SUMOylation of Pancreatic Glucokinase Regulates Its Cellular Stability and Activity*

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Background: Glucokinase is a key player in carbohydrate metabolism, but how this enzyme is regulated by post-translational modifications is largely unknown.

Results: Glucokinase is SUMO-modified in vitro and in pancreatic β-cells, increasing its activity and stability.

Conclusion: SUMOylation of glucokinase is a novel form of modification, regulating its cellular stability and activity.

Significance: SUMO conjugation of glucokinase may have an important regulatory function in pancreatic β-cells.

Glucokinase is the predominant hexokinase expressed in hepatocytes and pancreatic β-cells, with a pivotal role in regulating glucose-stimulated insulin secretion, illustrated by glucokinase gene mutations causing monogenic diabetes and congenital hyperinsulinemic hypoglycemia. A complex tissue-specific network of mechanisms regulates this enzyme, and a major unanswered question in glucokinase biology is how post-translational modifications control the function of the enzyme. Here, we show that the pancreatic isoform of human glucokinase is SUMOylated in vitro, using recombinant enzymes, and in insulin-secreting model cells. Three N-terminal lysines unique for the pancreatic isoform (Lys-12/Lys-13 and/or Lys-15) may represent one SUMOylation site, with an additional site (Lys-346) common for the pancreatic and the liver isoform. SUMO-1 and E2 overexpression stabilized preferentially the wild-type human pancreatic enzyme in MIN6 β-cells, and SUMOylation increased the catalytic activity of recombinant human glucokinase in vitro and also of glucokinase in target cells. Small ubiquitin-like modifier conjugation represents a novel form of post-translational modification of the enzyme, and it may have an important regulatory function in pancreatic β-cells.

Blood glucose levels are normally maintained within a very tight range (4–8 mM) by regulated homeostatic mechanisms involving the endocrine pancreas, liver, and peripheral tissues. Glucokinase (GK) is a 52-kDa enzyme abundantly expressed in pancreatic β-cells and hepatocytes, as well as in neuronal/neuroendocrine cells (1–3). GK catalyzes the first and rate-limiting step in glucose metabolism by converting α-D-glucose to glucose 6-phosphate. The enzyme is activated by glucose binding, which in pancreatic β-cells (isoform 1) results in glucose-stimulated insulin secretion (4) and in hepatocytes (isoform 2) leads to enhanced glucose uptake, glycolysis, and glycogen synthesis (5). Mutations in the GK gene (GCK) can result in three different forms of diabetes, including GCK-MODY or MODY2 (6), severe diabetes of the newborn (neonatal diabetes) (7), and hyperinsulinemia of infancy with hypoglycemia (8), demonstrating the essential role of GK for normal glucose-stimulated insulin secretion.

A complex network of mechanisms differentially regulates the GK isoforms in pancreatic β-cells and hepatocytes. In the hepatocyte, the GK regulatory protein inhibits GK activity and regulates its nuclear → cytoplasmic translocation in response to intracellular changes of metabolites (5, 9). Interaction with and catalytic activation of GK by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2/FBPase-2) has been demonstrated in both the liver (10) and the pancreas (11). Moreover, GK is part of a regulatory multiprotein complex in the outer mitochondrial membrane of rat hepatocytes and mouse β-cells/islets that includes the pro-apoptotic protein BAD, whereby the phosphorylation status of BAD helps regulate the catalytic activity of GK (12, 13). Additional regulatory mechanisms have been addressed, including our observation that poly/ubiquitination of recombinant pancreatic human

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**SUMOylation of Glucokinase**

Glucokinase (hGK) in vitro targets the newly synthesized enzyme for proteosomal degradation (14).

In general, post-translational modifications of proteins by members of the small ubiquitin-like modifier (SUMO) family regulate diverse cellular processes, including protein-protein interactions, intracellular localization, and protein function. In pancreatic β-cells, SUMOylation regulates the function of key proteins involved in insulin secretion, e.g. the insulin gene transcription factors MaF (15) and PDX-1 (16), the islet cell autoantigen 512 (17), and the voltage-dependent K⁺ channel Kv2.1 (18). Recently, overexpression of SUMO-1 in pancreatic human and rodent β-cells was shown to impair glucose-stimulated insulin secretion (19).

In this work, we report for the first time that recombinant GK modified by SUMOylation in vitro and in insulin-secreting β-cell lines (INS-1 and MIN6). Both recombinant pancreatic and liver hGK were SUMOylated in vitro, with the pancreatic isoform as the preferred SUMO-1 substrate. Conjugation of SUMO-1 to recombinant pancreatic hGK resulted in an allostERIC activation of the enzyme, and overexpression of SUMO-1 or stimulation of SUMOylation in MIN6 cells increased the cellular stability and activity of the enzyme. Thus, our biochemical and molecular studies suggest that SUMOylation of GK represents a novel form of post-translational modification, which may have an important regulatory function in pancreatic β-cells.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Cell Lines**—Generation of cDNA encoding liver hGK isomorphic and Lys > Arg mutants of both the liver and pancreatic isoforms was performed as described previously (14). For expression of wild-type (WT) and mutant GK in mammalian cells, the pcDNA3.1/V5-His-TOPO vector was used. All constructs were verified by automated DNA sequencing.

