Human Mutation within Per-Arnt-Sim (PAS) Domain-containing Protein Kinase (PASK) Causes Basal Insulin Hypersecretion*[S]

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Background: The glucose sensor PAS kinase (PASK) plays a fundamental role in pancreatic islet physiology.
Results: A single amino acid substitution in the human PASK kinase domain stimulates enzyme activity and increases insulin secretion at low glucose.
Conclusion: A rare, naturally occurring mutation in PASK modulates insulin release in man.
Significance: We provide direct genetic evidence for a role for PASK in controlling insulin secretion in man.

PAS kinase (PASK) is a glucose-regulated protein kinase involved in the control of pancreatic islet hormone release and insulin sensitivity. We aimed here to identify mutations in the *PASK* gene that may be associated with young-onset diabetes in humans. We screened 18 diabetic probands with unelucidated maturity-onset diabetes of the young (MODY). We identified two rare nonsynonymous mutations in the *PASK* gene (p.L1051V and p.G1117E), each of which was found in a single MODY family. Wild type or mutant PASKs were expressed in HEK 293 cells. Kinase activity of the affinity-purified proteins was assayed as autophosphorylation at amino acid Thr^{307} or against an Ugp1p-derived peptide. Whereas the PASK p.G1117E mutant displayed a ∼25% increase with respect to wild type PASK in the extent of autophosphorylation, and a ∼2-fold increase in kinase activity toward exogenous substrates, the activity of the p.L1051V mutant was unchanged. Amino acid

Gly^{1117} is located in an α helical region opposing the active site of PASK and may elicit either: (*a*) a conformational change that increases catalytic efficiency or (*b*) a diminished inhibitory interaction with the PAS domain. Mouse islets were therefore infected with adenoviruses expressing wild type or mutant PASK and the regulation of insulin secretion was examined. PASK p.G1117E-infected islets displayed a 4-fold decrease in glucose-stimulated (16.7 *versus* 3 mM) insulin secretion, chiefly reflecting a 4.5-fold increase in insulin release at low glucose. In summary, we have characterized a rare mutation (p.G1117E) in the *PASK* gene from a young-onset diabetes family, which modulates glucose-stimulated insulin secretion.

Per-ARNT-Sim (PAS)^5 domain-containing serine/threonine protein kinase (PASK) is the only protein kinase so far known in mammals to harbor a PAS domain (1, 2). By contrast, many members of this family (mostly histidine kinases) have been described and extensively characterized in prokaryotes. PAS domains appear to act as sensors of diverse physical environmental stimuli (light, oxygen partial pressure, and redox poten-

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[5] The abbreviations used are: PAS, Per-ARNT-Sim; Per, Period (*Drosophila melanogaster* circadian protein); ARNT, aryl hydrocarbon receptor nuclear translocator; Sim, single-minded (*D. melanogaster*); PASK, Per-ARNT-Sim domain-containing protein kinase; MODY, maturity-onset diabetes of the young; Ugp1p, UDP-glucose pyrophosphorylase protein; p.L1051V, mutation of leucine residue 1051 to valine; p.G1117E, mutation of glycine residue 1117 to glutamic acid; GSIS, glucose-stimulated insulin secretion; *Pask$^{-/-}$*, homozygous *Pask* knock-out; *Pask$^{+/+}$*, homozygous *Pask* wild type; hPASK, human PASK protein; Pdx-1, pancreatic duodenum homeobox-1; MIN6 β cells, mouse insulinoma β cells; Akt, serine/threonine protein kinase Akt; PKA, protein kinase A; MODY, maturity-onset diabetes of the young; Luc, luciferase.

tial) and chemical signals, functioning as interaction hubs (3). In an attempt to clarify the physiological functions of PASK, whole body knock-out mice were generated (4), but their phenotype was initially described overall as normal (5). Subsequently, these mice were shown to display impaired glucose-stimulated insulin secretion (GSIS) (6). As a consequence, plasma insulin levels in $Pask^{-/-}$ mice were slightly but significantly lower with respect to wild type mice. On the other hand, when fed a high-fat diet, $Pask^{-/-}$ mice showed improved glucose tolerance and increased insulin sensitivity. Furthermore, these animals were protected from high-fat diet-induced metabolic symptoms including obesity, hepatic steatosis, and insulin resistance (6).

We have previously investigated a possible role for PASK in pancreatic endocrine cells and observed that, in MIN6 β cells and rat islets, $Pask$ mRNA expression and kinase activity increase at high glucose concentrations. Furthermore PASK is necessary for the glucose-stimulated expression of both pre-pro-insulin (*INS*) and pancreatic duodenum homeobox-1 (*Pdx-1*) genes (7).

Interestingly, it was later demonstrated that palmitate inhibits the induction by glucose of *Pask* expression in rat pancreatic islets and that *hPASK* overexpression is capable of preventing the inhibitory effect of palmitate on insulin and PDX1 expression (8). Examination of the response of $Pask^{-/-}$ mice to hypoxia has suggested that Pask may be involved in the neural regulation of the hypoxic ventilatory response (9).

We have recently shown that, in human pancreatic islets, *PASK* mRNA expression is increased at high glucose concentrations, and that *PASK* mRNA levels are decreased in human pancreatic islets derived from type 2 diabetic patients compared with nondiabetic donors (10). At the same time, we measured *Pask* mRNA levels in FACS-purified mouse pancreatic α and β cells separately. Of note, α cells expressed almost 3-fold more *Pask* mRNA with respect to β cells. Moreover, plasma glucagon was significantly higher in $Pask^{-/-}$ versus $Pask^{+/+}$ mice. By analyzing the regulation by glucose of glucagon secretion in islets extracted from $Pask^{-/-}$ mice we also identified a defective repression of glucagon secretion in response to high glucose concentrations (10).

PASK contains a structurally validated PAS domain at its N terminus. The NMR structure of the PAS domain from human PASK reveals a left-hand glove-like fold capable of binding small organic compounds containing one or two aromatic rings (11). The fold of the PASK PAS domain appears to be more flexible as compared with other PAS domains. In fact, the PASK PAS domain can directly bind and inhibit the kinase domain, even when supplied in *trans* (11). We have thus proposed that binding of a ligand to the PAS domain releases the kinase domain enabling substrate binding and catalysis.

So far, the endogenous physiological cofactor for the PAS domains of PASK has not been defined. Phosphatidic acid and monophosphorylated phosphoinositides have recently been found to bind and affect PASK activity *in vitro* (12), but their physiological significance is yet to be demonstrated. The *Saccharomyces cerevisiae* PASK orthologs Psk1 and Psk2 are able to influence the fate of glucose between storage (as glycogen) and utilization for the cell wall biosynthesis (as glucan) by phos-

phorylating UDP-glucose pyrophosphorylase (Ugp1) and relocating the enzyme to the cell periphery (13, 14). Numerous *in vitro* substrates for PASK have been described in mammals even very recently (12, 15). One of the first to be reported was glycogen synthase, which can interact with PASK and is negatively regulated by PASK phosphorylation (16). Nevertheless, the physiological significance of these PASK targets has not been fully elucidated as yet.

