OPEN ACCESS

University of Dundee

University of Dundee

Evaluation of approaches to generation of tissue-specific knock-in mice

Bayascas, Jose R.; Sakamoto, Kei; Armit, Laura; Arthur, J. Simon C.; Alessi, Dario R.

Published in:
Journal of Biological Chemistry

DOI:
10.1074/jbc.M606789200

Publication date:
2006

Licence:
CC BY

Document Version
Publisher's PDF, also known as Version of record

Link to publication in Discovery Research Portal

Citation for published version (APA):
Bayascas, J. R., Sakamoto, K., Armit, L., Arthur, J. S. C., & Alessi, D. R. (2006). Evaluation of approaches to generation of tissue-specific knock-in mice. *Journal of Biological Chemistry*, *281*(39), 28772-28781. https://doi.org/10.1074/jbc.M606789200

General rights
Copyright and moral rights for the publications made accessible in Discovery Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from Discovery Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain.
- You may freely distribute the URL identifying the publication in the public portal.

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 17. Dec. 2022

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 39, pp. 28772–28781, September 29, 2006
© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

Evaluation of Approaches to Generation of Tissue-specific Knock-in Mice*[S]

Received for publication, July 17, 2006 Published, JBC Papers in Press, August 3, 2006, DOI 10.1074/jbc.M606789200

Jose R. Bayascas[‡1], Kei Sakamoto[‡], Laura Armit[§], J. Simon C. Arthur[‡], and Dario R. Alessi[‡]

From the [‡]MRC Protein Phosphorylation Unit and [§]School of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom

We explored three approaches to create tissue-specific knock-in mice by generating knock-in mice in which a substrate-docking site of the PDK1 protein kinase was ablated in Cre-expressing tissues in a way that prevented activation of one of its substrates, p70 ribosomal S6 kinase (S6K), but not another (protein kinase B (PKB)). Employing two of the approaches, termed the "heterozygous" and "minigene" methods, we generated mice in which Cre-expressing skeletal and cardiac muscle produced the mutant rather than wild type PDK1. Consistent with this, injection of these mice with insulin only induced activation of PKB but not S6K in muscle tissues. We have also demonstrated that insulin-stimulated glucose uptake proceeds normally in knock-in mice, consistent with the notion that PKB mediates this process. In contrast to conditional knock-out of PDK1 in muscle, the knock-in mice did not develop dilated cardiomyopathy, suggesting that PKB plays a key role in protecting mice from heart failure. The third knock-in strategy that was evaluated, termed the "inversion" method, did not proceed with high efficiency. We discuss the merits and disadvantages of each of the conditional knock-in approaches, along with the applications for which they may be most suited, and suggest how they could be further refined.

The ability to generate targeted mutation of proteins in mice by using knock-in methodologies is a powerful approach to investigating the physiological roles of disease-causing mutations and the functional roles of domains (1–4). The knock-in approach also has significant advantages over conventional knock-out approaches that are currently more widely used to analyze the function of genes (reviewed in Ref. 5). To date most knock-in mutations have been carried out in a manner that leads to the mutant gene being present in all cells; however, this can have drawbacks, for instance premature lethality or unwanted phenotypes. Embryonic lethality in conventional knock-outs can be overcome by employing Cre/LoxP recombinase methodology, in which the exons to be deleted are flanked with 34-base pair DNA recognition sites (LoxP) for the P1 bacteriophage Cre recombinase (6). These mice are crossed to transgenic mice that express Cre in specific tissues or developmental stages. In tissues expressing Cre, efficient recombination between the LoxP sequences takes place, resulting in the excision of the intervening DNA sequence and ablation of gene expression (6).

The ability to generate conditional knock-in mice using Cre/LoxP technology would be very beneficial; however, the methodology is less well characterized. In Fig. 1 we summarize three approaches that could be employed to generate tissue-specific knock-in mice. In the first strategy termed the "heterozygous" method, a mouse is generated in which one allele comprises the wild type exon flanked with LoxP sites, whereas the second allele contains the knock-in exon and is not flanked with LoxP sites. In tissues that express Cre, the wild type exon will be excised leaving only expression of the knock-in allele (Fig. 1*A*). The potential disadvantages of this method are that in non-Cre-expressing tissue both the mutant and wild type protein are expressed, and in the Cre-expressing tissues the knock-in protein may be expressed at half of the normal levels. To avoid expression of mutant protein in non-Cre-expressing tissues, we used the "minigene" method. In this method, an allele is constructed in such a way that in the absence of Cre, the wild gene is expressed through its natural promoter and exons that occur prior to the knock-in mutation, whereas expression of the remaining exons occurs through a minigene DNA cassette that is flanked by LoxP sequences (Fig. 1*B*). The construct is designed so that Cre-mediated recombination leads to the excision of the minigene cassette, resulting in the expression of the knock-in mutant instead (Fig. 1*B*). A third approach, called the "inversion" method, relies upon the finding that a modification of sequence and orientation of LoxP sequences leads to inversion rather than excision of the DNA sequence flanked by the LoxP sequences upon Cre-mediated recombination (7). In the inversion method, the knock-in exon is cloned in an antiparallel orientation after the wild type exon and the wild type and inverted knock-in exons are flanked with the modified LoxP sites (Fig. 1*C*). In the absence of Cre, the wild type exon will be expressed and the sequence within the inverted knock-in exon should be spliced-out of the pre-mRNA. In the presence of the Cre recombinase, DNA inversion would result

* This work was supported by grants from the Association for International Cancer Research (to D. R. A.), Diabetes UK (to D. R. A.), the Medical Research Council (to D. R. A.), the Moffat Charitable Trust (to D. R. A.), and by the pharmaceutical companies supporting the Division of Signal Transduction Therapy (AstraZeneca, Boehringer-Ingelheim, GlaxoSmithKline, Merck & Co. Inc., Merck KGaA, and Pfizer). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

[1] Supported by a long-term fellowship from the European Molecular Biology Organization as well as a Marie Curie fellowship. To whom correspondence should be addressed: MRC Protein Phosphorylation Unit, School of Life Sciences, University of Dundee, Dow St., Dundee DD1 5EH, United Kingdom. Tel.: 44-1382-384-241; Fax: 44-1382-223-778; E-mail: j.bayascas@dundee.ac.uk.

This is an Open Access article under the CC BY license.

in the knock-in exon being expressed rather than the wild type (Fig. 1*C*). A similar approach has recently been used to mimic a chromosomal translocation that occurs in human cancers (8).

In this study we have evaluated these approaches of generating conditional knock-in mice, using a mutation in 3-phosphoinositide-dependent protein kinase-1 (PDK1)[2] as an example. PDK1 plays an important role in insulin and growth factor signaling pathways by phosphorylating and activating the key effector kinases protein kinase B (PKB, also known as Akt) and ribosomal S6 kinase (S6K) (9). Our previous biochemical and structural studies, summarized in Fig. 1*D*, indicate that PDK1 possesses a substrate docking site termed the "PIF pocket" that is required for PDK1 to phosphorylate S6K but not PKB (10–12). Consistent with this, in knock-in embryonic stem (ES) cell lines in which both copies of the PDK1 gene are altered to express a form of PDK1 retaining catalytic activity, but in which the PIF-pocket site is disrupted by mutation of Leu-155 to Glu, IGF1 induced normal activation of PKB but not S6K (13). It is however not possible to generate global $PDK1^{L155E/L155E}$ knock-in mice, as the embryos of this phenotype die at embryonic day 11, displaying multiple phenotypes (14). In this study we investigated whether the conditional knock-in strategies outlined above can be used to generate mice that express the mutant PDK1[L155E] rather than wild type PDK1 in muscle tissues.

EXPERIMENTAL PROCEDURES

Materials—Protein G-Sepharose, streptavidin-Sepharose, [α-^{32}P]ATP and [γ-^{32}P]ATP were purchased from Amersham Biosciences. Protease-inhibitor mixture tablets and Taq polymerase were purchased from Roche Applied Science. Restriction enzymes were from New England Biolabs. Precast SDS-polyacrylamide bis-Tris gels were from Invitrogen. Human insulin from Novo-Nordisk was obtained from Ninewells Pharmacy, Dundee, United Kingdom.

