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Abstract

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 beta-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 beta-geo fusion gene encoding beta-galactosidase reporter activity. Here we show that no 5-bromo-4-chloro-3-indolyl-ss-d-galactopyranoside (X-gal) staining was observed in islet beta-cells derived from Paskin knockout mice, irrespective of the ambient glucose concentration, whereas adenoviral expression of the lacZ gene in beta-cells showed strong X-gal staining. No induction of PASKIN mRNA could be detected in insulinoma cell lines or in islet beta-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 beta-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 beta-cells and that glucose-stimulated insulin production is independent of PASKIN.
Glucose-stimulated insulin production in mice deficient for the PAS kinase PASKIN

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Short title: PASKIN-independent insulin production

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ABSTRACT

The 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 knock-out mice by targeted replacement of the kinase domain with the β-geo fusion gene encoding β-galactosidase reporter activity. Here we show that no X-Gal staining was observed in islet β-cells derived from Paskin knock-out 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 following i.p. glucose injection were indistinguishable between Paskin wild-type and knock out 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.
INTRODUCTION

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 which might be regulated in cis by binding of so far unknown (metabolic?) ligands to the PAS domain (3). Following 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 the 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 \textit{in vivo} data were provided in this report.

We previously generated PASKIN null mice by targeted replacement of the kinase domain of the mouse \textit{Paskin} gene by a \textit{lacZ-neo} fusion construct in embryonic stem cells \( (7; 8) \). Surprisingly, PASKIN expression is strongly upregulated in post-meiotic 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 knock-out mice. To examine the role of PASKIN in glucose-stimulated insulin production, we used pancreatic β-cells derived from wild-type and knock out mice and performed glucose tolerance tests in these mice.
RESEARCH DESIGN AND METHODS

PASKIN-deficient mice. The generation and genotyping of PASKIN knock-out mice was described previously (7). Heterozygous Paskin\textsuperscript{+/−} mice were crossed with the C57BL/6 inbred strain for ten generations and then bred to homozygosity for the knock-out allele, containing the lacZ reporter gene. All animal handling followed the guidelines for the use and care of laboratory animals of the Bundesamt für Veterinärwesen and was approved by the Kantonales Veterinäramt Zürich (Nr. 192/2003).

Islet isolation and β-cell culture. Male Paskin\textsuperscript{+/+} and Paskin\textsuperscript{−/−} mice were sacrificed by cervical dislocation, the pancreas was excised and islets were either manually picked or isolated by gradient density centrifugation as described before (9). Briefly, 2 ml ice-cold collagenase solution (1 PZ-U/ml NB-8, 10 mM HEPES, 3.3 mg/ml DNaseI, 10 mM CaCl\textsubscript{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 to 14 minutes in a 37°C shaking waterbath. The digestion was stopped by adding ice-cold FCS-quenching buffer (10% FCS in 22 mM HEPES, HBSS). After two washing steps with BSA-quenching buffer (0.5% BSA in 22 mM HEPES, HBSS) and following centrifugation at 1200 rpm/2 minutes/4°C, the digested pancreas was filtered through medical gauze. Following centrifugation as above, the pellet was resuspended either in 35 ml RPMI1640 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 (1200 rpm/25 minutes/4°C) and the islets were collected from the interphase, diluted 1:1 with BSA-quenching buffer, centrifuged (1200 rpm/10 minutes/4°C), washed once in HBSS and resuspended in RPMI1640 supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, 11 mM d-glucose (Invitrogen, Basel, Switzerland). Medium was changed every 24 hours for 2 to 3 days until the islets spread and flattened on 35 mm plates coated with
extracellular matrix (ECM) 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 RPMI1640 as above. Before glucose stimulation, islets were starved in medium containing 1.6 mM glucose for 30 minutes, 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 hours at 37°C. The supernatant was collected for insulin measurements and the islets were prepared for X-Gal staining. Insulin concentrations were determined by RIA using the insulin-CT kit according to the instructions provided by the manufacturer (Schering, Baar, Switzerland).