Here we describe two rare nonsynonymous mutations of the human *PASK* gene (chromosome 2q37), each of which has been identified in a single MODY family of unelucidated etiology. MODY is a dominantly inherited form of nonautoimmune early onset diabetes (17). Only one mutation, p.G1117E, seemed to be associated with early onset diabetes in a small family, but did not show a complete co-segregation with diabetes in this family, arguing against a direct disease-causing effect. We cloned and purified recombinant PASK proteins carrying the mutations and analyzed their catalytic activity compared with wild type PASK. We demonstrate that kinase activity is increased for one of the two mutants. Subsequently, we demonstrated that glucose-stimulated insulin secretion from mouse islets transduced with adenovirus expressing the PASK p.G1117E mutant was significantly impaired compared with wild type PASK. We thus propose a model whereby dysregulated PASK may contribute either positively or negatively to the diabetic phenotype, and/or may modulate the impact of other as yet unidentified pathological mutations. Importantly, these findings demonstrated the ability of a naturally occurring PASK mutant in man to regulate insulin secretion.

EXPERIMENTAL PROCEDURES

Subjects and Mutation Identification—We studied 18 young-onset diabetic probands of French families (except one of Mauritian ancestry) with clinically defined MODY on three criteria: diabetes diagnosed before age 30 (except for three subjects who were diagnosed at ages 33 and 36 years; median of age at diagnosis: 21.5 years), no requirement of exogenous insulin in the two first years, and an autosomal dominant inheritance of type 2 diabetes (18). These probands were negative for *GCK*/MODY-2 or *HNF1A*/MODY-3 mutations; and 15 were negative for a mutation in *HNF4A*/MODY-1 and *PDX1*/MODY-4, which are a very rare cause of MODY in the French patients (18, 19). They were also tested negative for serological markers of type 1 diabetes. The 17 coding exons of the *PASK* gene were screened for mutations by double strand direct sequencing from a genomic DNA sample of the patients, using a standard protocol (19). Up to 719 adult normoglycemic individuals of French origin were sequenced to assess the prevalence of the identified mutations. The local ethics committee approved the genetic study and all participating patients or their parents gave written informed consent for genetic testing.

Cell Culture and Reagents—Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml of penicillin, and 100 IU/ml of streptomycin.

Anti-myc Immunoprecipitation and AMARA Peptide Kinase Assay—HEK 293T cells were transfected using the calcium phosphate method with plasmids encoding C-terminal c-*myc*-tagged PASK wild type and mutant. Anti-c-*myc* (Roche Diagnostics) immunoprecipitation and AMARA (20) peptide kinase assays were conducted as described (7).

Mutagenesis—Plasmid JR688, *i.e.* pcDNA3-PASK wild type-c-*myc*, encoding wild type human PASK with a c-*myc* tag and plasmid JR703, *i.e.* pcDNA3-PASK kinase inactive p.K1028R-c-*myc*, have previously been described (2). pcDNA3-PASK p.L1051V-c-*myc* and pcDNA3-PASK-p.G1117E-c-*myc* mutant plasmids were generated using a QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions. C-terminal FLAG-HA-tagged human PASK was subcloned into the pQXCIN vector (BD Biosciences). PASK p.K1028R, p.L1051V, and p.G1117E mutants were generated by site-directed mutagenesis using the SOEing PCR-based method (21).

Immunoprecipitation of PASK-FLAG-HA Variants from HEK 293T Cells—HEK 293T cells transiently transfected with the indicated PASK-FLAG-HA plasmid were lysed in lysis buffer (50 mM Hepes, pH 7.6, 150 mM NaCl, 20 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 10 mM sodium fluoride, 0.1% Triton X-100 supplemented with phosphatase inhibitor mixtures 1 and 2 (Sigma) and protease inhibitor mixture (Sigma)). Cell lysates were centrifuged and incubated for 2 h (for PASK autophosphorylation) or overnight (for kinase assays) at 4 °C with anti-FLAG M2 affinity gel (Sigma). The immune complexes were washed five times with lysis buffer. For determination of PASK autophosphorylation, PASK-FLAG-HA variants were eluted using SDS-PAGE sample buffer, separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-FLAG (Sigma) and anti-pPASK (phospho-Akt substrate antibody; Cell Signaling Technology). Immunoblots were analyzed using the LI-COR Odyssey™ imaging system and results are expressed as a ratio of the signal obtained with anti-pPASK antibody to that obtained with the anti-FLAG antibody. For purification of PASK-FLAG-HA variants for kinase activity assays, variants were eluted with 1 mg/ml of FLAG peptide. Samples of the final eluates were subjected to Western (immuno-) blotting with anti-FLAG antibody (Sigma) and quantified and normalized using the LI-COR Odyssey imaging system.

PASKtide Kinase Assay—PASK activity was measured by its ability to phosphorylate PASK synthetic peptide (PASKtide: KKKHTKTHS*TYAFE). The reactions were started by the addition of assay buffer (50 mM Hepes, pH 7.5, 1 mM DTT) containing 200 μM [^{32}P]ATP (PerkinElmer Life Sciences), 5 mM MgCl$_2$, and 200 μM PASKtide to equal amounts of the indicated PASK-FLAG-HA variants. Reactions were incubated at 30 °C for 15 min and were terminated by applying the reaction mixture on 1-cm squares of P81 paper followed by transfer to 1% (v/v) orthophosphoric acid. P81 papers were allowed to air dry and radioactivity was measured using liquid scintillation counting.

Preparation of Recombinant Adenoviruses and Viral Infection—Recombinant adenoviruses expressing wild type and mutant forms of human PASK (kinase inactive mutant p.K1028R, and the two rare coding human variants p.L1051V and p.G1117E) and control adenovirus expressing green fluorescent protein (Ad-GFP) were prepared using the AdEasy system (22) as previously described (23).

Animals—CD1 mice were housed with five mice per cage in a pathogen-free facility and were fed *ad libitum* with a standard mouse chow diet. Female mice were used at 10–12 weeks of age and sacrificed by cervical dislocation as approved by the United Kingdom Home Office Animal Scientific Procedures Act, 1986.

Islet Isolation and GSIS—Islets were aseptically isolated by collagenase digestion of the pancreas of 15–20-week-old female CD1 mice and purified as described (24). After mice were sacrificed by cervical dislocation, collagenase (Serva, 1 mg/ml in RPMI) was injected into the pancreatic duct (5 ml/mouse) (25). The distended pancreas was then incubated at 37 °C for 10 min. Digested pancreata were washed two to three times with RPMI and islets were separated from exocrine tissues by centrifugation (2500 $\times$ *g* for 20 min) in Histopaque (Sigma) at different densities (26) before handpicking. Isolated islets were further incubated in RPMI medium supplemented with 2 mM glutamine, 100 units/ml of penicillin, and 100 units/ml of streptomycin and 10% (v/v) heat-inactivated fetal bovine serum for 24–72 h before adenoviral infection and measurement of *in vitro* insulin secretion. Islets were infected as described (27) with adenoviruses encoding for PASK or enhanced green fluorescent protein (GFP) at a multiplicity of infection of 100 and cultured for 72 h prior to use. For *in vitro* measurement of glucose- and KCl-stimulated insulin secretion, 10 size-matched islets were preincubated for 1 h at 37 °C in Krebs-Ringer buffer (in mmol/liter: 130 NaCl, 3.6 KCl, 1.5 CaCl$_2$, 0.5 MgSO$_4$, 0.5 KH$_2$PO$_4$, 2 NaHCO$_3$, 10 HEPES, and 0.1% (w/v) BSA, pH 7.4) containing 3 mmol/liter of glucose, with gentle agitation (120 rpm). Islets were further incubated for 2 h at 37 °C in KRB solution in the presence of 3 or 16.7 mmol/liter of glucose and supernatant fractions containing secreted insulin were collected. For total insulin extraction, islets were lysed in acid/ethanol/Triton solution (1.5% (v/v) HCl, 75% (v/v) ethanol, 0.1% (v/v) Triton X-100). Secreted and total insulin levels were measured using radioimmunoassay with competitive ^{125}I-labeled insulin (Millipore, Watford, UK). Triplicate experiments were performed for each condition.