INS cells were grown in RPMI 1640 Glutamax medium supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol. MIN6 cells were grown in DMEM (4.5 g/liter glucose) supplemented with 15% heat-inactivated FCS. Both media included penicillin/ streptomycin (100 units/ml). All cell culture reagents were purchased from Sigma-Aldrich. INS-1 and MIN6 cell lines (INS-1 and MIN6). Both recombinant pancreatic and liver hGK were SUMOylated in vitro, with the pancreatic isoform as the preferred SUMO-1 substrate. Conjugation of SUMO-1 to recombinant pancreatic hGK resulted in an allostERIC activation of the enzyme, and overexpression of SUMO-1 or stimulation of SUMOylation in MIN6 cells increased the cellular stability and activity of the enzyme. Thus, our biochemical and molecular studies suggest that SUMOylation of GK represents a novel form of post-translational modification, which may have an important regulatory function in pancreatic β-cells.

In Vitro SUMOylation Assay—All recombinant proteins, except RanBP2/FG (Enzo Life Sciences (Farmingdale, NY)), were from LAL Biotech International (Rockville, MD). The SUMOylation reactions in a standard assay were run for 1 h at 37 °C in a 20-μl reaction mixture, including 1 μg of recombinant hGK (~1 μM), 150 ng of Aos1/Uba2 (E1) (~65 nm), 1 μg of SUMO-1 (~4 μM), 250 ng of Ubc9 (E2) (~0.7 μM), in buffer containing 20 mM Hesper (pH 7.4), 5 mM MgCl₂, and 4 mM ATP. When testing the effect of RanBP2 (E3 ligase), reactions included 100 ng of Ubc9 (~270 nm) and varying amounts of RanBP2/FG. Negative control reactions were performed without ATP, SUMO-1, Aos1/Uba2, Ubc9, or hGK. Samples were analyzed by SDS-PAGE and immunoblotting using GK (H-88, Santa Cruz Biotechnology, or HPAA007034, Sigma) or SUMO-1-specific antibodies (Abs) (GMP-1, Invitrogen).

Catalytic Activity of Recombinant Liver and Pancreatic hGK—The in vitro SUMOylation reactions were performed by standard assay, in the presence or absence of SUMO-1. The buffer of the SUMO-1 stock solution was added in equivalent amounts to the reactions lacking SUMO-1. In the assay where recombinant GK was preincubuted in the presence/absence of a SUMO protease (Ubl-specific protease) (Invitrogen) for 1.5 h at 30 °C (1 unit of protease per 2 μM of GK) prior to SUMOylation, the buffer of the SUMO protease stock solution was added in equal amounts to the control reaction. Subsequently, half of all SUMOylation reactions were analyzed by SDS-PAGE and immunoblotting (GK Ab) to confirm efficient SUMOylation of hGK. The remaining samples (~0.5 μg) were preincubated with 50 mM D-glucose for 7 min, and GK activity was measured spectrophotometrically at 37 °C as described previously (20). His tag-based isolation of SUMOylated GK was unsuccessful due to insufficient recovery, possibly related to preferential isolation of unconjugated SUMO-1 and also auto-SUMOylated Ubc9 (21). Pancreatic hGK was included as a positive control when investigating the activity of SUMOylation-deficient mutants and the liver GK isomorphic.

Mass Spectrometry—The in vitro SUMOylation reaction was performed as described but was up-scaled (5–10 reactions). Samples were pooled, concentrated (Microcon concentrators, Millipore (Billerica, MA)), and loaded into 1 well on a SDS-polyacrylamide gel. After Coomassie staining, unmodified and SUMOylated hGK bands were excised, in-gel trypsin digested, and processed as described previously (22).

In a direct approach to map SUMOylation sites in pancreatic hGK, we replaced WT SUMO-1 with an E93R SUMO-1 mutant (Enzo Life Sciences) in the standard in vitro SUMOylation assay to avoid the presence of a long SUMO-1 C-terminal polypeptide remnant on tryptic peptides, which complicates their identification by mass spectrometry (MS) analyses (23, 24). Tryptic peptides from the SUMOylated sample were analyzed by microcapillary LC-MS/MS on a hybrid dual pressure linear ion trap/Orbitrap MS spectrometer (LTQ Orbitrap Velos) as described elsewhere (25).

