Original Article

Glucose-Stimulated Insulin Production in Mice Deficient for the PAS Kinase PASKIN

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The Per-ARNT-Sim (PAS) domain serine/threonine kinase PASKIN, or PAS kinase, links energy flux and protein synthesis in yeast and regulates glycogen synthase in mammals. A recent report suggested that PASKIN mRNA, protein, and kinase activity are increased in pancreatic islet β-cells under hyperglycemic conditions and that PASKIN is necessary for insulin gene expression. We previously generated *Paskin* knockout mice by targeted replacement of the kinase domain with the β-*geo* fusion gene encoding β-galactosidase reporter activity. Here we show that no 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) staining was observed in islet β-cells derived from *Paskin* knockout mice, irrespective of the ambient glucose concentration, whereas adenoviral expression of the *lacZ* gene in β-cells showed strong X-gal staining. No induction of PASKIN mRNA could be detected in insulinoma cell lines or in islet β-cells. Increasing glucose concentrations resulted in PASKIN-independent induction of insulin mRNA levels and insulin release. PASKIN mRNA levels were high in testes but undetectable in pancreas and in islet β-cells. Finally, blood glucose levels and glucose tolerance after intraperitoneal glucose injection were indistinguishable between *Paskin* wild-type and knockout mice. These results suggest that *Paskin* gene expression is not induced by glucose in pancreatic β-cells and that glucose-stimulated insulin production is independent of PASKIN. *Diabetes* 56: 113–117, 2007

The Per-ARNT-Sim (PAS) domain proteins often serve as environment sensors, regulating the cellular metabolism and behavior of microorganisms in response to, among others, oxygen or light. In nitrogen-fixing *Rhizobium* species, for example, the oxygen sensor protein FixL contains a heme group within its PAS domain. Oxygen bound to heme inhibits the histidine kinase domain. Under oxygen-free conditions, kinase activity is de-repressed and activates FixJ, the master transcriptional inducer of genes involved in nitrogen fixation.

We and others previously identified a novel mammalian PAS protein, termed PASKIN (1), or PAS kinase (2). The domain architecture of PASKIN resembles that of FixL. PASKIN contains two PAS domains (PAS A and PAS B) and a serine/threonine kinase domain related to AMP kinases that might be regulated in *cis* by the binding of thus far unknown (possibly metabolic) ligands to the PAS domain (3). After de-repression, autophosphorylation in *trans* results in the "switch-on" of the kinase domain of PASKIN (2). The budding yeast PASKIN homologs PSK1 and PSK2 phosphorylate three translation factors and two enzymes involved in the regulation of glycogen and trehalose synthesis, thereby coordinately controlling translation and sugar flux (4). Under stress conditions (nutrient restriction combined with high temperature), PASKIN kinase activity results in downregulation of protein synthesis and carbohydrate storage in yeast. In mammalian cells, PASKIN-dependent phosphorylation inhibits the activity of mammalian glycogen synthase (5).

A recent report suggested that PASKIN kinase activity followed by mRNA and protein expression is increased in MIN6 cells and in isolated pancreatic β-cells after exposure to high glucose concentrations (6). Increased PASKIN activity appeared to be required for glucose-dependent transcriptional induction of the pancreatic duodenum homeobox 1 (PDX-1) transcription factor, leading to transcriptional induction of preproinsulin but not glucokinase or uncoupling protein 2 gene expression. The authors concluded that decreases in PASKIN activity in β-cells might contribute to some forms of type 2 diabetes (6). However, no in vivo data were provided in the report.