Immunohistochemistry—CD1 mouse isolated islets were infected with PASK-expressing or GFP-expressing only (null) adenovirus and treated for sectioning with a cryostat as described in Ref. 28. After permeabilizing sections in 0.1% Triton X-100 overnight at 4 °C, immunostaining was carried out using the following antibodies: mouse monoclonal anti-glucagon antibody (1:200; Sigma), polyclonal anti-swine insulin (1:200; DakoCytomation, Ely, UK), and Alexa 564- or 633-coupled secondary antibodies (1:1000; Molecular Probes), and coverslips were mounted in VECTASHIELD (Vector Laboratories) hardset medium with DAPI. Isolated infected islet sections were then imaged using a Leica SP2 upright confocal microscope with an HC PL APO 20 $\times$ 0.70 CS objective and the following laser lines: Arg ion (488 nm), HeNe (633 nm), 561 nm diode and UV (350 nm) controlled by Leica LAS AF Lite™ software. Acquired images were analyzed using Volocity™ software (Improvision).
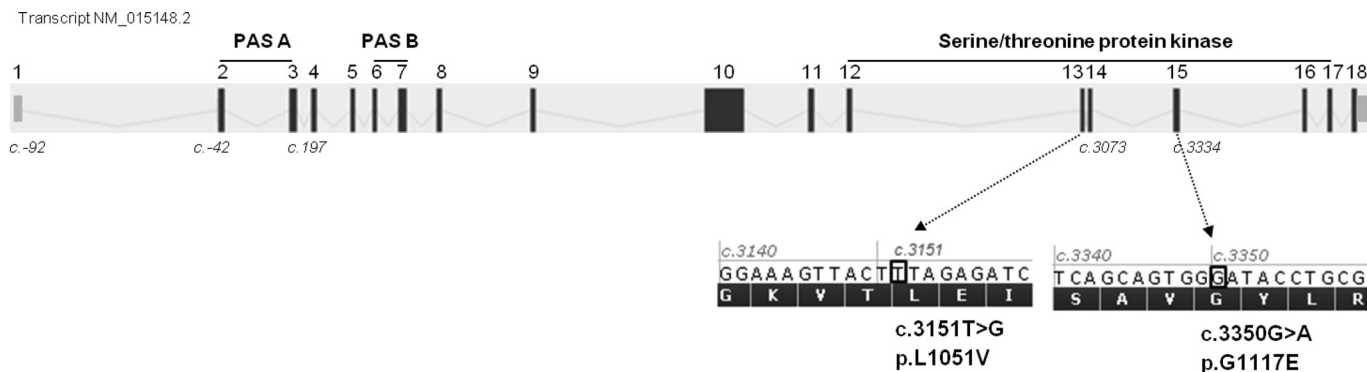
FIGURE 1. **Exon/intron and corresponding protein domain structure of the human *PASK* gene (chromosome 2q37) with the two rare mutations in evidence.** The exons are depicted by *black boxes* (the *gray* indicates untranslated exons) and are numbered from 1 to 18, according to PASK transcript NM_015148.2. The *numbers below* exons 1–3, 13, and 15 indicate the position of the first nucleotide in the cDNA sequence of transcript NM_015148.2, and in *parentheses* the position of the first amino acid codon for those exons. Edited from the Ensembl human gene annotation (Ensembl release 62, April 201). The characterized protein domains are *underlined above* the gene diagram. The location of the two identified mutations (c.3151T>G/p.L1051V and c.3350G>A/ p.G1117E) is depicted within exons 13 and 15 of the *PASK* gene, with the position of the nucleotide change indicated as well as the corresponding amino acid substitution.

Glucagon Secretion—CD1 mouse isolated islets were either left uninfected as a control or infected with adenoviruses encoding either for PASK wild type and mutants or GFP, for 48 h at a multiplicity of infection of 100 units/cell before glucagon assay. To measure glucagon secretion in the same conditions as described above for insulin secretion, islets (15–20 per condition, size-matched) were preincubated in modified Krebs-Ringer bicarbonate Hepes (KBH) solution (mmol/liter: 130 NaCl, 3.6 KCl, 1.5 $CaCl_2$, 0.5 $MgSO_4$, 0.5 KH_2PO_4, 2 $NaHCO_3$, 10 Hepes, 1.5 $CaCl_2$, and 0.1% (w/v) BSA (pH 7.4 with NaOH), equilibrated for 20 min with O_2/CO_2 (95:5 (v/v)) at 37 °C) containing 3 mmol/liter of glucose for 1 h min before being incubated in KBH solution containing 3 or 17 mM glucose for a further 2 h at 37 °C with gentle agitation. To measure glucagon secretion at permissive glucose concentration (0.5 mM) islets were preincubated in KBH containing 10 mmol/liter of glucose for 30 min at 37 °C with gentle agitation, before being transferred into KBH solution containing 10 or 0.5 mM glucose for 1 h at 37 °C with gentle agitation. In both sets of experiments (3–17 mM and 10–0.5 mM) at the end of the incubation, supernatant (0.5 ml) containing secreted glucagon was collected and total glucagon content was extracted from cells or islets into 0.5 ml of acid/ethanol/Triton solution (1.5% (v/v) HCl, 75% (v/v) ethanol, 0.1% (v/v) Triton X-100) for glucagon measurement using an immunoradioassay with ^{125}I-labeled glucagon (Glucagon RIA kit; Millipore).

Arterial Delivery of Adenoviral Vectors and Islet Isolation— Male Wistar rats (225–275 g) (Charles River Laboratories, Wilmington, MA) were housed on a 12-h light/dark cycle with free access to standard laboratory rat diet and water. All procedures were approved by the Institutional Committee for the Protection of Animals at the Centre Hospitalier de l'Université de Montréal. Wistar rats were subjected to laparotomy under general anesthesia. After ligation of the portal vein, the superior aorta, and the hepatic, mesenteric, and right and left renal arteries, adenoviruses (4×10^{10} pfu/pancreas) encoding luciferase (Luc), wild type hPASK (WT), or hPASK mutants (G1117E and L1051V) were injected in the celiac trunk of the inferior aorta as previously described (8, 29). Rat islets of Langerhans were isolated as described (8), pre-cultured for 16 h at 2.8 mmol/liter

glucose, and then exposed for 24 h to 2.8 or 16.7 mmol/liter of glucose.

Quantitative Real-time PCR—Total RNA was extracted from aliquots of 150 islets each and real-time PCR was carried out using Rotor-Gene SYBR Green PCR kit (Qiagen Inc., Mississauga, ON) as described (8). Specific rat primer sets for *prepro-insulin 2* pre-mRNA r*Pre-Ins2* (forward, AGCGTGGATTCT-TCTACACACC; reverse, AAGGATTCTCACTCCTTCT-TGG), r*Pdx-1* (forward, GAACCGGAGGAGAATAAGAGG; reverse, AGTCAAGTTGAGCATCACTGC), and *β-actin* (forward, TGAAGTGTGACGTTGACATCC; reverse, AGAGTG-AGGCCAGGATAGAGC) were designed using Primer3 (30). Results are expressed as the ratio of target mRNA to *β-actin* mRNA. Data are expressed as mean ± S.E. Gene expression levels at 16.7 mmol/liter of glucose were compared with the values at 2.8 mmol/liter of glucose for each construct using unpaired Student's *t* test. $p < 0.05$ was considered significant.