**Equilibrium Binding of Ubc9 to hGK**—The chromatographic hold up assay was performed essentially as described by Charbonnier et al. (26). This assay evaluates fractions of free and bound protein species at equilibrium binding conditions and thus allows the detection of fast-dissociating complexes. 10 μg of GST-tagged GK or RanBP2 (positive control for Ubc9 binding) was immobilized to 2 μl of settled glutathione magnetic beads (Thermo Scientific, Waltham, MA). Unbound fusion protein was removed, and magnetic beads were washed in 125 mM Tris, 150 mM NaCl (pH 8). The protein-bound beads were then supplemented with analyte as follows: 2 μg (8.5 μM) of Ubc9 protein. After binding for 30 min while shaking, elution of free plus bound Ubc9 (with GST-tagged GK or RanBP2) was achieved with 50 mM reduced glutathione, whereas elution with double distilled H₂O only recovered free (unbound) Ubc9. Samples were analyzed by SDS-PAGE and immunoblotting (anti-Ubc9).
Catalytic Activity of GK in MIN6 β-Cells—The cellular activity of hGK was measured in MIN6 cells co-transfected with plasmids encoding the deconjugating sentrin-specific protease SENP1 (17357, Addgene) or SENP1m (inactive SENP1 as a negative control, 17358, Addgene) (27). Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The cellular activity of endogenous mGK was measured in MIN6 cells in the presence of either SENP inhibitors (SUMO-1 and -2 aldehydes (Enzo Life Sciences)) or control buffer (SENP inhibitor stock solution). Cells were lysed in buffer containing 25 mM Hepes (pH 7.4), 25 mM KCl, 5 mM MgCl₂, and protease inhibitors by mechanical shearing (cell cracker, EMBL). Lysates were cleared by high speed centrifugation (22,700 × g for 15 min). GK activity was measured in 100 µg of total protein essentially as described before (28) at a saturating d-glucose concentration (60 mM).

Pulse-Chase and Immunoprecipitation of hGK in MIN6 β-Cells—MIN6 cells were transiently transfected using Lipofectamine 2000 (Invitrogen). Post-transfection (48 h), cells were rinsed (PBS), followed by a 1-h incubation in Met/Cys and serum-free DMEM. Cells were then supplemented with 5 µCi/ml [35S]Met/[35S]Cys for 30 min (pulse), rinsed, and further incubated in DMEM containing an excess (50×) of cold Met and Cys (chase). Cells were removed at various time points (0–12 h), lysed, and immunoprecipitated (anti-V5 Ab (R920-25, Invitrogen)) as described previously (28). Radioactive gels were developed by autoradiography, and protein signals were quantified by densitometric analysis (Image Gauge Version 4.0 software, Fuji Film, Tokyo, Japan). For nonradioactive IP, post-transfection, cells were lysed in RIPA buffer containing 20 mM N-ethylmaleimide and protease inhibitors. Lysates were boiled (95 °C) for 10 min and left for 1 h on a rotating wheel at 4 °C, prior to the addition of anti-V5 Ab. After immunoprecipitation, immunocomplexes were washed (RIPA buffer) and proteins subsequently eluted and analyzed by SDS-PAGE and immunoblotting.

Isolation of SUMOylated rGK from INS-1 Cells—SUMOylated proteins were isolated from cytosolic fractions of INS-1 cells by an SIM-agarose (BostonBiochem, Cambridge, MA). Fifteen µl of SIM-agarose beads were pre-washed in buffer (0.1 M Tris-HCl (pH 8.0), 0.5 M NaCl, 0.01% sodium azide). Two hundred µg of total protein from cytosolic INS-1 fractions, purified using NE-PER™ nuclear and cytoplasmic reagents (Pierce) in the presence of SENP inhibitors (SUMO-1 and -2 aldehydes) and 10 mM N-ethylmaleimide, was used for adsorption to the SIM resin. After 20 min of binding, the beads were washed four times with 1 ml of 50 mM Tris (pH 7.5) with 0.1% BSA. Bound protein was eluted with 2× SDS sample buffer and analyzed by SDS-PAGE and immunoblotting using anti-GK and anti-SUMO-1 (GMP-1).

Two-dimensional Electrophoresis—The two-dimensional electrophoresis gels were run using the ZOOM IPG® Runner™ System (Invitrogen). Samples (~300 µg of total cytosolic protein, purified using NE-PER™ nuclear and cytoplasmic reagents in the absence or presence of SENP inhibitors, or 1 µg of recombinant hGK protein) were desalted and buffer-exchanged on Zeba spin columns (Pierce) and diluted in a sample buffer containing 8 M urea, 2% (w/v) Chaps (GE Healthcare), 1% (v/v) IPG ampholyte solution (pH 3–10 NL, GE Healthcare), and 20 mM DTT. The first dimension was run at 200 V for 20 min, 450 V for 20 min, 750 V for 20 min, and 2000 V for 70 min. The second dimension was run in a gradient of 4–12% (w/v) pre-cast NuPAGE acrylamide gel (Invitrogen). Samples to compare were lysed, electrophoresed, blotted, and developed in parallel. For two-dimensional gel electrophoresis analysis, standard marker proteins (Sigma) with known pl and molecular weight values were used to calibrate the two-dimensional scale.

Protein-Protein Docking Analysis—The interaction of hGK (Protein Data Bank code 1v4s) and Ubc9 (Protein Data Bank code 1pzv) was determined by the coupled KDBOCK and HEX-DOC algorithms; water molecules were not included in the docking procedure. The coordinates obtained for the complex represent the best fit obtained by the HEX-DOC algorithm, and the surface presentation and contact residues are shown using PyMOL (29).