We previously generated PASKIN null mice by targeted replacement of the kinase domain of the mouse *Paskin* gene by a *lacZ-neo* fusion construct in embryonic stem cells (7,8). Surprisingly, PASKIN expression is strongly upregulated in postmeiotic germ cells during spermatogenesis, as revealed by both β-galactosidase staining as well as mRNA blotting. In fact, PASKIN mRNA levels in testis are several magnitudes higher than in all other organs tested. No other "sensory" organs, including pancreas, carotid bodies, or photoreceptor cells, stained positive for β-galactosidase. At least under laboratory conditions, fertility as well as sperm production and sperm motility were not affected in PASKIN knockout mice. To examine the role of PASKIN in glucose-stimulated insulin production, we used pancreatic β-cells derived from wild-type and knockout mice and performed glucose tolerance tests in these mice.

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HBSS, Hank's balanced salt solution; PAS, Per-ARNT-Sim; PDX-1, pancreatic duodenum homeobox 1; X-gal, 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside.

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RESEARCH DESIGN AND METHODS

The generation and genotyping of PASKIN knockout mice was described previously (7). Heterozygous *Paskin*$^{+/-}$ mice were crossed with the C57BL/6 inbred strain for 10 generations and then bred to homozygosity for the knockout allele containing the *lacZ* reporter gene. All animal handling followed the guidelines for the use and care of laboratory animals of the Federal Veterinary Office and was approved by the Cantonal Veterinary Office Zürich (no. 192/2003).

Islet isolation and β-cell culture. Male *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ mice were killed by cervical dislocation, the pancreata were excised, and islets were either manually picked or isolated by gradient density centrifugation as described previously (9). Briefly, 2 ml of ice-cold collagenase solution (1 pièze unit per ml NB-8, 10 mmol/l HEPES, 3.3 mg/ml DNaseI, and 10 mmol/l $CaCl_2$ in Hank's balanced salt solution [HBSS], pH 7.4) was injected into several sites of the pancreas, which was then incubated for 10–14 min in a 37°C shaking water bath. The digestion was stopped by adding ice-cold FCS-quenching buffer (10% FCS in HBSS containing 22 mmol/l HEPES). After two washing steps with BSA-quenching buffer (0.5% BSA in HBSS containing 22 mmol/l HEPES) and after centrifugation at 1,200 rpm for 2 min at 4°C, the digested pancreata were filtered through medical gauze. After centrifugation as described above, the pellet was resuspended in either 35 ml RPMI-1640 media for manual picking or in 5 ml 1.119 g/ml Histopaque (Sigma, Buchs, Switzerland) in a 50-ml tube and overlaid with 5 ml 1.100 g/ml histopaque, 10 ml 1.077g/ml histopaque, and 10 ml HBSS. The tube was centrifuged (1,200 rpm for 25 min at 4°C), and the islets were collected from the interphase, diluted 1:1 with BSA-quenching buffer, centrifuged (1,200 rpm for 10 min at 4°C), washed once in HBSS, and resuspended in RPMI-1640 supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mmol/l HEPES, and 11 mmol/l D-glucose (Invitrogen, Basel, Switzerland). Medium was changed every 24 h for 2–3 days until the islets were spread and flattened on 35-mm plates coated with extracellular matrix derived from bovine corneal endothelial cells (Novamed, Jerusalem, Israel). To control for β-galactosidase expression, islets were infected with adenovirus constitutively expressing the *lacZ* reporter gene (9).

Insulin determination. Islets were cultured in pyruvate-free RPMI-1640 as described above. Before glucose stimulation, islets were starved in medium containing 1.6 mmol/l glucose for 30 min, medium was replaced, and the islets incubated for another hour. The medium was replaced by fresh medium containing the desired glucose concentrations, and the islets were incubated for 6 h at 37°C. The supernatant was collected for insulin measurements, and the islets were prepared for 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) staining. Insulin concentrations were determined by radioimmunoassay using an insulin-CT kit (Schering, Baar, Switzerland) according to the instructions provided by the manufacturer.