Statistics—Data are expressed as mean ± S.E. Significance was tested by two samples of unpaired or paired Student's *t* test using Excel, or by analysis of variance using GraphPad Prism 4.0. A value of $p < 0.05$ was considered significant.

RESULTS

Identification of Two Mutations in Human PASK Gene—By sequencing all 17 coding exons of *PASK* in 18 MODY patients, who are registered in our French collection of MODY families as being of unknown molecular etiology for the disease, we identified two rare heterozygous nonsynonymous mutations: c.3151T>G (in exon 13)/p.L1051V and c.3350G>A (in exon 15)/p.G1117E (Fig. 1). Three synonymous variants (p.K1019K, p.H1061H, and p.S1086S) were also observed. The p.L1051V mutation (Fig. 2*B*) was found in a patient diagnosed with diabetes at 20 years requiring an oral hypoglycaemic treatment by sulfonylurea, but did not show a cosegregation with diabetes in the proband family; only three diabetic relatives out of seven affected subjects over three generations are carriers of the mutation, as well as one 42-year-old normoglycemic individual. The mutation was also found in one out of 371 adult normoglycemic individuals of French origin tested as controls (minor allele frequency: 0.13%).
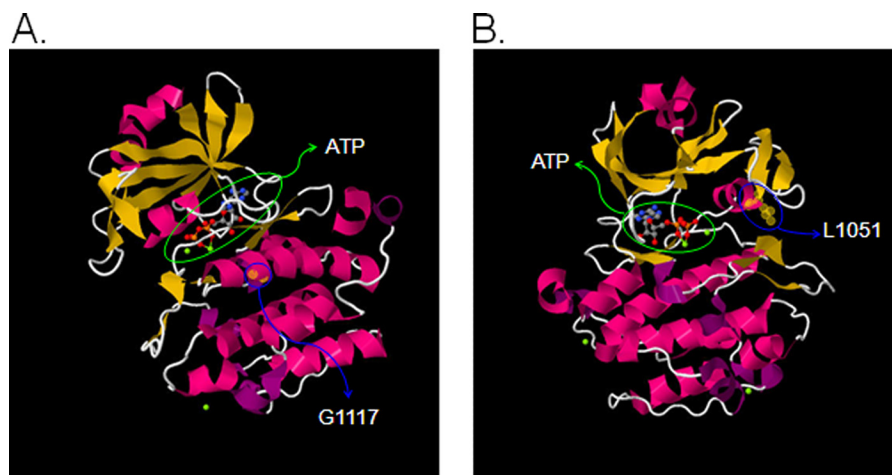
FIGURE 2. **The recently resolved crystal structure of the PASK kinase domain bound to ADP with mutated residues Leu[1051] and Gly[1117] shown.** In the *left panel* is a view from the back of a PASK kinase domain (amino acids 977–1300) crystal structure (PDB code 3DLS) with the position of residue Gly[1117] and the ADP bound molecule in evidence. An equivalent image is shown in the *right panel* for Leu[1051].

The p.G1117E mutation (Fig. 2*A*) has been identified in another MODY family, and is present in four relatives over three generations. Three of the mutation carriers were diagnosed with either impaired fasting glucose (fasting plasma glucose between 6.10 and 7.0 mmol/liter) at ages 5 and 14 or overt diabetes at 28 years, whereas a child inherited the mutation but showed euglycemia at 9 years. However, a 13-year-old girl presenting with impaired fasting glucose (fasting plasma glucose = 6.22 mmol/liter) does not carry the p.G1117E mutation. Of note, this mutation appears to be very rare, as found in only one of 719 adult normoglycemic controls (minor allele frequency: 0.07%); the mutation carrier showed a normal fasting plasma glucose of 5.20 mmol/liter at age 70 years. This mutation was not present in 492 adult diabetic patients from our French type 2 diabetes family collection.

PASK p.G1117E Mutant Shows Increased in Vitro Kinase Activity—To test initially whether we could measure any change in the kinase activity of PASK p.L1051V and p.G1117E mutants with respect to the wild type or the kinase-inactive mutant p.K1028R, we expressed the four PASK proteins with a C-terminal c-*myc* tag individually in HEK 293 cells. We then performed *in vitro* kinase assays using the AMARA peptide (sequence: AMARAASAAALARRR) [20]. We observed (Fig. 3*A*) an almost 2-fold increase in phosphate incorporation catalyzed by the PASK p.G1117E mutant relative to the wild type protein. We did not observe any significant change in PASK p.L1051V activity compared with wild type. This preliminary result prompted us to conduct further measurements.

In light of the above findings we therefore generated plasmids encoding C-terminal dual FLAG-HA-tagged wild type PASK, the kinase-inactive mutant p.K1028R, and the two human variants p.L1051V and p.G1117E. We expressed these proteins in HEK 293T cells, affinity-purified them using anti-FLAG-conjugated beads, and measured PASK autophosphorylation by Western blotting. We have previously identified Thr[307] as a PASK autophosphorylation site that can be recognized by an antibody that was originally generated against the phospho-Akt substrate motif R*XX*(S*/T*) (not shown). Using this antibody, and following normalization to total FLAG levels,

autophosphorylation of PASK p.G1117E was found to be increased by ∼25% compared with wild type PASK. In agreement with the results described above, there was no significant difference in autophosphorylation levels between wild type PASK and p.L1051V PASK (Fig. 3*B*). Next, we measured the activity of these two human variants, as well as wild type and kinase inactive PASK against a peptide substrate (PASKtide: KKKHTKTHS*TYAFE), which corresponds to the PASK phosphorylation site in Ugp1, a *bona fide* PASK substrate in yeast. The three PASK variants were expressed in HEK 293T cells and purified using FLAG-affinity purification followed by elution with a FLAG peptide. The expression levels of the PASK variants were normalized after purification using anti-FLAG signal levels. Autophosphorylation of the normalized PASK variants was also measured, with the p.G1117E variant displaying increased autophosphorylation relative to wild type PASK (Fig. 4). *In vitro* kinase assays were performed using the purified PASK variants and PASKtide. Similar to the results described above, p.G1117E PASK activity was significantly increased (∼25%; $p < 0.05$), whereas p.L1051V PASK activity was unchanged relative to the wild type protein (Fig. 4). Taken together, these results suggest that mutation of glycine 1117 to glutamic acid might lead to an increase in PASK kinase activity.
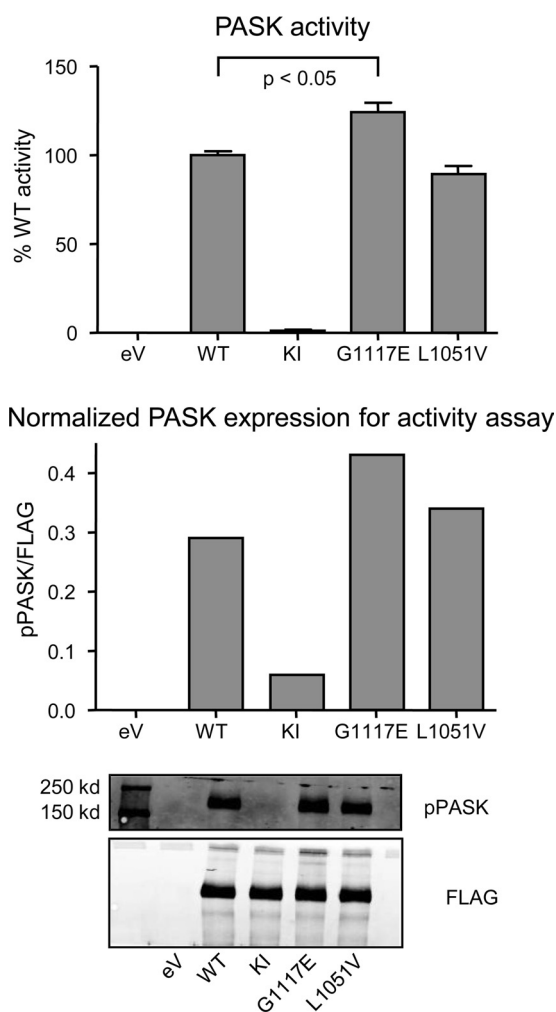
PASK Mutation G1117E Affects Glucose-stimulated Insulin Secretion from Mouse Pancreatic Islets but Does Not Have an Effect on Glucagon Secretion—To test whether the above increases in the activity of the p.G1117E mutant kinase might impact on glucose- or depolarization (KCl)-stimulated insulin secretion we next generated adenoviruses to permit their expression in isolated mouse pancreatic islets (supplemental Fig. S1). As shown in Fig. 5, and as we have previously reported [28], treatment of intact isolated islets with adenoviruses at nontoxic levels leads to infection chiefly of the first one-two layers of cells, comprising the surface of the islets. Consistent with our earlier findings, expression preferentially targets β cells over α cells despite the abundance of the latter at the islet surface [31]. Islets expressing the PASK p.G1117E mutant displayed enhanced basal (at 3 mM glucose) secretion (Fig. 6*A*), and