Statistical Analysis—All data are expressed as means ± S.D., and experiments were performed at least on three independent occasions unless otherwise specified. Statistical analyses were performed using the Student’s t test, and a p value <0.05 was considered significant.

RESULTS

hGK Is Modified in Vitro by SUMO-1 Protein Conjugation—Purified recombinant pancreatic and liver hGK were used as substrates in an in vitro SUMOylation assay, containing the SUMO-activating and -conjugating enzymes E1 (Aos1/Uba2) and E2 (Ubc9). The pancreatic isofrom was observed to be a better substrate than the liver isoform (Fig. 1A), with a dominant band at ~70 kDa and an additional weaker band observed at ~80 kDa. The ~70-kDa band was detected at low Ubc9 concentrations (Fig. 1B) compared with the ~80-kDa band (Fig. 1B, lanes 5–8), suggesting more than one target site in pancreatic hGK with variable accessibility for the conjugating machinery. No SUMOylation was observed in the negative control reactions performed in the absence of ATP, SUMO-1, or hGK (Fig. 1A) or E1 and E2 (data not shown).

SUMOylation of Pancreatic hGK Is Enhanced by the SUMO E3 Ligase RanBP2—The efficiency of SUMO-1 conjugation of pancreatic hGK was enhanced by the presence of RAN-binding protein 2 (RanBP2ΔFG) in vitro using recombinant enzymes (Fig. 1, D and E). By contrast, SUMOylation of recombinant liver hGK was less affected by the presence of RanBP2 (Fig. 1E).

Pancreatic hGK Is Modified in MIN6 Cells by SUMO-1 Conjugation—Pancreatic V5-hGK was also found to be SUMOylated in MIN6 cells when co-transfected with SUMO-1 (Fig. 1, F–H). Immunoisolation of a higher molecular mass V5-hGK-specific band of ~70 kDa, which disappeared when co-transfected with SENP1 (Fig. 1, F and H) and increased in intensity when co-transfected with RanBP2 (Fig. 1, G and H), was demonstrated by IP of radiolabeled V5-hGK and by nonradioactive IP (Fig. 1, F and G, and H, respectively). We could only detect the SUMOylated GK band by using HA antibody and not by V5 antibody, most probably because the accessibility of the V5 epitope at the unstructured N-terminal tail of GK is reduced upon SUMOylation.
Recombinant hGK Binds Ubc9—Because pancreatic hGK was observed to be a better SUMOylation target, we investigated the equilibrium binding of Ubc9 to GST-tagged pancreatic versus liver hGK, immobilized on glutathione beads, using a chromatographic hold up assay (26). Using RanBP2 as positive control for Ubc9 binding (30), the amount of bound versus free Ubc9 was analyzed by SDS-PAGE (Fig. 2). The assay revealed that Ubc9 binds equally to both pancreatic and liver isoforms. Binding was most evident for higher molecular mass forms of Ubc9, both to hGK and the positive control RanBP2, most likely corresponding to Ubc9 oligomers or dimers, also observed by others (31). We also confirmed the interaction between hGK and Ubc9 by protein-protein docking analyses, and the contact residues at the interface were identified as Thr-118, Lys-172, Gly-175, Gly-178, and Gln-286 in hGK, common to both isoforms (supplemental Fig. S1, A and B).

Pancreatic hGK Is SUMOylated at Lys-346 and Possibly Also at N-terminal Lysines—The pancreatic isoform was shown to be a better substrate for SUMO-1 conjugation than the liver isoform (Fig. 1A). The two hGK isoforms differ only in the 15 N-terminal residues encoded by exon 1a (pancreatic) and 1b (liver). The exon 1a-encoded sequence includes three closely
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A pancreatic hGK is SUMOylated in vitro and in MIN6 β-cells. A, pancreatic hGK is a better substrate for in vitro SUMO-1 conjugation than liver hGK. SUMOylation of pancreatic and liver hGK (1 µM) was performed by standard assay for 1 h at 37 °C using the recombinant enzymes indicated and as described under “Experimental Procedures.” SUMOylation in the absence of ATP, SUMO-1, or hGK represented negative controls. Samples were analyzed by SDS-PAGE and immunoblotting using anti-GK (Fig. 1, A–C), pancreatic hGK is SUMOylated in MIN6 β-cells, F, MIN6 cells transiently transfected with the indicated plasmids were computed, using the numbers only as a guiding reference (Fig. 4, A–C). The identity of spot 4 is more uncertain, but it might represent mono-ubiquitinated rGK based on its two-dimensional gel electrophoresis migration pattern relative to unmodified rGK as characterized previously for ubiquitinated recombinant pancreatic hGK (14). The increase in spot 4 intensity is assumed to be due to an increased availability of unmodified GK (spot 1) in the presence of SENP inhibitors. To confirm the efficiency of the SENP inhibitors on other SUMOylated proteins, MIN6 cells overexpressing SUMO-1 or SUMO-2/-3 proteins were lysed in the presence or absence of these inhibitors (Fig. 4D), and we confirmed their activity by demonstrating increased accumulation of SUMO-1-conjugated proteins, when present. SUMO-2/3 conjugation was unaffected by the presence of the SENP inhibitors (Fig. 4D).