X-gal staining and β-galactosidase assay. Excised mouse testes or cultured, glucose-treated islets were fixed with 0.2% glutaraldehyde, 5 mmol/l EGTA, 2 mmol/l $MgCl_2$, and 0.1 mol/l Na-phosphate buffer, pH 7.3, permeabilized with 0.01% Na-deoxycholate, 0.02% NP-40, 2 mmol/l $MgCl_2$, and 0.1 mol/l Na-phosphate buffer, pH 7.3, and incubated at 37°C in X-gal solution: 5 mmol/l $K_3Fe(CN)_6$, 5 mmol/l $K_4Fe(CN)_6$, 2 mmol/l $MgCl_2$, 0.02% NP-40, 0.01% Na-deoxycholate, 0.1 mol/l Na-phosphate buffer, pH 7.3, and 0.1% X-gal. β-Galactosidase activity was determined in tissue extracts by the ONPG (*o*-nitrophenyl-b-D-galactopyranoside) assay and normalized to protein content as described previously (10).

Cell culture and glucose stimulation. Mouse spermatogonia GC-1 and spermatocyte GC-2 (11), human testicular germ cell tumor NCCIT, and embryonal carcinoma NTERA-2 cl.D1 cell lines (provided by S. Schweyer, Göttingen, Germany); mouse insulinoma MIN6 (provided by W. Moritz, Zürich, Switzerland, and J. Rutter, Salt Lake City, UT) and the B1-MIN6 subline (provided by P.A. Halban, Geneva, Switzerland); and the rat insulinoma INS-1E cell line (provided by C.B. Wollheim, Geneva, Switzerland) were cultured in high-glucose Dulbecco's modified Eagle's medium (Sigma) as described previously (12). The glucose concentration was lowered to 3 mmol/l for 16 h before incubation of cells with 3 or 30 mmol/l glucose for 6 h.

mRNA quantification. Total RNA from mouse organs or cultured cells was isolated as described previously (11). Mouse PASKIN, insulin, and L28 mRNA was determined by RT–quantitative PCR. Briefly, 8 μg of total RNA was reverse-transcribed with StrataScript III (Stratagene, Amsterdam, the Netherlands), and quantitative PCR was performed in duplicate with 8% of the cDNA reaction mixture, using a SYBR Green quantitative PCR reagent kit on a MX3000P PCR light cycler according to the manufacturer's instructions (Stratagene). Primers (synthesized by Microsynth, Balgach, Switzerland) used were: PASKIN: hPASKINfwd 5′-ggaactgctccagtttcctg-3′ and hPASKINrev 5′-ggatctccgcttaatgacca-3′, mPASKINfwd 5′-agggtccaagaattgacgtg-3′ and mPASKINrev 5′-tgactgctcaccatcctctg-3′, and rPASKINfwd 5′-tgtggactgcagtg gagaag-3′ and rPASKINrev 5′-caaagaggtccaagccagag-3′; ribosomal protein L28: hL28fwd 5′-ggaactgctccagtttcctg-3′ and hL28rev 5′-ggatctccgcttaatgacca-3′, mL28fwd 5′-ggcaaaggggtcgtggtagt-3′ and mL28rev 5′-tcagggcggtacttgttctt-3′, and rL28fwd 5′-cggagcccaataatctgaag-3′ and rL28rev 5′-ttgatggtggtcctcacgta-3′; PDX-1: mPDX-1fwd 5′-gaaatccaccaaagctcacg-3′ and mPDX-1rev 5′-ttcaacatcactg ccagctc-3′; glucokinase: mGckfwd 5′-tatgaagaccgccaatgtga-3′ and mGckrev 5′-tttc cgccaatgatcttttc-3′, and rGckfwd 5′-cagtggagcgtgaagacaaa-3′ and rGckrev 5′-cttggtccaattgaggagga-3′; insulin: mmInsfwd 5′-tggcttcttctacacacccaag-3′ and mmInsrev 5′-acaatgccacgcttctgcc-3′, and rmIns1fwd 5′-caccttttgtggtcctcacct-3′ and rmIns1rev 5′-ccagttggtagagggagcag-3′; mPKfwd 5′-ttctgtctcgctaccgacct-3′ and mPKrev 5′-cctgtcaccacaatcaccag-3′, and rPKfwd 5′-cgttcagcccagcttc tatc-3′ and rPKrev 5′-cctgtcaccacaatcaccag-3′. Dilution series of the corresponding plasmids or gel-isolated PCR products were used to obtain standard curves.