A.

B.
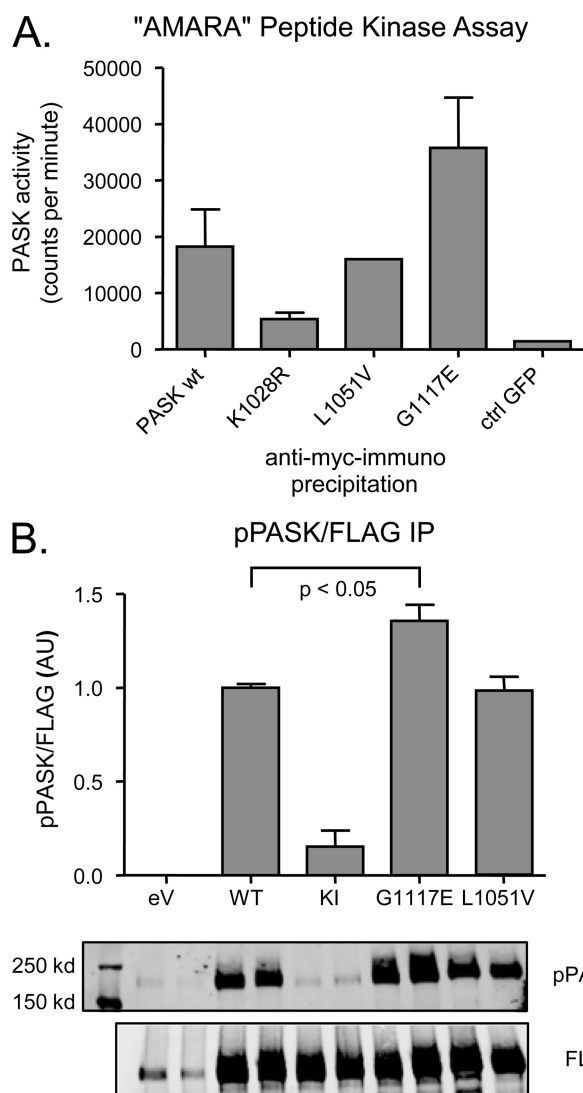
FIGURE 3. **Substitution of PASK Gly[1117] with glutamic acid enhances PASK activity on AMARA peptide and autophosphorylation.** PASK p.G1117E displayed a ~2-fold increase with respect to wild type PASK in kinase activity toward AMARA peptide (*A*) and a ~25% increase in the extent of autophosphorylation (*B*) as measured by Western blotting with an antiphospho-PASK threonine 307 antibody. The activity of the L1051V mutant was unchanged *versus* the wild type enzyme (*n* = 3). *eV*, empty vector; *WT*, PASK wild type; *KI*, PASK p.K1028R (kinase inactive); *G1117E*, PASK p.G1117E; *L1051V*, PASK p.L1051V.

FIGURE 4. **PASK p.G1117E increased catalytic activity is confirmed on PASKtide (Ugp1p-derived peptide).** Wild type PASK, kinase inactive (*KI*), and the two mutants were purified from HEK 293T cells using FLAG immuno-precipitation and elution. Expression levels were normalized using FLAG and phospho-PASK signal levels (*bottom panels*). The normalized levels were used to do an *in vitro* kinase assay using a peptide derived from UGP1 (PASKtide). The kinase assay was performed in duplicate for three different experiments (from the same purification procedure). Data for each separate experiment are normalized to wild type PASK activity and combined.

a significant (*p* < 0.05) decrease in the fold-stimulation of insulin release in response to high glucose (17 *versus* 3 mM glucose), *versus* wild type PASK-expressing islets, from ~16 to ~4-fold (Fig. 6*B*). The p.L1051V mutant exerted no significant effects on secretion at either glucose concentration tested. Neither mutant affected KCl-induced secretion. To exclude a possible effect of PASK pG1117E on glucagon secretion, which might explain the observed increase in basal insulin secretion (at 3 mM glucose), we then measured glucagon secretion from islets infected with adenoviruses encoding wild type or pG1117E mutant PASK and treated exactly as for the glucose-stimulated insulin secretion assay. Analyzed by one-way analysis of variance, no significant differences were observed for glucagon release at 3 or 17 mM between islets overexpressing wild type PASK with respect to islets infected

with adenoviruses encoding either the pG1117E PASK mutant or GFP alone (Fig. 6*C*). Similarly, no differences in glucagon secretion were observed for islets treated with the indicated viruses and incubated at 0.5 or 10 mM glucose (Fig. 7*A*), not where the fold-differences between the groups were significantly different (Fig. 7*B*). Under the same conditions, we also measured insulin secretion (10 and 0.5 mM glucose) from the islets and observed no significant differences among the groups (supplemental Table S1).