Antibodies—The following antibodies were raised in sheep and affinity-purified on the appropriate antigen. The PDK1 antibody was raised against the sequence RKIQEVWRQQYQSNPDAAVQ corresponding to residues 540–559 of mouse PDK1; the ERK2 antibody was raised against the full-length human protein; the PKB total antibody used for immunoblot analysis was raised against the sequence RPHFPQFSYSASGTA (residues 466–480) of rat PKB; and the phospho-PKB Ser-473 was raised against the peptide PHFPQFpSYSAS. The total GSK3α was raised against residues 471–483 of rat GSK3α (QAPDATPTLTNSS) and the total GSK3β against the full-length human protein; the total S6K1 antibody was raised against residues 25–44 of the human protein (AGVFDIDLDQPEDAGSEDEL); the phospho-S6 protein Ser-235 antibody was raised against the residues 229–242 of human S6 protein (AKRRRLpSSLRASTS); the total S6K2 used to measure the activity after immunoprecipitation was raised against residues 479–495 of the mouse S6K2 protein; and the total p90 ribosomal S6 kinase (RSK) antibody used to immunoprecipitate all RSK isoforms was raised against residues 712–734 of the human p90 RSK2 protein (RNQSPVLEPVGRSTLAQRRGIKK). The total PKBα antibody used to immunoprecipitate PKBα was a mouse monoclonal antibody raised against residues 1–149 of human PKB and was purchased from Upstate Biotechnology Inc. (catalog no. 05-591). The phospho-PKB Thr-308 (catalog no. 9275), phospho-GSK3α/β Ser-9/21 (catalog no. 9336), phospho-S6K Thr-389 (catalog no. 9205), and total S6 ribosomal protein (catalog no. 2212) were purchased from Cell Signaling Technology. The pan-PDK1 site antibody purchased from Cell Signaling Technology (catalog no. 9379) was found to recognize the phosphorylated T-loop Thr-229 of S6K in cell extracts (15). Secondary antibodies coupled to horseradish peroxidase were from Pierce.

General Methods and Buffers—Restriction enzyme digests, DNA ligations, site-directed mutagenesis, PCR, Southern blotting, and other recombinant DNA procedures were performed using standard protocols. All DNA constructs were verified by DNA sequencing, which was performed by The Sequencing Service, School of Life Sciences, University of Dundee, United Kingdom, using DYEnamic ET terminator chemistry (Amersham Biosciences) on Applied Biosystems automated DNA sequencers. Lysis buffer consisted of: 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (by mass) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol, and "Complete" proteinase inhibitor mixture (one tablet/50 ml).

Construction of the Targeting Vectors—For construction of the minigene and inversion cassette targeting constructs, 129sv mouse BAC (bacterial artificial chromosome) clone containing exons 2–6 of the mouse PDK1 gene was used as a backbone to PCR amplify the 5′ homology arm from a EcoRI/NdeI 5-kb region containing exon 2, and the 3′ homology arm from a HindIII/SpeI 2 kb region 3′ to exon 4. Both the 5′ and the 3′ homology arms were the same in both constructs. Appropriate primers were used to introduce a new EcoRV restriction site at the 3′-end of the 5′-arm for Southern screening purposes. The phosphoglycerate kinase (PGK)-thymidine kinase (TK)-pA cassette was included and the end of the 3′-arm for negative selection. For the minigene method, a targeting vector was constructed to insert a minigene cassette and a neomycin resistance cassette marker flanked by LoxP sites after exon 2 in the PDK1 gene and to mutate the codon in exon 4 of the endogenous PDK1 gene encoding Leu-155 to a Glu (Fig. 2*B*). The PDK1 minigene was PCR-derived from a PDK1 expressed sequence tag (GenBank™ accession number AA989806) and consisted of the cDNA corresponding to exons 3–14 and the intronic sequence 5′ to exon 3 to allow splicing. For the inversion cassette method, a targeting vector was constructed to replace the exons 3 and 4 in the PDK1 gene by an inversion cassette that contains the exon 3 and 4 fragment followed by a shorter and L155E-mutated version of the same exons 3 and 4-containing region cloned in an antiparallel orientation (supplemental Fig. 1). The inversion cassette was flanked by Lox66-

[2] The abbreviations used are: PDK1, 3-phosphoinositide-dependent protein kinase-1; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; EDL, extensor digitorum longus; ERK, extracellular-regulated kinase; ES, embryonic stem; GSK, glycogen synthase kinase; Mck, muscle creatine kinase; PIF, PDK1-interacting fragment; PKB, protein kinase B; RSK, p90 ribosomal S6 kinase; RT, reverse transcriptase; S6K, p70 ribosomal S6 kinase.

and Lox71-modified LoxP sites in opposite orientations (7). The targeting construct also contained a neomycin resistance cassette flanked by a second Lox71 site. Further details of the sequence of these targeting constructs are available by request. The final knock-in constructs were purified on a cesium chloride gradient and were linearized using NotI before electroporation.

ES Cell Targeting—E14.1 129/ola mouse ES cells were grown in Dulbecco's modified Eagle's medium containing high glucose supplemented with 15% fetal calf serum, 0.1 mM non-essential amino acids, antibiotics (100 units of penicillin G, 100 μg/ml streptomycin), 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, and 25 ng/ml murine leukemia inhibitory factor. During the initial selections ES cells were maintained on a feeder layer of mitotically inactivated G418-resistant primary embryonic fibroblasts derived from MTK-Neo mice (16). Targeting of the constructs to obtain heterozygous ES cells was performed using standard procedures (17). Briefly, following electroporation, cells were plated in the absence of G418 for 48 h and then grown in the presence of 0.2 mg/ml G418 and 2 μM gancyclovir (thymidine kinase negative selection) for 10–14 days. Colonies were picked and cultured in the absence of G418 and gancyclovir. Targeted cell lines were identified by Southern blotting using an EcoRV digest of genomic DNA and a 0.7-kb 5′-probe (Fig. 2*B* and supplemental Fig. 1). For the minigene cassette construct, 12 of 228 clones analyzed were confirmed as correctly targeted (5% efficiency), and for the inversion cassette screening 18 of 230 cell lines screened were positive (8% efficiency).

To test the efficiency of inversion in the $PDK1^{IC/+}$ ES cells, 1×10^7 cells were transfected with 30, 45, and 60 μg of pMC-CrePuro, which encodes for puromycin resistance and Cre recombinase, by electroporation using a Bio-Rad GENE PULSER II, set at 240 V and 500 μF. The pMC-CrePuro plasmid was prepared by cloning the blunted AscI fragment of pKO SelectPuro (Stratagene) containing the puromycin gene under the phosphoglycerate kinase promoter into the blunted HindIII site of pMC-Cre (18). The cells were plated out onto gelatinized tissue culture plastic, and puromycin was added to the medium at a concentration of 2 μg/ml during days 2–4. On day 4 the cells were trypsinized and seeded at low density (1000 cells/10-cm dish), and colonies were picked in duplicate into 96-well tissue culture plates. One plate was subjected to PCR analysis using primers ic1 (5′-CACATCAGGAAGCCACTGGCTTA-AGC-3′), ic2 (5′-CTTCCTCTTGCAAAACCACACTGCTC-G-3′), and ic3 (5′-GTGCTCTGTGTGGAGAACATTCTGG-A-3′) as described in supplemental Fig. 1. The other plate was used for expansion of positive ES cells. PCR yielded a product of 260 bp for the recombinant noninverted allele and a 314-bp product for the inverted one. Some of the clones were further confirmed by Southern analysis as described in supplemental Fig. 1.