**X-Gal staining and β-galactosidase assay.** Excised mouse testes or cultured, glucose-treated islets were fixed with 0.2% glutaraldehyde, 5 mM EGTA, 2 mM MgCl₂, 0.1 M Na-phosphate buffer pH 7.3, permeabilised with 0.01% Na-deoxycholate, 0.02% NP-40, 2 mM MgCl₂, 0.1 M Na-phosphate buffer pH 7.3, and incubated in X-gal solution (5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂, 0.02% NP-40, 0.01% Na-deoxycholate, 0.1 M Na-phosphate buffer pH 7.3, 0.1% X-Gal) at 37°C. β-Galactosidase activity was determined in tissue extracts by the ONPG assay and normalized to protein content as described before (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 (provided by S. Schweyer, Göttingen, Germany), mouse insulinoma MIN6 and the B1-MIN6 subline (provided by W. Moritz, Zürich, Switzerland; J. Rutter, Utah, USA; and P.A. Halban, Geneva, Switzerland, respectively), and rat insulinoma INS-1E (provided by C.B. Wollheim, Geneva, Switzerland) cell lines were cultured in high glucose Dulbecco's modified Eagle's
medium (Sigma) as described previously (12). The glucose concentration was lowered to 3 mM for 16 hours before incubation of cells with 3 mM or 30 mM glucose for 6 hours.

**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-qPCR. Briefly, 8 µg of total RNA was reverse transcribed with StrataScript III (Stratagene, Amsterdam, The Netherlands) and qPCR was performed in duplicates with 8% of the cDNA reaction mixture using a SYBRGreen qPCR 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'-ggaactgtccagtttcctg-3' and hPASKINrev 5'-ggaactgtccgtaatgacca-3'; mPASKINfwd 5'-agggtccagaaggtcgaagag3' and mPASKINrev 5'-tgaactgtccagatctctctg-3'; rPASKINfwd 5'-tgggactgtcaggtgagtgtgagtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtgagtgtg
goat serum (NGS) in PBS and stained with polyclonal guinea-pig anti-insulin antibodies (Sigma) diluted 1:100 in 1% NGS in PBS for 30 minutes at 37°C. Bound antibodies were detected with FITC-conjugated goat anti-guinea-pig antibodies at 1:20 dilution (Sigma). The ECM plates were mounted with Kaiser's glycerol gelatine (Merck, Darmstadt, Germany) and analyzed by fluorescence microscopy.

Intraperitoneal glucose tolerance tests. Glucose tolerance tests were performed in 6-8 and 14-16 weeks old male mice. Mice were fasted overnight (16 hours) and injected intraperitoneally with a saline glucose solution of 2 g/kg body weight. Blood was obtained by puncturing the tail vein and glucose levels were measured using ACCU-CHEK Aviva (Roche Diagnostics, Basel, Switzerland). Measurements were performed before and 30, 60, 90, 120 and 150 minutes 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, following exposure to high glucose levels (30 mM) for 6 hours, we could not confirm PASKIN mRNA induction in mouse MIN6 cells or rat INS-1E cells, while insulin secretion by MIN6 cells 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 testis-derived mouse spermatogonia GC-1 and spermatocyte GC-2 nor human testicular germ cell tumor cell lines NTERA and NCCIT significantly increased PASKIN mRNA expression following treatment with 30 mM glucose (data not shown). Generally, the 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 knock-out 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<sup>−/−</sup> mice with X-Gal did not reveal any signal when compared with wild-type mice (Fig. 1A). In contrast, β-cells infected with adenovirus expressing a lacZ reporter gene (Fig. 1A), or testes derived from the same
knock-out but not from the wild-type mice (Fig. 1B), readily stained blue. The identity of the primary β-cells was confirmed by immunostaining for insulin using immunofluorescence and immunohistochemistry (Fig. 1C). In another set of experiments, pools of islets were prepared from *groups of* 6 *Paskin*^+/+ or *Paskin*^−/−* mice, split into aliquots of 20 islets, exposed to various glucose concentrations for 6 hours, extracted and assayed for specific β-galactosidase activities using an *in vitro* assay. As shown in Fig. 1D, 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* 8 *Paskin*^+/+ or *Paskin*^−/−* mice, split, exposed to high or low glucose concentrations for 6 hours, and mRNA levels were determined by real-time RT-PCR. However, neither glucose-induction of insulin mRNA (Fig. 2A) nor insulin release into the supernatant (Fig. 2B) 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. 2C). 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. 2D). Of note, the copy number of PASKIN mRNA in
testis is approx. 1x10^6/µg total RNA whereas it was below the detection limit in pancreatic islets (data not shown).