Immunofluorescence. Flattened islet β-cells on extracellular matrix dishes were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.5% TritonX-100, blocked with 10% normal goat serum in PBS, and stained with polyclonal guinea pig anti-insulin antibodies (Sigma) diluted 1:100 in 1% normal goat serum in PBS for 30 min at 37°C. Bound antibodies were detected with fluorescein isothiocyanate–conjugated goat anti–guinea pig antibodies at 1:20 dilution (Sigma). The extracellular matrix plates were mounted with Kaiser's glycerol gelatin (Merck, Darmstadt, Germany) and analyzed by fluorescence microscopy.

Intraperitoneal glucose tolerance tests. Glucose tolerance tests were performed in 6- to 8-week-old and 14- to 16-week old male mice. Mice were fasted overnight (16 h) and injected intraperitoneally with a saline glucose solution of 2 g/kg body wt. Blood was obtained by puncturing the tail vein, and glucose levels were measured using Accu-Chek Aviva (Roche Diagnostics, Basel). Measurements were performed before and 30, 60, 90, 120, and 150 min after glucose injection.

RESULTS

PASKIN mRNA is not induced by high-glucose treatment of cultured cell lines. PASKIN mRNA has been previously suggested to be induced by glucose in pancreas-derived MIN6 cells (6). However, after exposure to high glucose levels (30 mmol/l) for 6 h, we could not confirm PASKIN mRNA induction in mouse MIN6 cells or rat INS-1E cells, whereas insulin secretion by MIN6cells or pyruvate kinase mRNA in INS-1E cells were readily increased under high glucose concentrations. Real-time RT-PCR also did not reveal any glucose-dependent increase in expression of PDX-1 or the two mouse or rat insulin genes (data not shown). A similar pattern of constitutive insulin and PDX-1 gene expression, as well as the glucose-dependent pyruvate kinase gene expression in INS-1E cells, has been reported previously (13). Moreover, neither the testis-derived mouse spermatogonia GC-1 and spermatocyte GC-2 nor the human testicular germ cell tumor cell lines NTERA and NCCIT significantly increased PASKIN mRNA expression after treatment with 30 mmol/l glucose (data not shown). Generally, PASKIN mRNA levels were very low in all of these cell culture models, suggesting that they might not represent appropriate models for PASKIN expression.

The mouse *Paskin* gene is not transcriptionally activated by high glucose in cultured islets. To obtain a physiologically more relevant cell culture model, pancreatic islets were isolated from *Paskin* wild-type mice as well as from *Paskin* knockout mice containing a *lacZ-neo* fusion gene replacing exons 10 to 14 of the *Paskin* gene (7). Expression and activity of the β-galactosidase reporter gene in these mice was consistent with PASKIN mRNA levels, as analyzed by Northern blotting, demonstrating adequate representation of *Paskin* gene activity (7). However, staining of β-cells derived from *Paskin*$^{-/-}$ mice with X-gal did not reveal any signal compared with wild-type mice (Fig. 1*A*). In contrast, β-cells infected with adenovirus expressing a *lacZ* reporter gene (Fig. 1*A*), or testes derived from the same knockout but not from the