FIGURE 1. Pancreatic hGK is SUMOylated in vitro and in MIN6 β-cells. A, pancreatic hGK is a better substrate for in vitro SUMO-1 conjugation than liver hGK. SUMOylation of pancreatic and liver hGK (1 µM) was performed by standard assay for 1 h at 37 °C using the recombinant enzymes indicated and as described under “Experimental Procedures.” SUMOylation in the absence of ATP, SUMO-1, or hGK represented negative controls. Samples were analyzed by SDS-PAGE and immunoblotting using anti-GK and anti-SUMO-1. WB, Western blot. B, in vitro SUMOylation of recombinant pancreatic hGK (1 µM) by standard assay but at increasing concentrations of Ubc9 (E2): 0.05, 1.25, 2.5, 5, 12.5, 25, 37.5, or 50 ng/µl (27–270 nM) (lanes 1–8, respectively). C, time course for the in vitro SUMOylation of recombinant pancreatic hGK (1 µM) as in A; however, only half-reaction loaded on the gel. D, RanBP2 acts as a SUMO E3 ligase for the in vitro SUMOylation of pancreatic hGK. In vitro SUMOylation of hGK (1 µM) as in A but in the presence of 0 (lane 1), 0.125 (17 nM) (lane 2), and 2.5 ng/µl (42 nM) (lane 3), or 5 ng/µl (84 nM) (lanes 4 and 5) of RanBP2 recombinant enzyme. E, in vitro SUMOylation of recombinant pancreatic or liver hGK (1 µM) by standard assay as described under “Experimental Procedures,” using the indicated recombinant enzymes, in the presence or absence of 100 ng (84 nM) of RanBP2. Samples were analyzed by SDS-PAGE and immunoblotting using anti-GK. F–H, pancreatic hGK is SUMOylated in MIN6 β-cells. F, MIN6 cells transiently transfected with the indicated plasmids were labeled with [35S]Met/[35S]Cys for 30 min followed by immunoprecipitation of total V5-tagged hGK using anti-V5 Ab. Negative controls were transfections without hGK or SUMO-1 plasmids or co-transfecting with SENP1 plasmid. G, RanBP2 acts as a SUMO E3 ligase for the SUMOylation of pancreatic hGK in MIN6 β-cells. MIN6 cells transiently transfected with the indicated plasmids were labeled with [35S]Met/[35S]Cys for 30 min followed by immunoprecipitation of total V5-tagged hGK using anti-V5 Ab. H, demonstration of SUMOylation pancreatic hGK in MIN6 β-cells by nonradioactive IP. MIN6 cells were transiently transfected with the indicated plasmids followed by immunoprecipitation using anti-V5 Ab. SUMOylated hGK could be detected by immunoblotting using anti-HA. Bands marked with an asterisk in A, B, D, and E represent trace amounts of residual uncleaved GST-hGK and in H trace amounts of V5-hGK detected due to insufficient antibody stripping. Arrows indicate SUMOylated hGK. Samples in B–E and H were analyzed by SDS-PAGE and immunoblotting with anti-GK (B–E) or anti-HA/anti-V5 (H). Samples in F and G were analyzed by SDS-PAGE and autoradiography.
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most likely related to the large pool of nonconjugated SUMO-2/3 compared with SUMO-1 proteins and also reported elsewhere (34). The two-dimensional gel electrophoresis pattern of in vitro SUMOylated recombinant pancreatic hGK (± SUMO-1) may serve as a reference to the pattern observed for SUMOylated rGK in INS-1 cells. Despite pronounced streaking of recombinant GK, most likely due to sample preparation, a well defined spot at 70 kDa with a basic shift in pI was seen for the SUMOylated sample (Fig. 4E). It corresponds to spot 2, relative to spot 1, observed in INS-1 cells (Fig. 4, A and B). SUMOylated proteins were also isolated from cytosolic INS-1 cell fractions using a SIM-agarose (35) and included SUMOylated rGK (Fig. 4F, pulldown), shown by GK- and SUMO-1-immunoreactive bands with a size corresponding to mono-SUMOylated rGK.

SUMOylation of Pancreatic hGK Increases Its Catalytic Activity—Following in vitro SUMOylation of recombinant pancreatic hGK ± SUMO-1, the catalytic activity of the non-SUMOylated and SUMOylated hGK samples was measured and compared (Fig. 5A) revealed a 1.23-fold increase in the catalytic activity (n = 6, p = 0.009) of the SUMOylated hGK sample compared with the unmodified hGK sample (column 2 versus 1), which was attributed to SUMO-1 conjugation of GK and not an interaction of SUMO-1 with GK (column 3 versus 4). This degree of stimulation was measured in protein samples with an estimated level of ~8% SUMOylated enzyme (7.9 ± 1.2%, n = 6), indicating that the increase in homospecific activity of the SUMO-conjugated enzyme was even higher (possibly by a factor of ~3). Moreover, the increase in GK activity was even higher (~1.48-fold, n = 6, p = 0.0002) when the in vitro SUMOylation reaction was performed on GK preincubated with, versus without, a SUMO protease (Ulp) (Fig. 5B, column 1 versus 2). However, this increase was re-estimated to ~1.34-fold after adjusting for a potential contribution (inhibition) by the SUMO protease itself (Fig. 5B, column 3 versus 4).

