of glucose tolerance (22). These mice differ from both the Prep1−/− and the Prep1+/+ mice, which show a significant enhancement in insulin sensitivity and exhibit no tendency to develop abnormal glucose tolerance. Furthermore, Prep1-hypomorphic mice are protected from streptozotocin-induced diabetes in spite of their decreased β-cell mass and circulating insulin levels. Based on TUNEL assays, this protection was independent of differences in streptozotocin-induced β-cell apoptosis between the hypomorphic and control mice (data not shown). The increase in the sensitivity to insulin action on glucose disposal and on glucose uptake by the skeletal muscle, together with the reduced glu- cagon levels, may contribute to the stability of glucose tolerance in the Prep1-hypomorphic mice.

Another important aspect of our work is that Prep1 and Pbx1 have distinct roles in pancreas function in the adult. Indeed, Prep1 binds and stabilizes Pbx proteins in a cell-specific manner (15, 26, 38, 46). Consistently, we show that the level of Pbx1 in Prep1+/+ hypomorphic mice is decreased in the adult pancreas (Fig. 10A), though not in the skeletal muscle (Fig. 6). Therefore, the Prep1+/+ animals were expected to display a pancreatic phenotype similar to that of Pbx1−/− mice, i.e., disrupted architecture of the pancreatic islets and hypoin- sulinemia (22). Surprisingly, Prep1-hypomorphic mice were found to have normal islet architecture. However, they did show islet hypoplasia, accompanied by significantly reduced absolute insulin levels (both basal and postloading). Why the pancreatic phenotype of the Prep1+/+ mice, which includes a strong reduction of Pbx1 pancreas expression (Fig. 10A), is different from that of the Pbx1−/− heterozygous mice (22) is not completely clear. The deficiency of Prep1 might have been complemented by the overexpression of other family members. This is unlikely, however, as no increase of Prep2 or Meis proteins is observed in Prep1+/+ embryos (15). More simply, the reduction of Pbx1 in Prep1-hypomorphic mice may not be enough to generate the phenotype of the Pbx1−/− mouse. It is also possible that other TALE family members (e.g., one of the Meis genes) cooperate with Pbx1 in pancreas development and in functions where Prep1 is not involved. Finally, it is possible that the islet phenotype of the Prep1-hypomorphic mice is established as an adaptive response to their enhanced insulin sensitivity. Indeed, islets isolated from Prep1+/+ mice exhibit a normal secretory response to glucose in culture (Fig. 10B). A decreased β-cell mass in conditions of chronically improved insulin sensitivity has been previously observed in mice. For instance, pancreatic β-cell area is decreased in protein tyrosine phosphatase 1b-deficient mice owing to increased peripheral insulin sensitivity and reduced insulin requirement (25).

The present results may have clinical relevance. In individuals with normal glucose tolerance, insulin sensitivity and secretion span a wide range and are determined by a number of different genes (16, 28). Variability in some of these genes is known to provide an important contribution to major human disorders, including type 2 diabetes (16, 17). For example, Pdx1 point mutations cause impaired insulin secretion and a rare form of non-insulin-dependent diabetes formerly termed MODY4 (43). On the basis of the results of this study, Prep1 might also be involved in the pathogenesis of these diseases. Whether genetic variability at the Prep1 locus affects insulin sensitivity in humans is an important issue presently under investigation in the laboratory.

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