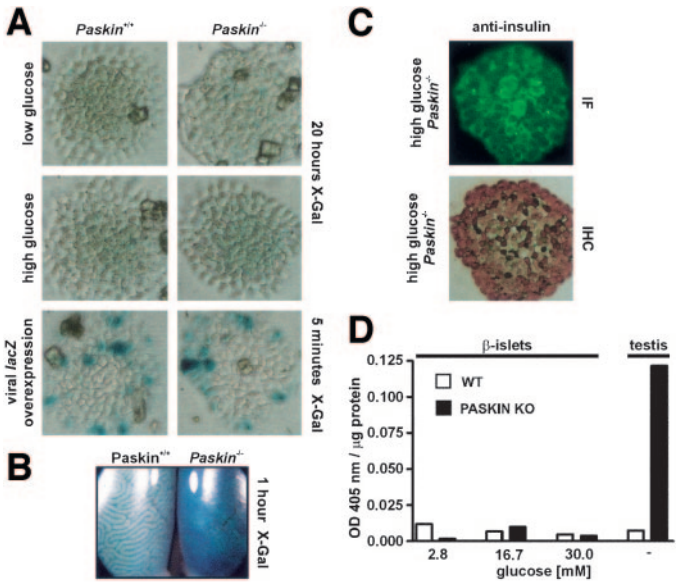

FIG. 1. *Paskin* gene expression in pancreatic islet β-cells derived from *Paskin* wild-type (WT) or knockout (KO) mice. Pancreata and testes were excised from the same male mice at 6–8 weeks of age. Islets were prepared, cultured under high- or low-glucose conditions, and stained as described in RESEARCH DESIGN AND METHODS. *A*: X-gal staining of islets to determine β-galactosidase activity derived from *lacZ* gene expression used as a reporter gene within the *Paskin* locus in *Paskin*$^{-/-}$ mice or derived from adenoviral *lacZ* expression as control. The different X-gal incubation times are indicated. *B*: Examples of a control X-gal staining of testes derived from the same animals. *C*: Examples of insulin immunodetection by immunofluorescence (IF) or immunohistochemistry (IHC), after X-gal staining, to confirm the identity and integrity of the β-cells. *D*: In another set of experiments, β-galactosidase activities were quantitatively determined by a colorimetric assay using protein extracts derived from pooled β-cells kept under different glucose concentrations as indicated, or from testes as control. The optical density (OD) 405 nm values were normalized to 1 μg of protein.