Generation of $PDK1^{MG/+}$ Mice and Genotyping Analysis—All animal studies and breeding performed in this study were approved by the University of Dundee ethical committee and performed under a UK Home Office project license. Heterozygous knock-in $PDK1^{MG/+}$ ES cell clones were microinjected into C57Black6 × Balb/c blastocysts, which were then reim-

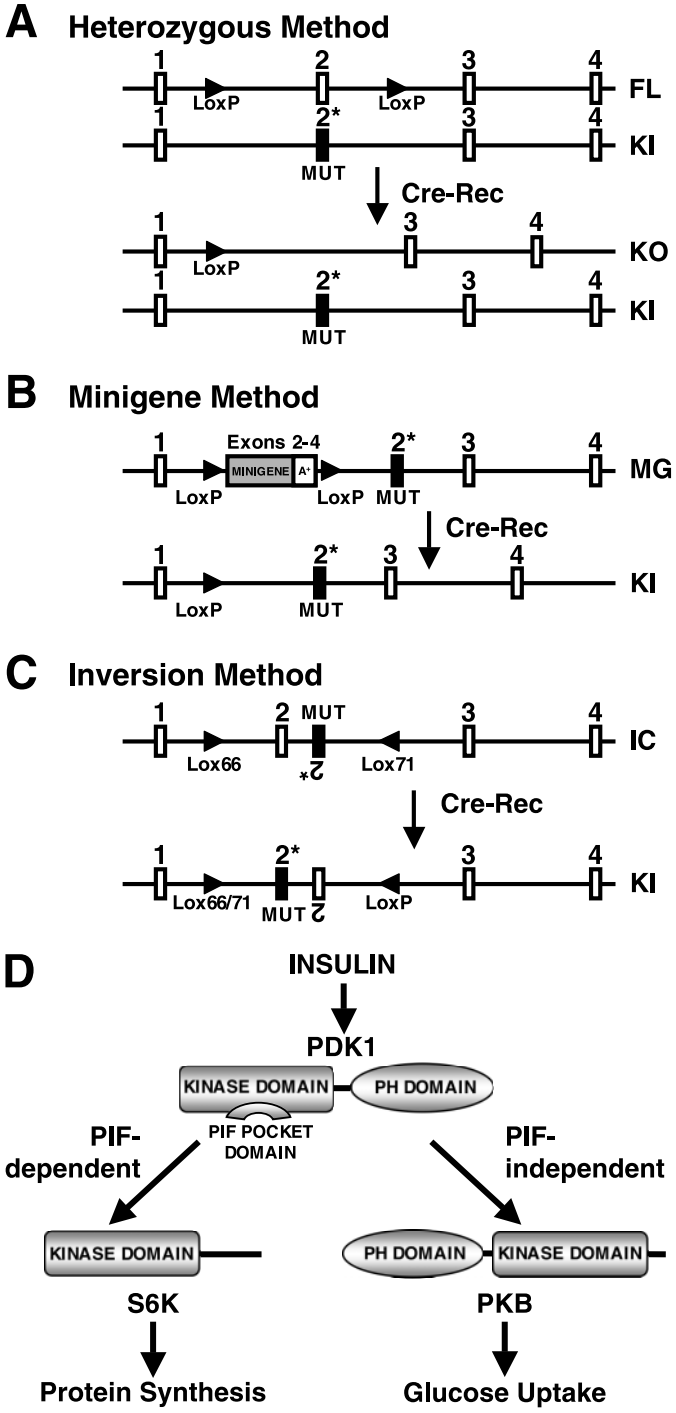

FIGURE 1. **Conditional knock-in strategies.** *A–C*, summary of the heterozygous (*A*), minigene (*B*), and inversion (*C*) conditional knock-in strategies outlined in the Introduction. A hypothetical gene consisting of four exons is depicted. The *white boxes* represent wild type exons, the *black boxes* represent mutated (*MUT*) exon 2 (which is also labeled with an *asterisk*), the *black triangles* represent wild type LoxP sites, and the modified Lox66 and Lox71 sites described previously (7) are indicated. The minigene cassette is depicted as a *gray box* corresponding to wild type exons 2–4 followed by a *white box* labeled with A^+, which corresponds to the endogenous 3′-untranslated region of the messenger and includes the polyadenylation transcription termination signal. *Cre-Rec*, Cre-mediated recombination; *KI*, knock-in; *MG*, minigene; *IC*, inversion cassette. *D*, mechanisms of activation of S6K and PKB by PDK1. Activation of S6K by PDK1 relies on the interaction of PDK1 with S6K through the PIF substrate-binding pocket, whereas the activation of PKB by PDK1 depends on the co-localization of both kinases at the plasma membrane mediated by their PH domains, a mechanism that is PIF pocket-independent (9).