Effect of PASK Mutations on Insulin and Pdx-1 Gene Expression—We next analyzed the impact of PASK mutations on prepro-insulin *Pre-Ins2* and *Pdx-1* gene expression. For this purpose and to ensure the expression of PASK variants throughout the islet syncytium (29), we injected the adenoviruses directly into the celiac artery of Wistar rats at the time of sacrifice prior to pancreas dissection and islet isolation. Islets were then isolated, allowed to recover overnight, and exposed to 2.8 or 16.7 mM glucose for 24 h. Overexpression of human
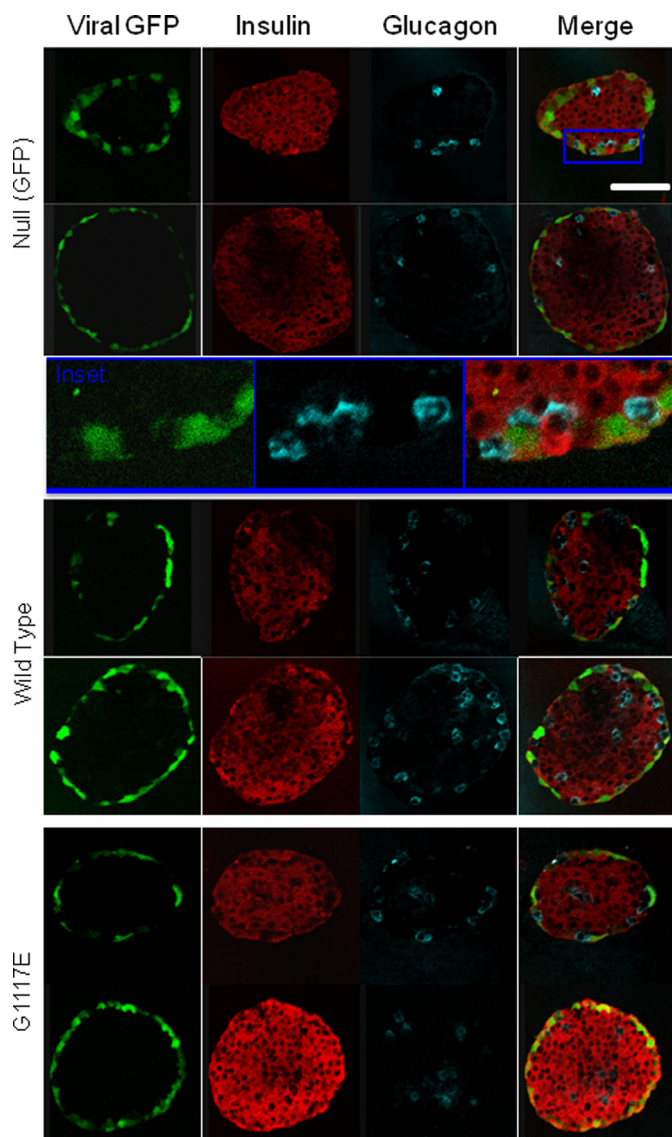
FIGURE 5. *In vitro* adenoviral infection penetration of mouse CD1-isolated islet cryosections. Immunostaining of CD1 mouse isolated islet sections with anti-insulin (*red*) and anti-glucagon (*blue*) antibody and imaging the level of penetration of adenoviral infection with GFP (*green*). CD1 mouse isolated islets were infected as described under "Experimental Procedures" with adenovirus co-expressing either GFP and PASK wild type (*wild type*), or GFP and PASK G1117E mutant (*G1117E*), or GFP only (*null/GFP*) and treated for cryosectioning as described in Ref. 28. After permeabilizing sections in 0.1% Triton X-100 overnight at 4 °C, immunostaining was carried out using the following antibodies: mouse monoclonal anti-glucagon antibody (1:200; Sigma), polyclonal anti-swine insulin (1:200; DakoCytomation), and Alexa 564- or 633-coupled secondary antibodies (1:1000; Molecular Probes), and coverslips were mounted in VECTASHIELD (Vector Laboratories) hardset medium with DAPI. Isolated infected islet sections were then imaged using a Leica SP2 upright confocal microscope with an HC PL APO 20 × 0.70 CS objective and the following laser lines: Arg ion (488 nm), HeNe (633 nm), 561 nm diode and UV (350 nm) controlled by Leica LAS AF Lite™ software. Acquired images were analyzed using Volocity software (Improvision). *Scale bar* = 100 μm.
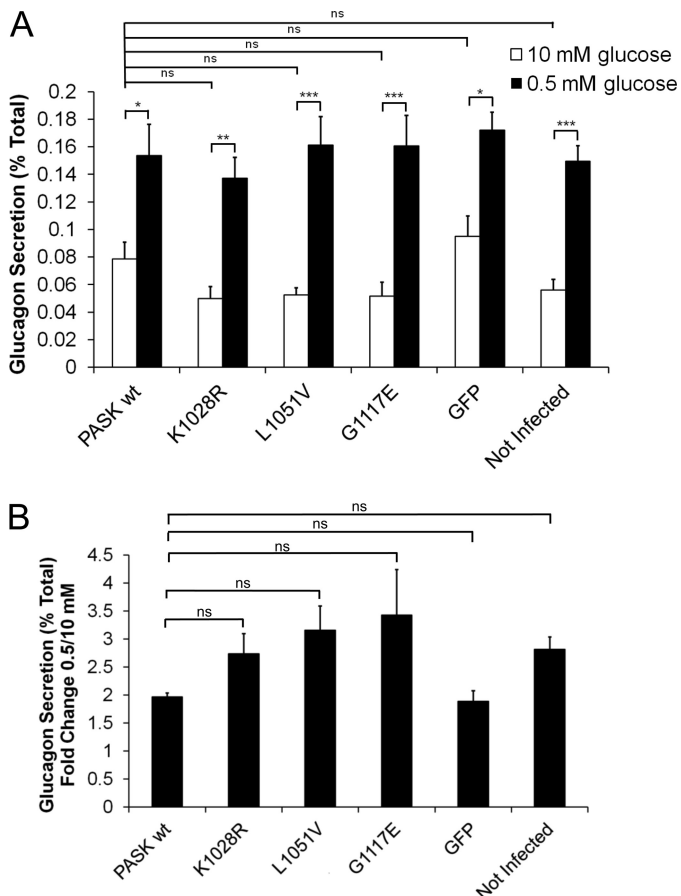
PASK wild type and mutant mRNAs was confirmed by quantitative real time PCR as shown in supplemental Fig. 2. We then measured the levels of insulin 2 pre-mRNA. We used primers encompassing an intron 2-containing pre-mRNA transcript that has a short half-life and therefore enables us to detect changes in expression that might otherwise be masked by the very long half-life of the mature insulin transcript (32, 33).
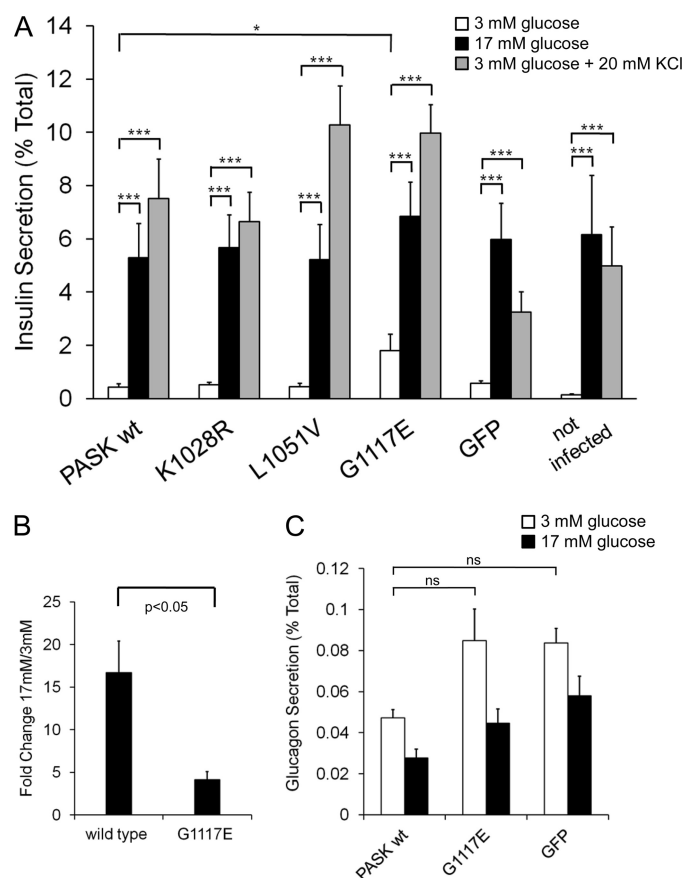
FIGURE 6. **Islets expressing PASK p.G1117E mutant display impaired insulin secretion, whereas glucagon secretion is not affected.** *A*, effect of mutations on glucose- and KCl-stimulated insulin secretion. Islets expressing PASK p.G1117E show enhanced basal secretion. At basal 3 mM glucose, PASK p.G1117E overexpressing islets secrete about 4 times more insulin compared with PASK wild type. $n = 3$ separate GSIS experiments and $n > 3$ separate islet preparation, 10 islets per point. $* = p < 0.05$; $*** = p < 0.001$. *B,* islets expressing PASK p.G1117E display impaired *in vitro* insulin secretion. Islets expressing PASK p.G1117E show a significant decrease in the fold-stimulation with glucose (16.7 *versus* 3 mM glucose) of insulin release *versus* PASK wild type expressing islets. The increase in insulin secreted (percent of total insulin) by the latter at 16.7 mM (stimulated insulin secretion) *versus* 3 mM glucose (basal insulin secretion) was 16.7 ($\pm$3.6)-fold, whereas it was only 4.1 ($\pm$0.9)-fold for the islets infected with PASK p.G1117E adenovirus. Such a distinctive difference was imputable to increased basal insulin secretion as shown in *panel A*. *C,* PASK p.G1117E mutant increases basal insulin secretion, but does not affect glucagon secretion at the same low glucose concentration. Islets were treated as for *A* and glucagon secretion was measured by radioimmunoassay ($n = 3$, 20–21 islets per point). *ns*, not significant.