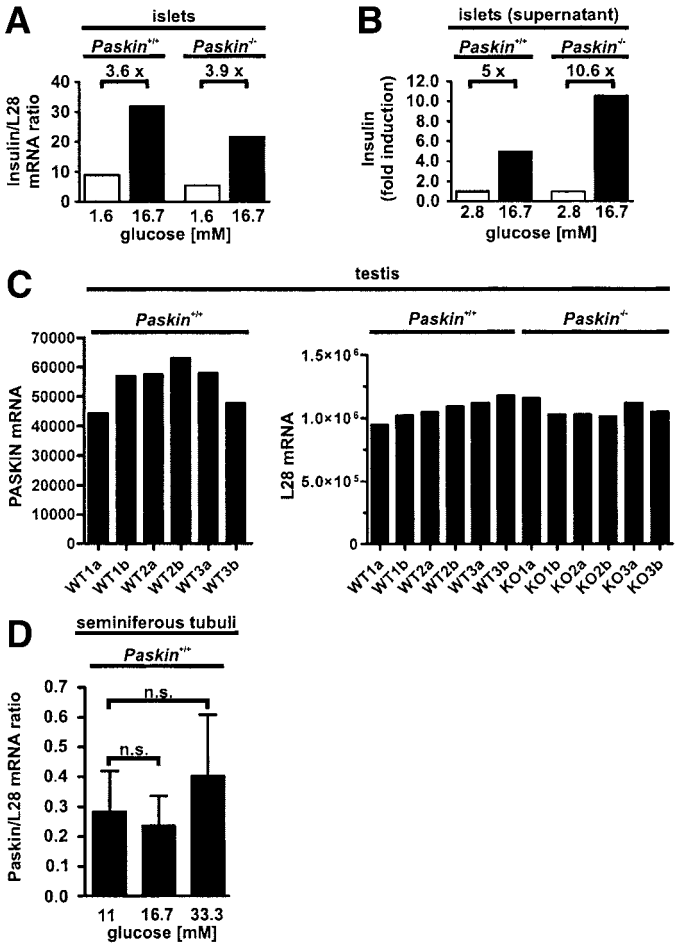

FIG. 2. Insulin gene expression and insulin secretion by pancreatic islet β-cells pooled from groups of eight *Paskin* wild-type (WT) or knockout (KO) mice. Pancreata and testes were excised from the same male mice at 6–8 weeks of age. Islets were prepared and then cultured under high- or low-glucose conditions, and insulin mRNA and protein secretion was determined by real-time RT-PCR and radioimmunoassay, respectively. Glucose induction of insulin mRNA (*A*) and insulin secretion (*B*) were equal in β-cells derived from *Paskin* wild-type and knockout mice. *C*: Example of simultaneous PASKIN mRNA determination in testes derived from the same mice as those used for islet preparation. "a" and "b" refer to left and right testis of the same animal. PASKIN mRNA was undetectable in knockout mice (not shown). *D*: Lack of PASKIN mRNA induction by glucose in cultured seminiferous tubules derived from 14-week-old mice. Data are the means ± SE of $n = 3$ independent experiments. n.s., not significant, with $P = 0.80$ and 0.65, respectively, using unpaired Student's t tests.

wild-type mice (Fig. 1*B*), readily stained blue. The identity of the primary β-cells was confirmed by immunostaining for insulin using immunofluorescence and immunohistochemistry (Fig. 1*C*). In another set of experiments, pools of islets were prepared from groups of six *Paskin*$^{+/+}$ or *Paskin*$^{-/-}$ mice, divided into aliquots of 20 islets, exposed to various glucose concentrations for 6 h, extracted, and assayed for specific β-galactosidase activities using an in vitro assay. As shown in Fig. 1*D*, only background levels could be detected in *Paskin*$^{-/-}$ islets, corresponding to *Paskin*$^{+/+}$ islets, which were not induced by treatment with high glucose concentrations. In contrast, simultaneously prepared testis extracts derived from *Paskin*$^{-/-}$ mice displayed at least 14-fold higher β-galactosidase activities than the background levels observed in *Paskin*$^{+/+}$ testes.

Insulin gene expression is independent of PASKIN in cultured primary β-cells. Because not only PASKIN mRNA levels, but also kinase activity, has been reported to be induced by high glucose (6), insulin gene expression could still be regulated in a PASKIN-dependent manner, even if the *Paskin* gene itself is not glucose-responsive. Therefore, pools of islets were prepared from groups of eight *Paskin*$^{+/+}$ or *Paskin*$^{-/-}$ mice, divided, and exposed to high or low glucose concentrations for 6 h, and mRNA levels were determined by real-time RT-PCR. However, neither glucose induction of insulin mRNA (Fig. 2*A*) nor insulin release into the supernatant (Fig. 2*B*) were dependent on the presence of a functional *Paskin* gene. The genotype of the mice was confirmed by simultaneous PASKIN RT-PCR using testis-derived RNA, which, as expected, had high PASKIN mRNA levels when isolated from *Paskin*$^{+/+}$ mice but undetectable levels when isolated from *Paskin*$^{-/-}$ mice (Fig. 2*C*). To test whether PASKIN mRNA could be induced by glucose in the major organ expressing PASKIN, seminiferous tubuli were excised from testis and cultured in vitro under various glucose conditions. Again, no significant glucose-dependent PASKIN mRNA induction could be observed by real-time RT-PCR (Fig. 2*D*). Of note, the copy number of PASKIN mRNA in testis is $\sim 1 \times 10^6$/μg total RNA, whereas it was below the detection limit in pancreatic islets (data not shown).