To determine a dose response of GK SUMOylation on GK activity increase, activity was measured on samples after varying SUMOylation reaction times (Figs. 1C and 5C). Although the activity of the non-SUMOylated GK sample decreased over time, indicating loss/degradation of unmodified GK after longer exposure to 37 °C, the activity of the SUMOylated sample was stable and slightly increased after a 40- and 60-min SUMOylation reaction time (Fig. 5C). Considering that still >90% of total GK in this sample is non-SUMOylated, a decline in GK activity, over time, would be expected if the SUMOylated form of GK is equally active as unmodified GK. On the contrary, a small increase in activity is observed, mediated by the remaining 8% SUMOylated GK of the total pool (Fig. 5C).

The activity of SUMOylated versus non-SUMOylated liver hGK was also investigated and confirmed that SUMOylation increases the activity of the liver isomorph as well (1.14-fold, n = 6, p = 0.0002) (Fig. 5D), however, not as efficiently as the pancreatic isomorph (Fig. 5, A and B). SUMOylation did not significantly increase the activity of SUMOylation-deficient mutants of both GK isofoms (Fig. 5D), confirming that both Lys-346 (Lys-345 in liver GK) and N-terminal Lys residues in pancreatic hGK are involved in SUMO-1 conjugation of GK.

FIGURE 3. Pancreatic hGK is SUMOylated at Lys-346 and possibly also at the N-terminal Lys residues Lys-12, Lys-13, and/or Lys-15. A, peptides from in vitro SUMOylated recombinant pancreatic hGK using the E93R SUMO-1 mutant, which were mapped for SUMOylation sites using tandem MS, identify Lys-346 as the SUMO-1 acceptor site, indicated by an asterisk in the peptide sequence. The MS/MS spectrum of the quadruply charged signature peptide Lys-346 to Arg-368 SUMOylated at Lys-346 and the sequence of the peptide, including the prominent fragment ions, are shown. For more details, see under “Experimental Procedures” and supplemental material. B and C, site-directed mutagenesis of hGK demonstrates loss of SUMOylation upon mutation of Lys-12, Lys-13, and Lys-15, and Lys-346 in vitro (B) and in MIN6 cells (C). B, in vitro SUMOylation of recombinant pancreatic WT hGK and the hGK mutants K346R, K12R, K13R, K15R (K12,13,15R), and K12R, K13R, K15R, K346R (K12,13,15,346R) (1 μM) by standard assay and with the appropriate negative controls as in Fig. 1A, analyzed by SDS-PAGE, and immunoblotted using anti-GK and anti-SUMO-1. Bands marked with an asterisk represent trace amounts of residual uncleaved GST-hGK. C, MIN6 cells were transiently transfected with the indicated plasmids followed by immunoprecipitation (IP) using anti-V5 Ab. SUMOylated WT pancreatic hGK was detected by SDS-PAGE and immunoblotting using anti-HA. Arrows indicate SUMOylated hGK. WB, Western blot.
SUMOylation Increases the Activity of Cellular GK—We further assessed if a similar increase in catalytic activity could be detected for the cellular enzyme, by transfecting MIN6 β-cells with constructs encoding pancreatic hGK, SUMO-1, and SENP-1 or SENP-1 mutant (inactive) (Fig. 5E). Cells were lysed, and the GK-specific activity measured was on an average ∼1.39-fold higher in the presence of inactive SENP (SENP-1m; mutant) than in the presence of active SENP-1 (Fig. 5E). Moreover, in untransfected MIN6 cells, the activity of mGK increased in the presence of increasing concentrations of SENP inhibitors versus control buffer (Fig. 5F), presumably due to increased steady-state level of cellular GK, illustrated by two-dimensional gel electrophoresis analyses of SENP inhibitor treated cytosolic INS-1 cell fractions (Fig. 4, A and B).