As expected, the control islets infected by an adenovirus encoding Luc showed a marked increase in insulin pre-mRNA in response to glucose (Fig. 8*A*). In islets overexpressing wild type PASK (WT), insulin pre-mRNA was increased at low glucose, consistent with our previous report (8). Islets infected with adenovirus encoding the PASK p.L1051V mutant (LV) do not show an increase of insulin pre-mRNA at low glucose but, at the same time, overexpression of this mutant does not prevent the expected increase at high glucose. On the other hand, islets infected with the adenovirus encoding PASK p.G117E (GE) mutant displayed the opposite effect: in fact they presented an increase of insulin mRNA at low glucose that contributes to abolish the increase observed with the Luc encoding adenovirus-infected islets. We also measured Pdx-1 message

FIGURE 8. **Effect of PASK mutations on *insulin* and *Pdx-1* gene expression.** Isolated rat islets were infected by arterial injection with adenoviruses encoding for Luc, PASK wild type (*WT*), PASK p.G1117E mutant (*G1117E*), or PASK p.L1051V mutant (*L1051V*). After 16 h at 2.8 mmol/liter of glucose, islets were exposed to 2.8 or 16.7 mmol/liter of glucose for 24 h. Prepro-insulin *Pre-Ins2* (*A*) and *Pdx-1* (*B*) mRNA levels were measured by real-time PCR and normalized to *β-actin* mRNA. Results are expressed as the ratio of rGene/rβ-actin mRNA and are mean ± S.E. of 4–6 independent experiments. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$, *versus* the respective 2.8 mM glucose condition.
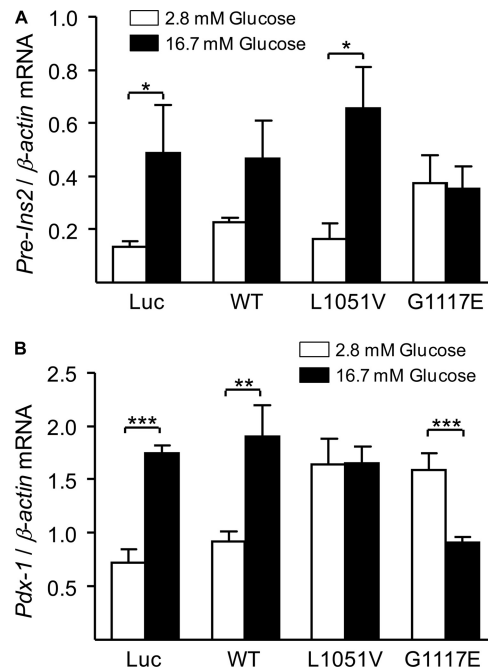
FIGURE 7. **Glucagon secretion assay from PASK wild type and mutants overexpressing islets.** PASK mutants do not affect glucagon secretion from *in vitro* infected islets at a permissive glucose concentration (0.5 mM). *A,* CD1 mouse isolated islets were infected with PASK mutant-overexpressing adenoviruses as described under "Experimental Procedures" and incubated for 1 h in 0.5 mM glucose after 30 min preincubation at an inhibitory glucose concentration (10 mM glucose). Islets infected with the PASK mutants do not display significant changes in glucagon secretion with respect to islets infected with PASK wild type-overexpressing adenovirus. Glucagon secretion is expressed as percentage of secreted hormone over total hormone content per sample. *B,* islets expressing PASK p.G1117E show an increase in the fold-release of inhibition with low glucose (0.5 *versus* 10 mM glucose) of glucagon secretion *versus* PASK wild type expressing islets. The increase in glucagon secreted (percent of total glucagon) by the G1117E mutant at 0.5 mM (released glucagon secretion) *versus* 10 mM glucose (inhibited glucagon secretion) was 3.4 (±0.8)-fold, whereas it was 1.9(±0.07)-fold for the islets infected with PASK wild type adenovirus. The significance of this increase was assessed with one-way analysis of variance and resulted as not significant (*ns*).

RNA (Fig. 8*B*) and, overall, this closely followed expression of the insulin gene with the exception of the effect of the PASK p.L1051V mutant at low glucose (it increased Pdx-1 but not insulin 2 mRNA level). Note that the lack of increase of the Pdx-1 message upon overexpression of PASK wild type is consistent with our previous observations (8)[6] and suggests that PASK increases Pdx-1 expression essentially by enhancing stability of the protein rather than by a transcriptional effect.

DISCUSSION

We describe here two very rare nonsynonymous mutations in *PASK*, each one occurring in a single family with uneluci-

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[6] M. Semache, G. Fontés, and V. Poitout, unpublished data.

dated MODY. Of these, only one mutation (p.G1117E) seemed to be associated with the young-onset diabetes phenotype in a small family of European origin. However, one child clinically defined as having impaired fasting glucose (*i.e.* a pre-diabetic condition) did not inherit the p.G1117E mutation from her diabetic father. Therefore, the true contribution of the *PASK* mutation to diabetes causality is unclear: this mutation may rather act as a modifier genetic factor.

We observed that the amino acid residues, which in the above described two rare mutations of PASK are changed with respect to the wild type protein, Leu[1051] and Gly[1117], are both located within the kinase domain of PASK. As it is clear from a crystal structure of the kinase domain (amino acids 977–1300) of human PASK binding an ADP molecule (34), Leu[1051] is found on a short loop connecting the hairpin β strand (βH2) to the extra α chain (αC) in the N-terminal lobe of the kinase domain (Fig. 2*B*). A two-stranded β-hairpin (termed βH1 and βH2) and a truncated αC helix represent a characteristic feature of the PASK kinase domain, shared also by PIM1, the serine/threonine kinase most structurally related to PASK. Immediately adjacent to the Leu[1051], residue Thr[1050] appears to contribute to the hydrophobic environment important for stabilizing the hairpin-like structure. It is not clear what the consequences would be of the substitution of residue Leu[1051] with a similarly hydrophobic valine, as is the case for PASK p.L1051V, because the role of the hairpin in regulating or influencing the kinase activity has not yet been elucidated. Residue Gly[1117] (Fig. 2*A*) was found at the C terminus of α helix αE facing outwards with respect to the catalytic pocket of the

kinase where the ATP molecule is bound. Gly[1117] is in the center of a large hydrophobic patch and substitution of the small and amphiphilic glycine with a bigger, acidic and hydrophilic residue such as glutamic acid might bring consequences like, for example, the breaking of a possible interaction of this α helix with the N-terminal regulatory PAS domains or with other unidentified binding partners. Other protein kinases including 3′-phosphoinositide-dependent protein kinase-1 and protein kinase A (PKA) possess a glutamic residue in this position, which suggests that the above substitution may not affect the kinase domain fold dramatically. Correspondingly, we observed kinase activity equal to the wild type for the PASK p.L1051V mutant and increased in the case of the p.G1117E mutant.