**Normal glucose tolerance in Paskin^-/- mice.** In order 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 diet *ad libitum* were determined by real-time RT-PCR. As shown in Fig. 3A (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. While mRNA derived from pancreas, as expected, generally was of worse quality than 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. 3A, bottom panel).

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

PASKIN is highly expressed in the testis and *Paskin* knock-out mice display no obvious phenotype and a normal fertility and lifespan (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 back-crossed our initial *Paskin* knock-out strain ten times with C57BL/6 inbred mice in order to obtain a more homogenous genetic background and repeated the experiments reported by da Silva Xavier et al. (6). We neither found PASKIN mRNA regulation by high glucose in various pancreatic β-cell or testicular cell lines, nor in isolated islets or tubuli seminiferi. 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.

While 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 do have no explanation for this discrepancy.

We obtained MIN6 cells from three distinct sources, but 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 knock-out 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 knock-out mice. We extended the determination of blood glucose levels to additional groups of mice fed with normal chow diet or fasted overnight, but again could not observe any significant difference in blood glucose concentrations. Thus these experiments demonstrated normal acute and chronic insulin
function in *Paskin* knock-out 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 (Tröger J., Eckhardt K., Wenger R.H.; 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. While we can exclude glucose from being a direct activator of PASKIN *in vitro* (Tröger J., Eckhardt K., Wenger R.H.; 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* knock-out mice. Like in yeast, PASKIN function might become apparent only under altered environmental conditions such as nutrient stress. Emphasis hence must be put on the identification of PASKIN-activating conditions. Apparently, high glucose alone is not sufficient to induce PASKIN.

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REFERENCES


FIGURE LEGENDS

FIG. 1. *Paskin* gene expression in pancreatic islet β-cells derived from *Paskin* wild-type or knock-out 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 reporter gene within the *Paskin* locus in the *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), following 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 various glucose concentrations as indicated, or from testes as control. The OD_{405} 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 8 *Paskin* wild-type (WT) or knock-out (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 insulin mRNA and protein secretion determined by real-time RT-PCR and RIA, respectively. Glucose induction of insulin mRNA (*A*) and insulin secretion (*B*) were equal in β-cells derived from *Paskin* wild-type and knock-out mice. *C*: Example of simultaneous PASKIN mRNA determination in testes derived from the same mice as used for islet preparation. "a" and "b" refer to left and right testis of the same animal. PASKIN mRNA was undetectable in knock-out mice (not shown). *D*: Lack of PASKIN
mRNA induction by glucose in cultured tubuli seminiferi derived from 14 week old mice (mean ± SEM of n = 3 independent experiments; n.s., not significant with p=0.80 and 0.65, respectively, using unpaired t-tests).

FIG. 3. Normal blood glucose concentrations and glucose tolerance in *Paskin* wild-type and knock-out mice. A: PASKIN and insulin mRNA levels in various organs derived from C57BL/6 mice determined by real-time RT-PCR. B: Intraperitoneal glucose tolerance tests in 6-8 or 14-16 weeks old *Paskin* wild-type or knock-out mice. C: Blood glucose concentrations in 6-8 or 14-16 weeks old *Paskin* wild-type or knock-out mice either fed *ad libitum* or fasted overnight for 16 hours (shown are mean ± SD values of the indicated number n of male mice).
Figure 2

A: Islets

- **Paskin**
  - 3.6 x
- **Paskin**
  - 3.9 x

B: Islets (supernatant)

- **Paskin**
  - 5 x
- **Paskin**
  - 10.6 x

C: Testis

- **Paskin**
  - Bar graph
- **Paskin**
  - Bar graph

D: Seminiferous tubuli

- **Paskin**
  - Bar graph

- n.s.

Glucose [mM]: 1.6, 16.7, 33.3
Figure 3

A

[Graph showing mRNA ratio for different tissues.]

B

6-8 week old mice

- Paskin\textsuperscript{−/−} (n=5)
- Paskin\textsuperscript{+} (n=6)

14-16 week old mice

- Paskin\textsuperscript{−/−} (n=7)
- Paskin\textsuperscript{+} (n=7)

C

6-8 week

- fed
- fasted

14-16 week

- fed
- fasted

<table>
<thead>
<tr>
<th>Blood glucose [mg/dL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 14 5 6 7 7 7 7</td>
</tr>
</tbody>
</table>

\textsuperscript{−/−} Paskin\textsuperscript{+}