SUMOylation Increases GK Stability in MIN6 Cells—We further investigated whether SUMOylation affects the steady-state level and stability of cellular GK by transiently co-transfecting MIN6 cells with WT hGK or SUMOylation-deficient mutants alone and in the presence or absence of SUMO-1 and Ubc9, similar to other studies (36–38). The recovery of total GK was followed by pulse-chase experiments (0–12 h) and immunoprecipitation and analyzed by SDS-PAGE and quantification of the immunoisolated proteins (Fig. 6). Co-transfection with SUMO-1 and Ubc9 significantly increased the recovery of the WT enzyme over time (Fig. 6A) compared with mutants K12R,K13R,K15R, K346R, and K12R,K13R,K15R,K346R (Fig. 6, B–D, respectively). Semilogarithmic plot analyses (Fig. 7) revealed a biphasic time course for the recovery of all hGK proteins. The WT demonstrated apparent half-life values of 4.6 and 12.8 h for the two recovery curves, which were increased to 6.4 and 15.6 h in the presence of SUMO-1 and Ubc9 (Fig. 7A). The SUMOylation-deficient mutants K12R,K13R,K15R (Fig. 7B), K346R (Fig. 7C), and K12R,K13R,K15R,K346R (Fig. 7D) also showed a biphasic time course for their recovery but with shorter half-lives than for WT hGK. As expected, the steady-state level of these mutant enzymes was less affected by the presence of SUMO-1 and Ubc9 than that seen for WT hGK. We also investigated the stability of WT hGK in the presence of either Ubc9 alone (supplemental Fig. S3A) or SUMO-1 alone (supplemental Fig. S3B). The half-life of WT hGK was not affected by Ubc9, and only a small increase in GK recovery was observed in the presence of SUMO-1 (t1/2 values of 4.9 and 13.7 h). Altogether, the results strongly indicate that SUMOylation stabilizes hGK and involves N-terminal Lys residues and Lys-346.
**SUMOylation of Glucokinase**

**DISCUSSION**

In this study, we report for the first time that the pancreatic isoform of hGK is SUMO-1-modified and that hGK SUMOylation results in an allosteric catalytic activation and an increased cellular stability.

**SUMOylation of Pancreatic hGK Involves a Cluster of N-terminal Lys Residues and Lys-346**—Although it is clear that a complex network of mechanisms is involved in the regulation of GK in pancreatic β-cells and hepatocytes, the mechanisms by which post-translational modifications control its enzyme function are not fully understood. Here, we report for the first time that GK is SUMOylated *in vitro* using recombinant enzymes, as well as in target cells, and with possible functional implications. Although both the recombinant pancreatic and liver enzymes were SUMO-conjugated *in vitro* (Fig. 1, A–E), the pancreatic isoform was clearly the preferred substrate for SUMO-1 (Fig. 1A). This was not attributed to an increased affinity of the pancreatic isoform for Ubc9, as both isoforms bound Ubc9 equally (Fig. 2) and share the interacting residues (supplemental Fig. S1B). The only sequence difference between the isoforms lies at the N terminus (32), pointing to Lys-12, Lys-13, and/or Lys-15 as SUMOylation site candidates. Only Lys-12 is part of a consensus SUMOylation motif following the criteria VILMAPF(K)XED (39, 40) and was also found conserved between human, rat, and mouse. The N-terminal lysines and their importance in GK SUMOylation was confirmed by

![Graphs and images related to the discussion](image-url)
SUMOylation of Glucokinase

SUMOylation of GK Affects Enzyme Stability and Activity—Although the majority of SUMOylated substrates are nuclear proteins, SUMOylation also affects a great variety of cellular proteins and functions outside the nucleus (47, 48). In this study, we have demonstrated SUMOylation of exogenous GK in MIN6 cells (Fig. 1, F–H) and of endogenous GK in INS-1 cells (Fig. 4, A, B and F). Our finding that the steady-state level and stability of the transfected pancreatic enzyme were increased by co-transfection with SUMO-1 and Ubc9 in MIN6 cells (Figs. 6A and 7A) supports a physiological relevance of SUMOylation in the regulation of GK stability, as seen for other proteins (37). This conclusion was further supported by experiments showing that the stability of the SUMOylation-deficient mutants were lower compared with the WT enzyme, and more importantly, their steady-state levels were barely affected by the presence of SUMO-1 and Ubc9 (Figs. 6, B–D, and 7, B–D). Interestingly, the quadruple mutant K12R,K13R,K15R,K346R lacking both the predicted SUMOylation sites demonstrated the shortest half-life and was also least affected by SUMO-1 and Ubc9 coexpression (Figs. 6D and 7D). Furthermore, in cytosolic fractions from INS-1 cells, including inhibitors of SENPs, the level of unmodified endogenous rGK increased relative to an internal control (Fig. 4, A and B), which further supports our observation that SUMOylation increases GK stability. In some cases, SUMOylation stabilizes target proteins by blocking ubiquitination at the same Lys residue(s) and thus prevents signal-induced ubiquitination and degradation (48). Previously, we found that pancreatic hGK is poly-/multiubiquitinated in vitro, targeting in part the newly synthesized enzyme for proteasomal degradation (14). That ubiquitin competes with SUMO for the same Lys residue(s) in GK is unlikely, because all the SUMOylation-deficient mutants demonstrated the wild-type-like ubiquitination role in the shuttling of proteins from the cytosol to the nucleus (44). Besides its cytoplasmic localization, GK has also been found in the nucleus of both hepatocytes and pancreatic β-cells (45, 46). In hepatocytes, the mechanism for the reversible cytoplasmic ↔ nuclear shuttling of GK involves the GK regulatory protein (45), whereas the mechanism for the nuclear import in the β-cell is still unclear. SUMOylation of pancreatic hGK could be involved in this translocation process.