Normal glucose tolerance in *Paskin*$^{-/-}$ mice. To rule out that the in vitro cultivation of islets inadequately reflected PASKIN function, in vivo experiments were performed. First, endogenous PASKIN and insulin expression levels in organs derived from mice fed normal chow

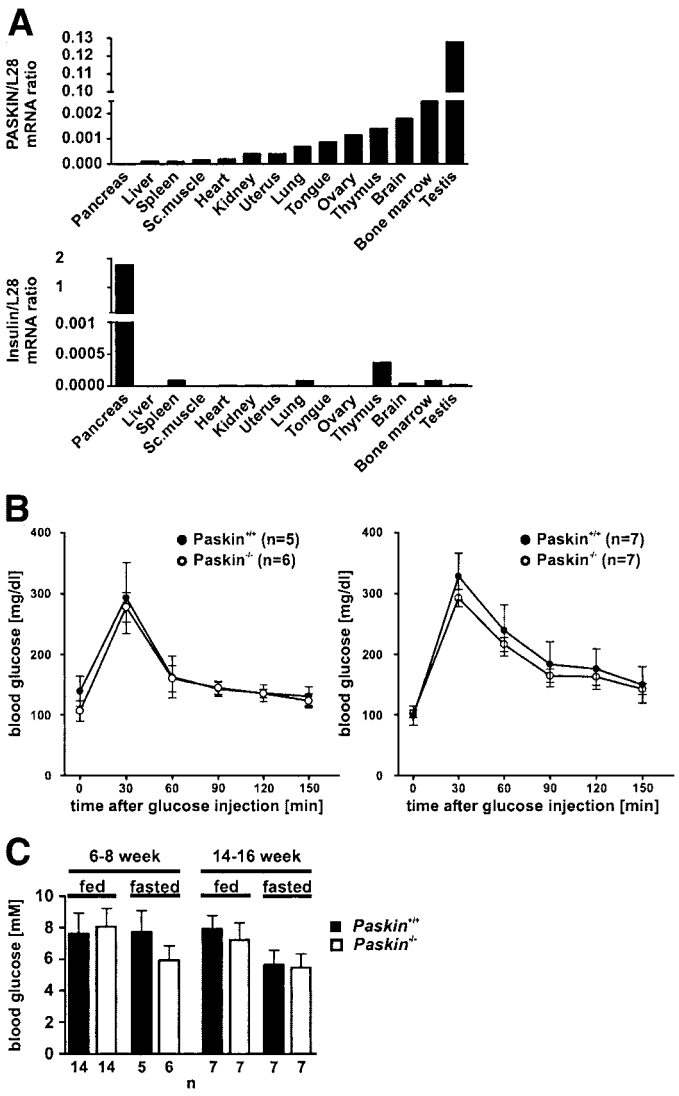

FIG. 3. Normal blood glucose concentrations and glucose tolerance in *Paskin* wild-type and knockout mice. *A*: PASKIN and insulin mRNA levels in different organs derived from C57BL/6 mice determined by real-time PCR. Sc. muscle, skeletal muscle. *B*: Intraperitoneal glucose tolerance tests in 6- to 8-week-old (*left panel*) or 14- to 16-week-old (*right panel*) *Paskin* wild-type or knockout mice. *C*: Blood glucose concentrations in 6- to 8-week-old or 14- to 16-week-old *Paskin* wild-type or knockout mice either fed ad libitum or fasted overnight for 16 h. Data are the means ± SD values of the indicated number (*n*) of male mice.

diet ad libitum were determined by real-time RT-PCR. As shown in Fig. 3*A* (top panel), PASKIN mRNA levels were at least 50-fold higher in the testis than in every other organ tested, confirming previous Northern blotting data (7). PASKIN mRNA was undetectable in pancreas. Although mRNA derived from pancreas, as expected, was generally of worse quality than that from other organs, PASKIN could not be detected even when 10-fold more RNA was used for the RT reactions. However, high insulin mRNA levels were found in the same pancreas cDNA samples (Fig. 3*A*, *bottom panel*).