We investigated the effects of the two mutations on PASK catalytic activity by expressing in HEK 293T cells and affinity purifying C-terminal c-*myc*-tagged PASK p.L1051V and PASK p.G1117E in parallel with PASK wild type and the kinase-inactive mutant p.K1028R (2). We observed a 2-fold increase of ^{32}P incorporation by PASK p.G1117E compared with PASK wild type, whereas we did not see any significant difference between PASK p.L1051V and the wild type. Similarly PASK p.G1117E autophosphorylation was enhanced by 25% with respect to wild type PASK. Again, PASK p.L1051V activity was not significantly changed in comparison to the wild type enzyme. We then assayed the four kinase activities on a peptide derived from the *S. cerevisiae* well characterized PASK *in vivo* protein substrate Ugp1, and we confirmed the previous results since we measured an increase of about 24% in the p.G1117E mutant activity compared with wild type, whereas PASK p.L1051V displayed no significant difference. Recently (12), it was reported that PASK autophosphorylation (measured as ^{33}P incorporation on PASK protein itself) does not always proportionally correlate to PASK kinase activity at least on *in vitro* protein substrates ribosomal protein S6 and eukaryotic elongation factor 1A1. In our hands, increased autophosphorylation of PASK (as detected by Western blotting with an anti-phosphothreonine residue 307 antibody) correlated with increased activity measured as phosphorylation of target peptide.

Considering the model previously proposed for PAS kinase activity regulation in *trans* by its N-terminal PAS domain, and more recent structural observations (34) pointing out that the PASK kinase domain appears to be stable in its active conformation, we propose a model whereby substitution of glycine residue 1117 with glutamic acid might disrupt the interaction between the kinase and PAS domains, thereby stimulating kinase activity in the absence of putative small regulatory molecules.

Upon infection with adenoviruses encoding PASK wild type or mutants, mouse islets overexpressing PASK p.G1117E displayed impaired glucose-stimulated insulin secretion with respect to islets that had been infected with a PASK wild type-expressing adenovirus. This was largely due to an increase in basal insulin release at 3 mM glucose. Suggesting that this may reflect a "left-shift" in the dose-response of PASK G1117E-infected β cells *versus* controls, no differences in insulin release were observed between the two at 0.5 mM glucose nor at 10 mM glucose ([supplemental Table S1](#)). Nevertheless, our data sug-

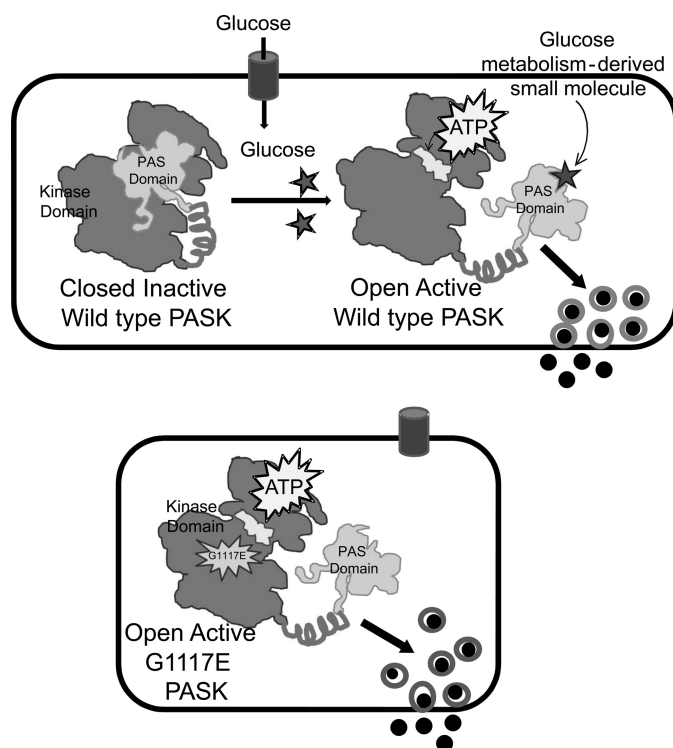

FIGURE 9. **Model.** Tight control of PASK kinase is necessary in β cells for physiological insulin secretion in response to nutrients (glucose). Substitution of glycine 1117 with glutamic acid brings along a positive dysregulation of PASK kinase activity, which may affect insulin secretion via an as yet unidentified connection to secretory signaling pathways.

gest that the increased basal insulin secretion in islets expressing PASK p.G1117E was intrinsic to β cells and was not due to a paracrine effect of glucagon, whose release remained unchanged (Figs. 6*C* and 7, *A* and *B*), likely reflecting the relatively poor infection of rodent α cells with the adenoviruses deployed here (28, 35). Of note, these findings are in contrast to our demonstration of an inhibition of glucagon secretion in response to infection of human islets with (wild type) PASK-expressing virus (10) likely reflecting both the greater proportion of α cells within human islets and differences in the tropism of the deployed adenoviruses for human *versus* mouse α cells. Consequently, the present studies do not rule out an independent and additional effect of the PASK p.G1117E mutant on glucagon secretion in man. Further analyses, using alternative approaches (including human islets), would be needed to explore this question. Likewise, the possible mechanisms involved in the action of the PASK p.G1117E mutant on basal insulin secretion will require further detailed studies in the future, although changes in the expression of key glucose-sensing genes (GLUT2, GCK (glucokinase), etc.) may conceivably play a role.

We previously demonstrated that PASK mRNA expression and kinase activity are positively regulated by glucose (7) in MIN6 β cells, and that PASK is necessary for glucose-responsive transcription of prepro-insulin and *PDX-1* genes. Interestingly, both PASK wild type and, to a greater extent, the PASK p.G1117E mutant, tended to elevate the expression of *PreIns2* mRNA in transduced islets at low (2.8 mM) glucose, such that the stimulatory effect of high glucose (16.7 *versus* 2.8 mM) on

this parameter was no longer apparent (Fig. 8*A*). Moreover, overexpression of PASK p.G117E tended to increase the expression of Pdx-1 at 2.8 mM glucose, in this case reversing the action of high glucose on Pdx-1 mRNA levels (Fig. 8*B*). Although the molecular mechanisms involved in each case are unclear, it is possible that common changes may be involved in both enhancement in the stimulation of the release of stored insulin (Fig. 6, *A* and *B*), and in the apparent activation of gene expression (Fig. 9). The extent to which these and other transcriptomic or post-transcriptional changes are involved in the stimulation of basal insulin secretion (Fig. 6*A*) remains to be examined.

In summary, we describe and characterize a naturally occurring PASK mutation, p.G1117E, which may be related to early age-onset diabetes. An increase in basal insulin secretion, as measured from mouse islets overexpressing this PASK variant, might in subjects either (*a*) contribute to the diabetic condition, for example, by causing the down-regulation of insulin receptors on target tissues, or (*b*) in part compensate for, or modulate, the impact of another, as yet unidentified, mutation that may more directly cause diabetes development and progression. Further studies, including whole exome analysis of the families studied, are currently underway to distinguish between these possible pathophysiological disease mechanisms.

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