FIGURE 5. SUMOylation increases the catalytic activity of recombinant pancreatic hGK in vitro and in MIN6 β-cells. A, first, hGK was SUMOylated in vitro in the presence or absence of SUMO-1 using the indicated recombinant enzymes, for 1 h at 37 °C by standard assay as described under “Experimental Procedures.” Thereafter, the catalytic activity was measured spectrophotometrically, at 50 mm glucose, on 0.5 μg of non-SUMOylated and SUMOylated pancreatic hGK (column 1 versus column 2) (**, p = 0.0009, n = 6). The activity of hGK was also measured in the absence and presence of SUMO-1 alone (columns 3 and 4, respectively), to exclude any contribution to the activity increase seen in column 2, by SUMO-1 interaction (noncovalent) with hGK. B, first, hGK was incubated in the presence (column 1) or absence (column 2) of SUMO protease (Ulp1), for 1 h at 30 °C. Thereafter, both samples were SUMOylated as described under A. The specific activity of the SUMOylated sample (column 2) was significantly higher (**, p = 0.0002, n = 6) compared with the SENP-1-treated sample (column 1). A slight inhibition of GK activity by SENP-1 alone (column 3 versus 4) was detected and accounted for (*, p = 0.036, n = 6), C, time course for the in vitro SUMOylation of recombinant pancreatic hGK (1 μM) as in A, in the presence or absence of SUMO-1, as indicated. The specific activity was measured spectrophotometrically as in A, and after the indicated SUMOylation time, the activity of GK was significantly higher in the SUMOylated versus the non-SUMOylated sample after 40 min (*, p = 0.028, n = 6) and after 60 min (**, p = 0.0038, n = 6). SUMOylation of samples were controlled by SDS-PAGE and immunoblotting using anti-GK. D, recombinant liver and pancreatic hGK and SUMOylation-deficient mutants of the liver (K345R) and pancreatic (K12R,K13R,K15R,K346R, K12,13,15,R346R) isoforms were SUMOylated in vitro, and their specific activity was determined spectrophotometrically, as in A. **, p = 0.0002, n = 5. E, SUMO-1 overexpression and conjugation increases the activity of exogenous pancreatic hGK in MIN6 β-cells. MIN6 cells were transiently transfected with the indicated plasmids and lysed by mechanical shear forces. The activity of GK was measured as described under “Experimental Procedures” in 100 μg of total proteins from high speed centrifuged cytosolic fractions and shown to be significantly higher (*, p = 0.039, n = 5) in cells transfected with the SENP1 mutant (inactive) compared with cells transfected with the SENP1 plasmid (active). F, inhibiting deSUMOylation increases the activity of endogenous pancreatic mGK in MIN6 β-cells. Cells were lysed by mechanical shear forces in the presence of control medium (light gray columns) or medium containing 0.5 or 1.0 μM SENP inhibitors (dark gray columns), and GK activity was measured as described in E. The activity of mGK was shown to be significantly higher (*, p = 0.047, n = 3) in MIN6 cells lysed in the presence of (1 μM) SENP inhibitors. Samples in E and F were analyzed by SDS-PAGE and immunoblotting using anti-GK and anti-actin. Mouse hexokinase activity in E and F was measured at 0.5 mm glucose and subtracted from the GK activity measured at 50 mm glucose. WB, Western blot.
pattern in vitro (data not shown). However, it is worth noticing that of the 15 diabetes-associated GCK mutations identified to date that affect a Lys residue (43), four have been functionally characterized and three have been reported to result in a thermally unstable protein (49, 50). Whether the instability is related to post-translational modifications, potentially due to a change in the total number of Lys residues, warrants further investigations.

Conjugation of recombinant pancreatic hGK by SUMO-1 in vitro was found to increase the catalytic activity of the enzyme by 20–30% (Fig. 5, A and B) at a partially (~8%) SUMO-conjugated enzyme (Fig. 1A). Interestingly, this stimulation in catalytic activity (Vmax) at a low nanomolar SUMO-1 concentration (~80 nM) is similar to what we reported for hGK by polyubiq-
Our biochemical and molecular studies suggest a physiological cell line. The SUMOylation sites were assigned to Lys-346, activity and stability, GK is regulated by SUMO-1 conjugation, which affects its secretion.

This activity increase is due to a more active SUMOylated GK, SUMOylation had been stimulated (Fig. 5, E and F). Whether this activity increase is due to a more active SUMOylated GK form, as seen for the recombinate enzyme (Fig. 5, A–C), or due to increased steady-state level of hGK protein (Fig. 6A) is uncertain.

In summary, we report here that the pancreatic isoform of GK is regulated by SUMO-1 conjugation, which affects its activity and stability, in vitro, and in the MIN6 insulin-secreting cell line. The SUMOylation sites were assigned to Lys-346, which is common to the pancreatic and liver isoforms, and to a cluster of lysine residues in the N terminus of pancreatic hGK. Our biochemical and molecular studies suggest a physiological role of SUMO conjugation in the regulation of GK in the pancreatic β-cell, as seen for other SUMOylated β-cell proteins (56). Further studies are needed to reveal how SUMOylation affects GK’s regulation of glucose metabolism and insulin secretion.

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