Glucose (2 g/kg body wt) was injected intraperitoneally into 6- to 8-week-old (n = 5–6 per group) or 14- to 16-week-old (n = 7 per group) *Paskin*$^{+/+}$ or *Paskin*$^{-/-}$ mice, and blood glucose levels were followed at 30-min intervals for 2.5 h. There was no significant difference in glucose tolerance between *Paskin* wild-type and knockout mice in either of the age groups (Fig. *3B*). To examine basal blood glucose levels, groups of 6- to 8-week-old or 14- to 16-week-old mice were either fed ad libitum or fasted overnight for 16 h. As shown in Fig. *3C*, there was again no significant difference between *Paskin* wild-type and knockout mice, irrespective of their food supply.

DISCUSSION

PASKIN is highly expressed in the testis, and *Paskin* knockout mice display no obvious phenotype and normal fertility and life spans (7). Therefore, a recent report suggesting that PASKIN is required for glucose-induced PDX-1 and insulin gene expression was quite unexpected (6). We thus backcrossed our initial *Paskin* knockout strain 10 times with C57BL/6 inbred mice to obtain a more homogenous genetic background and repeated the experiments reported by da Silva Xavier et al. (6). We did not find PASKIN mRNA regulation by high glucose in different pancreatic β-cell or testicular cell lines, nor did we find them in isolated islets or seminiferous tubules. One explanation for this difference might be that we used real-time RT-PCR rather than the probably less reliable Northern blotting for mRNA determination. Although we were unable to demonstrate glucose-dependent induction of PDX-1 and insulin mRNA expression, glucose readily induced insulin release into the supernatant of MIN6 cells, and glucose also stimulated pyruvate kinase gene expression in INS-1E cells, demonstrating adequate cell culture conditions. Currently, we have no explanation for this discrepancy. We obtained MIN6 cells from three distinct sources, but we obtained similar results with all batches. However, probably more important than the cultured cell lines are the results with cultured islets. Importantly, increasing the ambient glucose concentration resulted in a similar increase in insulin mRNA and insulin secretion, whether the β-cells were derived from *Paskin* wild-type or knockout mice. This experiment probably provides the most convincing evidence that glucose-stimulated insulin gene expression is independent of PASKIN. Another argument was provided by glucose tolerance tests, which showed equal blood glucose clearance in young as well as older *Paskin* wild-type and knockout mice. We extended the determination of blood glucose levels to additional groups of mice fed with normal chow diet or fasted overnight, but again we could not observe any significant difference in blood glucose concentrations. Thus, these experiments demonstrated normal acute and chronic insulin function in *Paskin* knockout mice in vivo, further supporting our notion that insulin expression is independent of PASKIN.

PASKIN clearly has a metabolic function in yeast, and the regulation of glycogen synthase supports the idea of a similar function also in mammals (4,5). In addition, we recently identified enzymes of the glycolytic pathway as potential PASKIN targets (J. Tröger, K. Eckhardt, R.H. Wenger, unpublished observations). Thus, even when the PASKIN mRNA expression levels are rather low in all tissues except testis, a PASKIN function outside of the testis certainly cannot be excluded. However, considering its structural architecture, PASKIN kinase activity probably represents the metabolically regulated effector rather than PASKIN mRNA and/or protein induction. Although we can exclude glucose from being a direct activator of PASKIN in vitro (J. Tröger, K. Eckhardt, R.H. Wenger, unpublished observations), it appears to be likely that

another, yet unidentified, metabolic ligand induces PASKIN kinase activity and target protein phosphorylation. Such an activator also would explain the lack of an obvious phenotype in *Paskin* knockout mice. Like in yeast, PASKIN function might become apparent only under altered environmental conditions, such as nutrient stress. Hence, emphasis must be put on the identification of PASKIN-activating conditions. Apparently, high glucose alone is not sufficient to induce PASKIN.

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