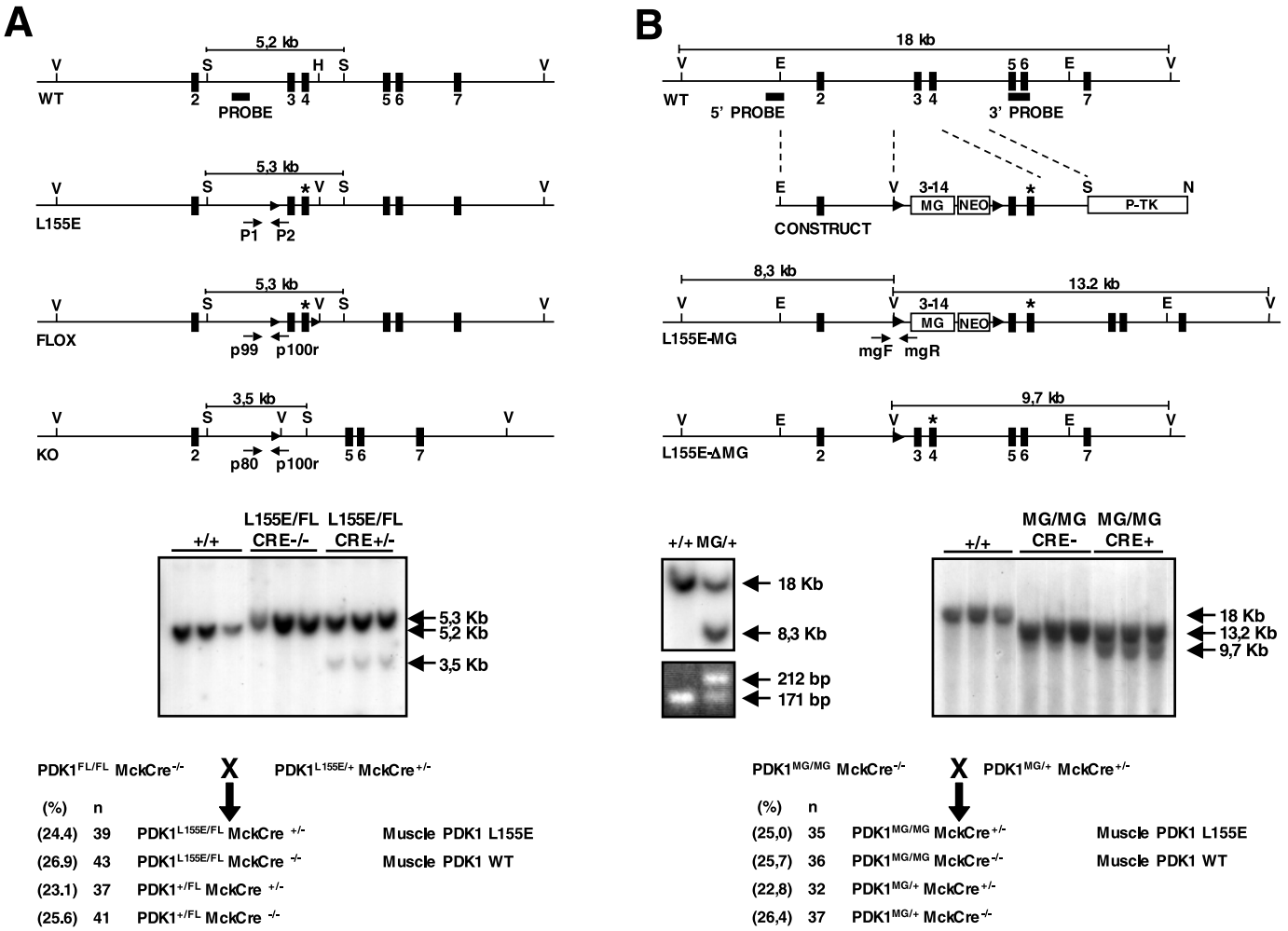

FIGURE 2. **Tissue-specific PDK1 L155E conditional knock-in strategies.** *A*, *top panel*, illustrates the 5′-end of the PDK1 gene from exons 2 to 7 (*WT*, wild type) and the different alleles used for the heterozygous strategy. The *black boxes* represent exons, and the *black triangles* represent LoxP sites. The position of the probe used to show the tissue-specific recombination is shown as a *black bar*. *Arrows* indicate the position of the PCR primers used to genotype mice. The knock-in allele containing the L155E mutation in exon 4 is marked with an *asterisk* and can be detected by genotyping using primers P1 and P2 as described previously (13). The conditional knock-out allele with the LoxP sites flanking exons 3 and 4 (*FLOX*) and the resulting knock-out allele (*KO*) in which exon 3 and 4 have been excised by Cre recombinase, resulting in the ablation of the expression of PDK1 beyond exon 2, were reported previously and can be distinguished by using genotyping primers p99, p80, and p100r (21). *Middle panel*, genomic DNA purified from cardiac muscle tissue from the indicated genotypes was digested with StuI and subjected to Southern analysis with the corresponding DNA probe. The wild type allele generates a 5.2-kb fragment, and both the L155E and the *FLOX* allele generate a 5.3-kb fragment, whereas the knock-out allele gives rise to a 3.5-kb fragment. *Bottom panel*, breeding strategy used for the generation of mice expressing the L155E mutation in skeletal muscle and heart, where *MckCre* denotes transgenic mice expressing the Cre recombinase under the muscular creatine kinase promoter. The number (*n*) and proportion (%) of mice of each genotype resulting from this breeding are indicated. *B*, *top panel*, diagram illustrating the targeting minigene construct, the 5′-end of the PDK1 gene from wild type (exons 2–7) and the allele modification generated. The position of the 5′-probe used to genotype-targeted cells and the position of the 3′-probe used to show the tissue-specific recombination are shown as *black bars*. The minigene cassette includes the natural polyadenylation signals, and the neomycin resistance marker cassette is followed by two further polyadenylation signals. An *asterisk* represents the position of the L155E mutation in exon 4. The positions of the mgF and mgR PCR primers used to genotype mice, described under "Experimental Procedures," are indicated by *arrows*. *L155E-MG* represents the targeted minigene conditional knock-in allele, and *L155E-ΔMG* is the Cre-targeted allele in which the minigene cassette and the neomycin cassette have been removed. *Middle panel*, *left*, genomic DNA purified from the indicated ES cell lines was digested with EcoRV and analyzed by Southern blot with the 5′-probe. The wild type allele generates an 18-kb fragment, whereas the *L155E-MG* allele gives rise to a 8.3-kb fragment. The same genomic DNA was PCR-amplified with primers mgF and mgR. The wild type allele produces a 171-bp fragment, whereas the *L155E-MG* allele generates a 212-bp product. *Middle panel right*, genomic DNA purified from cardiac muscle tissue from the indicated genotypes was digested with EcoRV and subjected to Southern analysis with the 3′-probe. The wild type allele generates an 18-kb fragment, the *L155E-MG* allele generates a 13.2-kb fragment, and the *L155E-ΔMG* allele gives rise to a 9.7-kb fragment. *Lower panel*, breeding strategy used for the generation of mice expressing the L155E mutation in skeletal muscle and heart.

planted into recipient female mice. Chimeric mice that had a high degree of ES cell contribution were identified by a champagne color and were then crossed to C57Black6 mice. Genotyping of PDK1$^{MG/+}$ mice was carried out by PCR of genomic DNA isolated from ear biopsy using primers P1 (5′-GGAACTTACTCTGTAGACCAGGCTG-3′) and P2 (5′-GACGTGTCCTAATACTACCACAAGTGGC-3′). As depicted in Fig. 2*B*, primers P1 and P2 result in a 171-bp product from the wild type allele and a 212-bp product from the targeted allele. PDK1$^{MG/+}$ mice were crossed to transgenic mice expressing Cre recombinase under muscle creatine kinase promoter (MckCre) (19) employing the strategy outlined in the legend for Fig. 2*B*. For the detection of Cre the following primers were employed: Cre1 (5′-AAATGGTTTCCCGCAGAACC-3′) and Cre10 (5′-

TAGCTGGCTGGTGGCAGATG-3′). For the heterozygous method, experimental PDK1$^{fl/L155E}$Cre$^{+/-}$ and control PDK1$^{fl/L155E}$Cre$^{-/-}$ mice were obtained as described in the legend for Fig. 2*A*. The floxed and knock-out alleles were detected with primers p80, p99, and p100r as described previously (20), and the L155E allele was detected with the p1 and p2 primers described by Collins *et al.* (13).

RNA Extraction and RT-PCR Analysis—Total RNA was isolated from the ventricles of mice with the indicated genotype (Fig. 3*D*) using a Total RNA isolation kit (Macherey-Nagel). The Access RT-PCR System (Promega) was employed to amplify a 331-bp PDK1 cDNA region containing exons 3 and 4 with primers MGRT F (5′-AGCACCCTGCCCAGCTGCCACCACAG-3′) and MGRT R (5′-GCCAATTTTGCGGATGTATTTAAGTAGCTC-3′), which flank the XmaI restriction polymorphic site and the triplet coding for the residue 155. The DNA band was cloned, and 30 independent clones obtained from three independent PCR reactions were sequenced.

Preparation of Tissue Extracts, Immunoblotting, and Protein Kinase Assays—Following an overnight fast, a bolus of insulin (150 milliunits/g) or saline solution was intraperitoneally injected to terminally anesthetized mice. At the indicated times, tissues were rapidly extracted, freeze-clamped in liquid nitrogen, stored at −80 °C, and homogenized to a powder in liquid nitrogen. A 10-fold mass excess of ice-cold lysis buffer was added to the powdered tissue, briefly vortexed, and then centrifuged at 4 °C for 10 min at 13,000 × *g* to remove insoluble material. The supernatant was snap-frozen in aliquots in liquid nitrogen and stored at −80 °C. The activation state of PKB or S6K (in Figs. 4 and 5) was assessed by either immunoblotting the extracts (20 μg) with the indicated phosphospecific antibodies or following the immunoprecipitation of these enzymes from cell extracts and assaying their activity using the Crosstide peptide (GRPRTSSFAEG) as described previously (21). In Fig. 3, PDK1 was affinity-purified on PIF-Sepharose from 0.5 mg of tissue lysates as described previously (13). Briefly, 10 μl of streptavidin-Sepharose was conjugated to 0.5 μg of biotinylated PIF peptide (biotin-C6 spacer-REPRILSEEEQEMFRDFAYIADWC) and incubated with the lysates. The beads were resuspended in 20 μl of lysis buffer to which 5 μl of 5× SDS sample buffer was added. The samples were electrophoresed and then immunoblotted. Glucose transport in isolated skeletal muscle was performed as described previously (22).

RESULTS

Generation of PDK1[L155E] Knock-in Mice—The goal of this study was to evaluate whether the heterozygous (Fig. 1*A*), minigene (Fig. 1*B*), and inversion cassette (Fig. 1*C*) methods could be employed to generate conditional knock-in mice that express mutant PDK1[L155E] rather than wild type PDK1 in muscle tissues. For the heterozygous method described previously, mice in which the wild type PDK1 allele was flanked with LoxP sites (PDK1$^{fl/fl}$) (20) were bred with PDK1$^{L155E/+}$ knock-in mice (14) to generate PDK1$^{fl/L155E}$ animals (Fig. 2*A*), which were in turn crossed with transgenic mice expressing the Cre recombinase under the Mck promoter (which induces expression of the Cre recombinase specifically in skeletal muscle and heart just

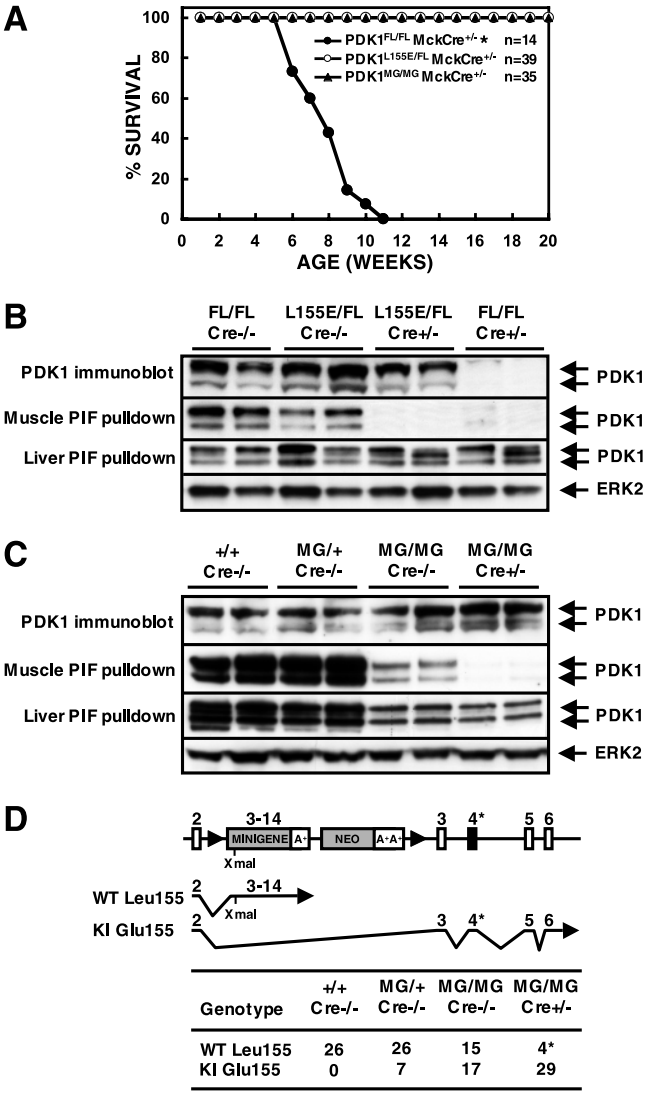

FIGURE 3. **Characterization of muscle-specific PDK1[L155E] mice.** *A*, the indicated mice were maintained under standard husbandry conditions; the percentage of surviving mice of each age is represented. *, indicates the survival of the muscle-specific PDK1 knock-out mice (PDK1$^{fl/fl}$MckCre$^{+/-}$); these data are derived from our previous study (24). No lethality of control PDK1$^{L155E/fl}$MckCre$^{-/-}$ or PDK1$^{MG/MG}$MckCre$^{-/-}$ was observed (data not shown). *B* and *C*, PDK1 was affinity purified from the skeletal muscles of mice of the indicated genotypes based on its ability to bind PIF-Sepharose as described under "Experimental Procedures." *Top panels*, display direct immunoblotting of muscle tissue lysates with the PDK1 antibody. *Middle panels*, PDK1 immunoblots of the PIF-Sepharose pulldown on either muscle or liver extracts. *Bottom panels*, immunoblots of the indicated muscle lysates with the ERK2 antibody are shown as control of protein loading. *D*, diagram depicting the PDK1 MG allele from exons 2–6, in which the *white boxes* represent exons, the *black box* the mutated exon 4 (labeled with an *asterisk*), and the *black triangles* LoxP sites. The minigene cassette (*MINIGENE*) including the 3′-untranslated region, the neomycin cassette (*NEO*), and the polyadenylation transcription termination sequences (A^+) are shown. The minigene cassette contains a novel XmaI restriction site at the 5′-end of the minigene, which enables the sequence of the mRNA transcribed from the minigene or the endogenous exon 3 to be distinguished. Total RNA purified from mouse heart tissue of the indicated genotypes was subjected to RT-PCR analysis by using primers MGRT F and MGRT R, as described under "Experimental Procedures," to generate a 331-bp product encompassing the XmaI polymorphic restriction site on exon 3 and the 155 mutated triplet on exon 4. The resultant PCR products were ligated onto the pCR-topo2.1 vector and transformed in *E. coli*, and ~30 independent clones were sequenced. The numbers of wild type Leu-155 and knock-in Glu-155 sequences obtained for each genotype are indicated. *Asterisk* indicates that the four wild type Leu-155 sequences obtained in PDK1$^{MG/MG}$MckCre$^{+/-}$ mice are likely to arise from the presence of non-muscle (endothelial and fat) cells within isolated heart muscle tissue.

prior to birth (19)). In the resulting PDK1$^{fl/L155E}$MckCre$^{+/-}$ mice, exons 3 and 4 would be deleted in muscle, ablating functional PDK1 expression, as this truncation prevents translation of the entire kinase catalytic domain and subsequent portion of the protein (21); and expression of PDK1 would occur only from the L155E knock-in allele, which is not flanked with LoxP sites. These animals were viable (Fig. 3*A*) and displayed no phenotype. For the minigene method the targeting construct and PDK1$^{MG/MG}$ mice were generated as described in the legend for Fig. 2*B* and under "Experimental Procedures." The PDK1$^{MG/MG}$ mice were crossed with MckCre mice (Fig. 2*B*), and the resultant PDK1$^{MG/MG}$MckCre$^{+/-}$ mice were viable (Fig. 3*A*), of normal size, and displayed no other apparent phenotype.

For the inversion method a targeting construct (described in supplemental Fig. 1) was generated. We observed a tendency for the DNA construct to undergo recombination in *Escherichia coli*, due to the presence of palindromic sequence. To circumvent this problem, the targeting construct was designed to minimize the amount of palindromic sequence. We next targeted ES cells with this construct and were able to isolate 18 independent clones of correctly targeted PDK1$^{IC/+}$ ES cells by Southern blot analysis (supplemental Fig. 1). Prior to generating mice with these ES cell lines, we verified whether transfection of three independently targeted ES cell lines with CRE recombinase induced the expected inversion of the DNA cassette contained within the modified LoxP sites. We analyzed over 400 PDK1$^{IC/+}$ ES cells that expressed the CRE recombinase but found that no inversion had occurred in any of the cells. We also found that in *E. coli* expressing the Cre recombinase, the efficiency of inversion of the DNA cassette within our construct, although detectable, was low. Because of the poor efficiency of inversion

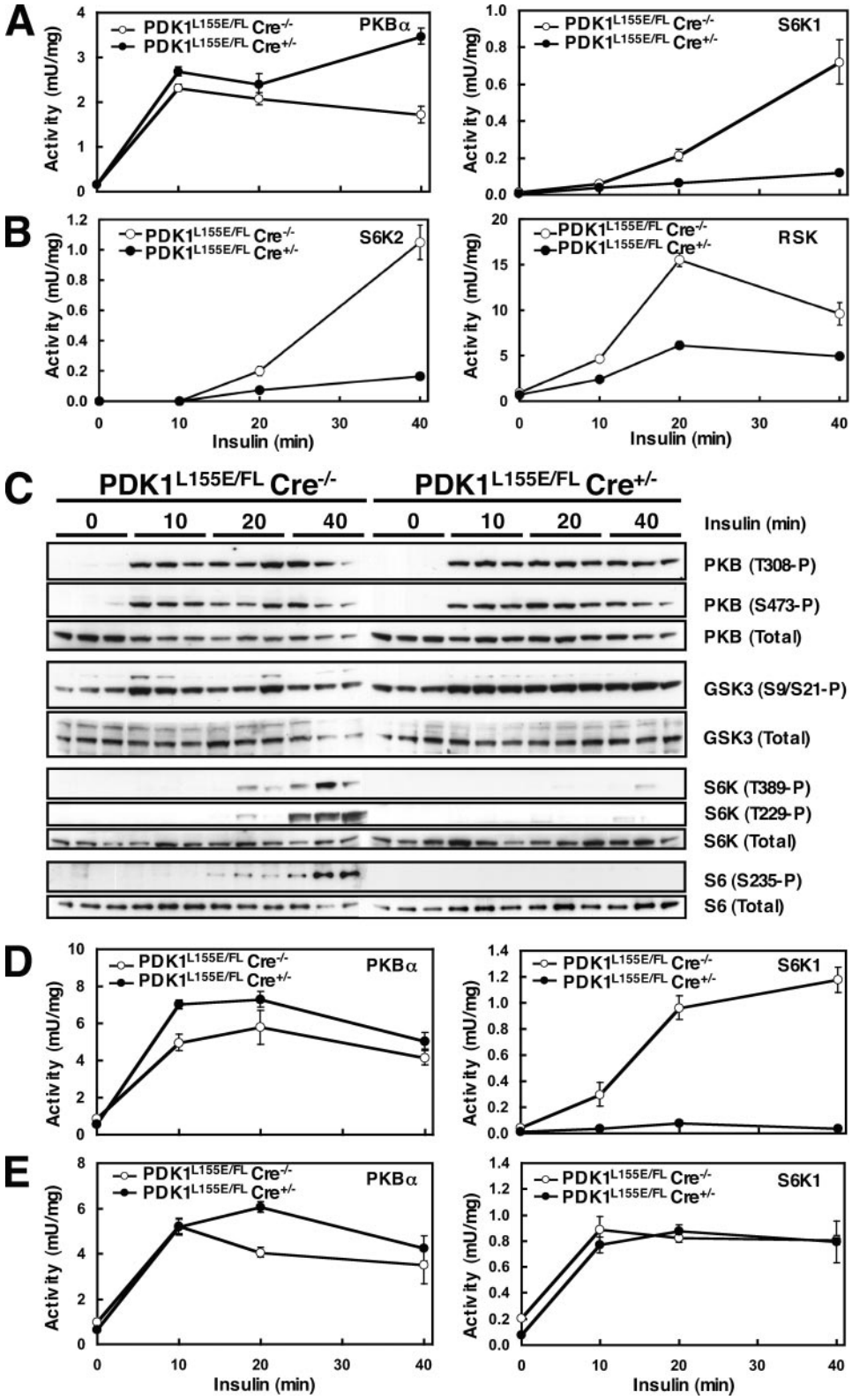

FIGURE 4. **PDK1 signaling in heterozygous knock-in mice.** Mice were fasted overnight and intraperitoneally injected with either saline (for the 0 min time point control) or 150 milliunits/g insulin for 10, 20, and 40 min. Skeletal muscle (*A–C*), cardiac muscle (*D*), or adipose tissue (*E*) was then rapidly extracted and frozen in liquid nitrogen. (*A, B, D,* and *E*) PKB, S6K1, S6K2, and RSK isoforms were immunoprecipitated from the indicated extracts, and the activity was measured using a quantitative peptide phosphorylation assay. Each point represents the mean activity ± S.E. of three different samples with each assayed in triplicate. *C*, the cell lysates from skeletal muscle extracts were immunoblotted with the indicated antibodies; each *lane* represents a different mouse.

observed, we were unable to pursue our analysis of the inversion method; this will be discussed further under "Discussion."

Expression of PDK1 in Knock-in Mice—We found that PDK1 is expressed at near wild type levels in skeletal muscle of PDK1$^{fl/fl}$MckCre$^{-/-}$ and PDK1$^{fl/155E}$MckCre$^{-/-}$ mice not expressing Cre recombinase. In PDK1$^{fl/155E}$MckCre$^{+/-}$ muscle, consistent with excision of the allele encoding wild type PDK1, the level of PDK1 protein was moderately lower than observed in the muscle of littermate PDK1$^{fl/155E}$MckCre$^{-/-}$ animals (Fig. 3*B*). To distinguish between the expression of wild type PDK1 and mutant PDK1[L155E], we incubated muscle extracts with Sepharose conjugated to the PIF peptide, which interacts strongly with the PIF pocket of wild type PDK1 but cannot interact with mutant PDK1[L155E] (10, 23). PDK1 could be affinity-purified from PDK1$^{fl/fl}$MckCre$^{-/-}$ and PDK1$^{fl/155E}$MckCre$^{-/-}$ muscle but not from the PDK1$^{fl/155E}$MckCre$^{+/-}$ tissue (Fig. 3*B*), confirming that mutant PDK1[L155E] rather than wild type PDK1 was expressed. As a control, we demonstrated that in the liver tissue of the PDK1$^{fl/155E}$MckCre$^{+/-}$ mice, which does not express Cre, PDK1 could be affinity-purified.

The analysis of PDK1 expression in muscle tissues of PDK1$^{MG/MG}$MckCre$^{-/-}$ and PDK1$^{MG/MG}$MckCre$^{+/-}$ mice indicated that PDK1 levels were slightly higher than observed in wild type control muscle (Fig. 3*C*). Using the PIF-Sepharose pulldown assay, we were unable to detect wild type PDK1 protein in the muscle of PDK1$^{MG/MG}$MckCre$^{+/-}$ mice. This indicated that we had succeeded in the goal of generating mice that express near normal levels of mutant PDK1[L155E] rather than wild type PDK1 in muscle tissues employing the minigene method. Unexpectedly, however, when analyzing the muscle tissue of littermate control PDK1$^{MG/MG}$MckCre$^{-/-}$ mice, despite the absence of Cre, we observed that the mutant PDK1[L155E] was also expressed at a significant level, as indicated by the lower level of PDK1 in the PIF-Sepharose pulldown assay (Fig. 3*C*). To analyze further the expression of wild type PDK1 and PDK1[L155E], the mRNA for PDK1 was amplified by RT-PCR from cardiac muscle and then

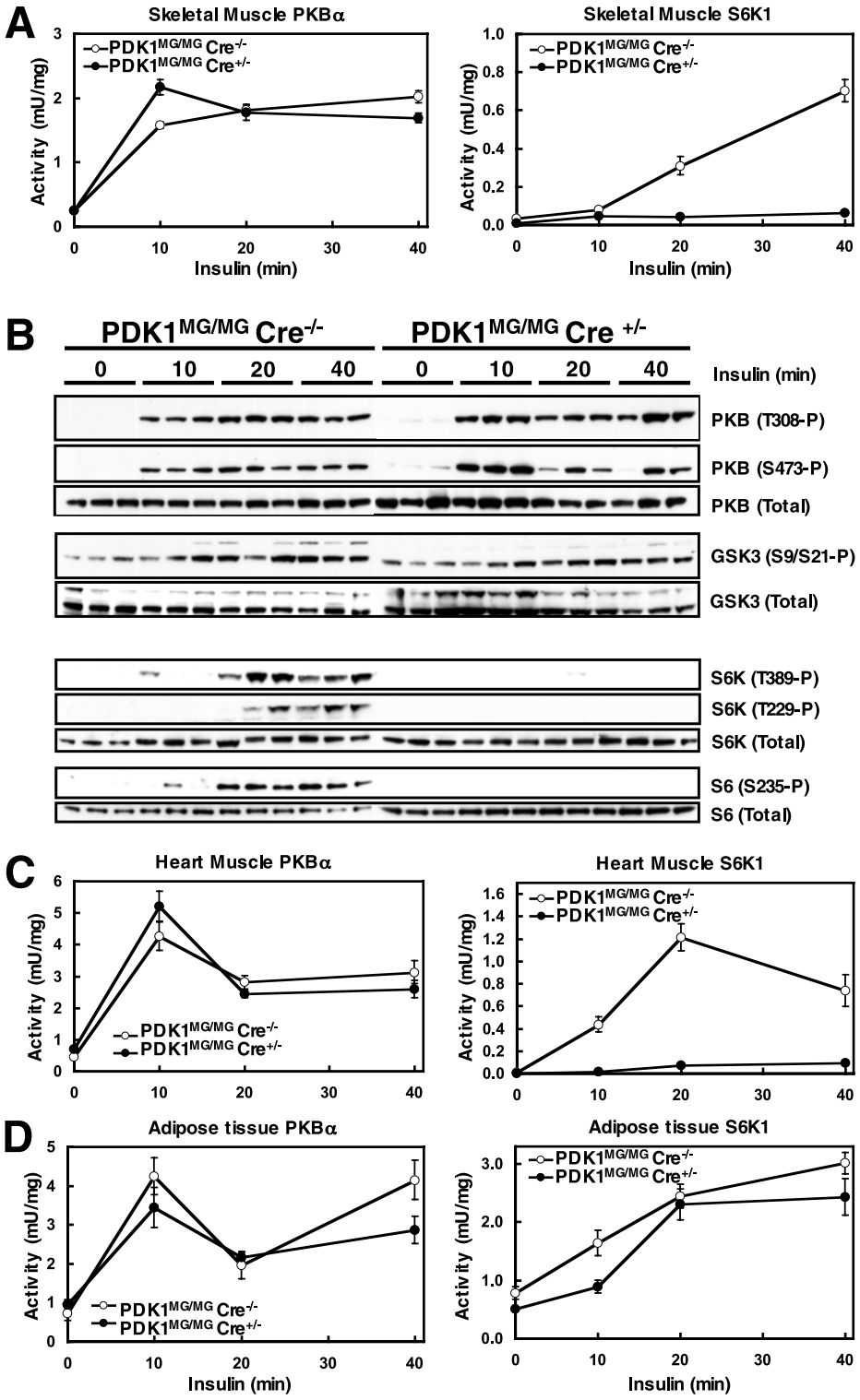

FIGURE 5. **PDK1 signaling in minigene knock-in mice.** Mice were fasted overnight and intraperitoneally injected with either saline (for the 0 min time point control) or 150 milliunits/g insulin for 10, 20, and 40 min. Skeletal muscle (*A* and *B*), cardiac muscle (*C*), or adipose tissue (*D*) was then rapidly extracted and frozen in liquid nitrogen. *A*, *C*, and *D*, PKB or S6K were immunoprecipitated from the indicated extracts, and the activity was measured using a quantitative peptide phosphorylation assay. Each point represents the mean activity ± S.E. of three different samples with each assayed in triplicate. *B*, the cell lysates from skeletal muscle extracts were immunoblotted with the indicated antibodies; each *lane* represents a different mouse.

cloned, and the region corresponding to the L155E mutation was sequenced. This revealed that in PDK1$^{MG/MG}$MckCre$^{-/-}$ mice, similar amounts of mRNA for wild type PDK1 (corresponding to splicing onto the minigene) and PDK1[L155E] (corresponding to splicing across the minigene from exons 2–3) were expressed (Fig. 3*D*). This indicates that despite the presence of three polyadenylation transcription termination sequences within the minigene/neomycin cassette (Fig. 3*D*), transcription did not efficiently terminate and continued into the knock-in region of the gene. Moreover, to obtain expression of PDK1[L155E] protein in non-Cre-expressing cells, splicing must have occurred in a manner that precisely excised the minigene and neomycin cassettes (Fig. 3*D*).

Analysis of Insulin-stimulated PKB and S6K Activity in Tissue-specific Knock-in Mice—The phosphorylation and activation of the PKB and S6K substrates of PDK1 is stimulated by insulin (9). If the tissue-specific knock-in strategy was successful, insulin should induce phosphorylation and activation of PKB but not S6K in Cre-targeted tissues, whereas both PKB and S6K should be activated in non-Cre expressing tissues. To test whether the heterozygous tissue-specific knock-in approach had worked, we starved control PDK1$^{fl/155E}$MckCre$^{-/-}$ and experimental PDK1$^{fl/155E}$MckCre$^{+/-}$ mice overnight so that insulin levels were low. The animals were injected with saline or insulin for 10, 20, and 40 min, after which PKB and S6K activity and phosphorylation were measured in Cre-expressing skeletal and cardiac muscle and in non-Cre-expressing adipose tissue. In the skeletal muscle of control PDK1$^{fl/155E}$MckCre$^{-/-}$ and experimental PDK1$^{fl/155E}$MckCre$^{+/-}$ mice, insulin induced similar and robust phosphorylation and activation of PKB as well as phosphorylation of one of its substrates, GSK3, at the site phosphorylated by PKB (Fig. 4, *A* and *C*). In the control PDK1$^{fl/155E}$MckCre$^{-/-}$ mice, insulin also induced a marked activation of S6K, which, as observed previously (20, 24), occurred more slowly than activation of PKB (Fig. 4*A*, *right panel*). In marked contrast, S6K was not significantly phosphorylated or activated by insulin in the experimental PDK1$^{fl/155E}$MckCre$^{+/-}$ mice (Fig. 4, *A* and *C*). Consistent with this observation, insulin failed to induce phosphorylation of the ribosomal S6 protein, an S6K substrate, in the muscle of PDK1$^{fl/155E}$MckCre$^{+/-}$ mice (Fig. 4*C*, *bottom panels*). PDK1 also activates other AGC kinases, including S6K2 and RSK, in a manner that is dependent upon the PIF pocket of PDK1 (13, 25). Consistent with this notion we found that S6K2 and RSK isoforms were poorly activated in skeletal muscle of PDK1$^{fl/155E}$MckCre$^{+/-}$ mice compared with littermate control PDK1$^{fl/155E}$MckCre$^{-/-}$ animals (Fig. 4*B*).

We also analyzed PKB and S6K activity in the heart muscle of experimental PDK1$^{fl/155E}$MckCre$^{+/-}$ mice, which also express the Cre recombinase. Similar to findings in skeletal muscle, we found that PKB was normally activated by insulin, whereas S6K was not significantly stimulated (Fig. 4*D*). However, in adipose tissue that does not express the Cre recombinase, insulin induced normal activation of S6K in the experimental PDK1$^{fl/155E}$MckCre$^{+/-}$ animals (Fig. 4*E*).

We also analyzed activation of PKB and S6K in the skeletal and heart muscle as well as adipose tissue of control PDK1$^{MG/MG}$MckCre$^{-/-}$ and experimental PDK1$^{MG/MG}$MckCre$^{+/-}$ mice. We found that in the experimental PDK1$^{MG/MG}$MckCre$^{+/-}$ animals, PKB was normally phosphorylated and activated in skeletal and heart muscle under conditions where S6K remained nonphosphorylated and was inactive (Fig. 5, *A–C*). In non Cre-expressing adipose tissue, insulin induced normal activation of S6K in the experimental PDK1$^{MG/MG}$MckCre$^{+/-}$ mice (Fig. 5*D*).

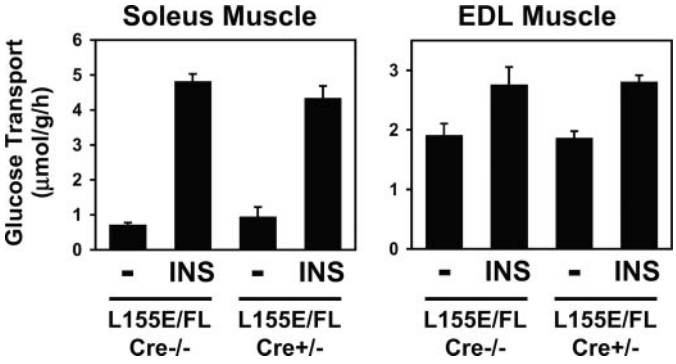

FIGURE 6. **Glucose transport in heterozygous knock-in muscle.** 2-Deoxyglucose uptake in isolated soleus and EDL muscles derived from the indicated mice was determined without (−) or with insulin (*INS*) as described previously (22). The data are presented as the mean ± S.E. for muscle isolated from 4–5 mice for each genotype.

Analysis of Insulin-stimulated Glucose Uptake in Tissue-specific Knock-in Mice—A key role of PKB in muscle tissues is thought to be the regulation of glucose transport in response to insulin. However, there are several reports suggesting that other PDK1-regulated kinases, including atypical PKC isoforms that require the PIF pocket of PDK1, may play a role in insulin-stimulated glucose uptake in muscle tissue (26). We therefore measured glucose uptake in isolated slow twitch soleus and fast twitch extensor digitorum longus (EDL) muscles. The muscles were incubated for 60 min in the presence or absence of 100 nM insulin followed by 10 min in [^{3}H]2-deoxyglucose. We observed that the basal levels of glucose transport were comparable in the PDK1$^{L155E/fl}$Cre$^{-/-}$ control and the PDK1$^{L155E/fl}$Cre$^{+/-}$ muscle-specific knock-in mice, both in the soleus and the EDL muscles, and that glucose uptake was stimulated by insulin to a similar extent in both the PDK1$^{L155E/fl}$Cre$^{-/-}$ and the PDK1$^{L155E/fl}$Cre$^{+/-}$ mice (5-fold in the soleus muscle and 1.5-fold in the EDL muscle (Fig. 6)). These results support the importance of PKB activation in stimulating glucose transport in response to insulin.

DISCUSSION

The results presented in this study indicate that both the heterozygous and minigene methodology, in combination with MckCre transgenic mice, can be successfully deployed to generate mice expressing mutant PDK1[L155E] rather than wild type PDK1 in heart and muscle tissues. This was demonstrated in the PIF-Sepharose pulldown assay (Fig. 3) and by measuring PKB and S6K activity and phosphorylation (Figs. 4 and 5). We had previously found that conditional knock-out mice lacking PDK1 in heart muscle in which neither PKB or S6K are activated develop dilated cardiomyopathy and die of heart failure at 5–11 weeks of age (24). In contrast, neither the PDK1$^{fl/L155E}$MckCre$^{+/-}$ nor PDK1$^{MG/MG}$MckCre$^{+/-}$ animals

develop any symptoms of heart failure[3] and survive normally for at least 1 year (Fig. 3*A*). These results suggest that lack of PKB activity is the main cause of heart failure in mice lacking cardiac PDK1. The ability to generate tissue-specific PDK1 knock-out and PDK1[L155E] knock-in mice will aid in investigations to dissect the distinct physiological roles of PKB and S6K.

A potential drawback of the heterozygous method (Fig. 1*A*) is that both the mutant and wild proteins will be expressed in non-Cre-expressing tissues. This is not a significant problem in the case of PDK1, where the PDK1[L155E] mutant is not functionally dominant over the wild type protein. However, if a mutant protein is dominant, then the heterozygous knock-in approach would not be desirable. We therefore developed the minigene method to circumvent this situation. Although we found that PDK1 expression was relatively normal in the $PDK1^{MG/MG}$ mice in the presence or absence of the Cre recombinase, our data demonstrated that despite the presence of three polyadenylation transcription termination sequences that were included to discourage transcription beyond the minigene and neomycin cassette (Fig. 2*B*), this was not sufficient to ablate transcription and subsequent splicing out of the entire minigene/neomycin cassettes (Fig. 3*D*). Recently, a minigene knock-in strategy similar to that employed in our study has been reported to introduce a mutation in the γ-aminobutyric acid receptor subunit in Cre-expressing neurones (27). In contrast to our observations, the authors of this study reported no expression of the knock-in mutation in non-Cre-expressing tissue (27). A possible explanation for this could lie in the inclusion of a small downstream nontranscribed sequence following the polyadenylation signal in the design of the minigene construct, which is derived from the 3′-end of the γ-aminobutyric acid receptor subunit gene. We would recommend inclusion of this small downstream nontranscribed sequence following the polyadenylation motif when utilizing the minigene method in the future. Alternatively, the difference in expression may be due to the use of two minigenes in the γ-aminobutyric gene. Another consideration when deploying the minigene method is whether gene expression might be affected by noncoding motifs in introns that are lost when constructing the minigene cassette.

The inversion cassette strategy was unsuccessful because of the poor efficiency of Cre-mediated inversion that we observed in $PDK1^{IC/+}$ ES cells. The method we followed to generate the construct made use of a modified LoxP sequence described previously (7) and has recently been employed to mimic a chromosomal fusion of the Ews-ERG oncogene that occurs in leukemia (8). Although this study demonstrated that the Ews-ERG fusion protein was produced in Cre-expressing lymphoid cells, the efficiency at which inversion occurred was not addressed. Even if Cre-mediated inversion had occurred in a very low proportion of lymphoid cells, these would have a huge proliferative advantage and the potential to induce leukemia. For the analysis of nondominant knock-in mutation in a tissue such as muscle, it is essential that Cre inversion proceeds efficiently in the majority of cells. After we had commenced our study, an alternative strategy to induce inversion of DNA sequences was described that makes use of dual wild type and modified LoxP sites on either end of the DNA sequence that is targeted for inversion (28). This method of inducing the inversion of DNA cassettes was reportedly efficient in Cre-expressing mouse tissues (28) and may work more reliably than the method we employed.

In summary, the precise strategy to create a tissue-specific knock-in mouse depends on whether the mutant protein is likely to have a dominant effect over the wild type protein. If as in the case of PDK1, the mutant protein does not interfere with the function of the wild type protein, then either the heterozygous or minigene strategies of generating conditional knock-in mice should yield satisfactory results. It is possible that the inclusion of additional polyadenylation transcriptional termination sequences following the minigene cassette would more efficiently ablate expression of mutant protein in non-Cre-expressing tissues. An advantage of the heterozygous knock-in method over the minigene method is that strains of mice expressing knock-in alleles and conditional LoxP-flanked knock-out alleles may have already been generated for previous studies, as was the case for PDK1, so that no new mouse strains need to be generated to create a tissue-specific knock-in mouse. Clearly, if these mice had not been previously generated, two strains of mice would be required for the heterozygous method, in contrast to only a single strain for the minigene and inversion cassette approaches. For knock-in mutations that are dominant, for example those that might result in cancer, then the inversion method may be preferable, as expression of the mutant protein would not occur in non-Cre-expressing tissues. The only concern with this method that needs to be addressed for certain studies would be the efficiency with which Cre-mediated inversion occurs in Cre-expressing tissues.

Acknowledgments—We thank Rod Hardy and Tony Pawson for suggesting the heterozygous method to generate tissue-specific PDK1[L155E] knock-in mice; Leah Ronaldson and Gail Fraser for assistance with genotyping of mice; and the antibody purification team (Division of Signal Transduction Therapy, University of Dundee), coordinated by Hilary McLauchlan and James Hastie, for generation and purification of antibodies.

[3] J. R. Bayascas, data not shown.

REFERENCES

1. Mikkola, H. K., and Orkin, S. H. (2005) *Methods Mol. Med.* **105,** 3–22
2. Goadsby, P. J. (2004) *Neuron* **41,** 679–680
3. Lester, H. A., Fonck, C., Tapper, A. R., McKinney, S., Damaj, M. I., Balogh, S., Owens, J., Wehner, J. M., Collins, A. C., and Labarca, C. (2003) *Curr. Opin. Drug Discovery Dev.* **6,** 633–639
4. McManus, E. J., Sakamoto, K., Armit, L. J., Ronaldson, L., Shpiro, N., Marquez, R., and Alessi, D. R. (2005) *EMBO J.* **24,** 1571–1583
5. Vanhaesebroeck, B., Rohn, J. L., and Waterfield, M. D. (2004) *Cell* **118,** 274–276
6. Nagy, A. (2000) *Genesis* **26,** 99–109
7. Zhang, Z., and Lutz, B. (2002) *Nucleic Acids Res.* **30,** e90
8. Forster, A., Pannell, R., Drynan, L. F., Codrington, R., Daser, A., Metzler, M., Lobato, M. N., and Rabbitts, T. H. (2005) *Nat. Methods* **2,** 27–30
9. Mora, A., Komander, D., Van Aalten, D. M., and Alessi, D. R. (2004) *Semin. Cell Dev. Biol.* **15,** 161–170
10. Biondi, R. M., Cheung, P. C., Casamayor, A., Deak, M., Currie, R. A., and

Alessi, D. R. (2000) *EMBO J.* **19,** 979–988
11. Biondi, R. M., Kieloch, A., Currie, R. A., Deak, M., and Alessi, D. R. (2001) *EMBO J.* **20,** 4380–4390
12. Biondi, R. M., Komander, D., Thomas, C. C., Lizcano, J. M., Deak, M., Alessi, D. R., and Van Aalten, D. M. (2002) *EMBO J.* **21,** 4219–4228
13. Collins, B. J., Deak, M., Arthur, J. S., Armit, L. J., and Alessi, D. R. (2003) *EMBO J.* **22,** 4202–4211
14. McManus, E. J., Collins, B. J., Ashby, P. R., Prescott, A. R., Murray-Tait, V., Armit, L. J., Arthur, J. S., and Alessi, D. R. (2004) *EMBO J.* **23,** 2071–2082
15. Collins, B. J., Deak, M., Murray-Tait, V., and Alessi, D. R. (2005) *J. Cell Sci.* **118,** 5023–5034
16. Stewart, C. L., Schuetze, S., Vanek, M., and Wagner, E. F. (1987) *EMBO J.* **6,** 383–388
17. Joyner, A. L. (1993) *Gene Targeting, A Practical Approach*, IRL Press
18. Gu, H., Zou, Y. R., and Rajewsky, K. (1993) *Cell* **73,** 1155–1164
19. Bruning, J. C., Michael, M. D., Winnay, J. N., Hayashi, T., Horsch, D., Accili, D., Goodyear, L. J., and Kahn, C. R. (1998) *Mol. Cell* **2,** 559–569
20. Lawlor, M. A., Mora, A., Ashby, P. R., Williams, M. R., Murray-Tait, V., Malone, L., Prescott, A. R., Lucocq, J. M., and Alessi, D. R. (2002) *EMBO J.* **21,** 3728–3738
21. Williams, M. R., Arthur, J. S., Balendran, A., van der Kaay, J., Poli, V., Cohen, P., and Alessi, D. R. (2000) *Curr. Biol.* **10,** 439–448
22. Sakamoto, K., McCarthy, A., Smith, D., Green, K. A., Grahame Hardie, D., Ashworth, A., and Alessi, D. R. (2005) *EMBO J.* **24,** 1810–1820
23. Balendran, A., Casamayor, A., Deak, M., Paterson, A., Gaffney, P., Currie, R., Downes, C. P., and Alessi, D. R. (1999) *Curr. Biol.* **9,** 393–404
24. Mora, A., Davies, A. M., Bertrand, L., Sharif, I., Budas, G. R., Jovanovic, S., Mouton, V., Kahn, C. R., Lucocq, J. M., Gray, G. A., Jovanovic, A., and Alessi, D. R. (2003) *EMBO J.* **22,** 4666–4676
25. Frodin, M., Antal, T. L., Dummler, B. A., Jensen, C. J., Deak, M., Gammeltoft, S., and Biondi, R. M. (2002) *EMBO J.* **21,** 5396–5407
26. Ishiki, M., and Klip, A. (2005) *Endocrinology* **146,** 5071–5078
27. Skvorak, K., Vissel, B., and Homanics, G. E. (2006) *Genesis* **44,** 345–353
28. Schnutgen, F., Doerflinger, N., Calleja, C., Wendling, O., Chambon, P., and Ghyselinck, N. B. (2003) *Nat. Biotechnol.* **21,** 562–565