Human glucokinase (hGK) is a monomeric enzyme highly regulated in pancreatic β-cells (isoform 1) and hepatocytes (isoforms 2 and 3). Although certain cellular proteins are known to either stimulate or inhibit its activity, little is known about post-translational modifications of this enzyme and their possible regulatory functions. In this study, we have identified isoforms 1 and 2 of hGK as novel substrates for the ubiquitin-conjugating enzyme system of the rabbit reticulocyte lysate. Both isoforms were polyubiquitinated on at least two lysine residues, and mutation analysis indicated that multiple lysine residues functioned as redundant acceptor sites. Deletion of its C-terminal α-helix, as part of a ubiquitin-interacting motif, affected the polyubiquitination at one of the sites and resulted in a completely inactive enzyme. Evidence is presented that poly/multibuquitination of hGK in vitro serves as a signal for proteasomal degradation of the newly synthesized protein. Moreover, the recombinant hGK was found to interact with and to be allosterically activated up to ~1.4-fold by purified free pentaubiquitin chains at ~100 nM (with an apparent EC50 of 93 nM), and possibly also by unidentified polyubiquitinated proteins assigned to their equilibrium binding to the ubiquitin-interacting motif site. The affinity of pentaubiquitin binding to hGK is regulated by the ligand (D-glucose)-dependent conformational state of the site. Both ubiquitination of hGK and its activation by polyubiquitin chains potentially represent physiological regulatory mechanisms for glucokinase-dependent insulin secretion in pancreatic β-cells.

The glucose-phosphorylating enzyme glucokinase (GK) (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.2) plays a pivotal role in the regulation of glycolytic flux in hepatocytes and pancreatic β-cells at physiological millimolar concentrations of glucose (Glc). This 50-kDa size monomeric hexokinase catalyzes the phosphorylation of D-glucose to form glucose 6-phosphate with MgATP2− as the phosphoryl donor and is characterized by a low affinity for Glc ([S]0.5 ~ 8.0 mM), a positive kinetic cooperativity of Glc binding (nH ~ 1.7), and no feedback inhibition by its product.

Besides its expression in hepatocytes (isoforms 2 and 3) as a cytoplasmic and nuclear enzyme, where its translocation and activity is regulated by the glucokinase regulatory protein and the metabolic state of the cell (1), human GK is also expressed in pancreatic β-cells (isoform 1, the neuroendocrine isoform) mainly as a soluble cytoplasmic enzyme. In pancreatic β-cells GK has been found to be partitioned between the cytoplasm and the insulin secretory granules as a peripheral membrane protein (2–4) and in a regulated manner, i.e. mediated by Glc/insulin. In pancreatic β-cells, GK acts as the glucose sensor (5), a concept supported by the finding that complete GK deficiency leads to neonatal diabetes (6, 7). More than 200 different mutations have been identified in the hGK gene (8), and most of them lead to reduced enzyme activity and are associated with mild diabetes, maturity-onset diabetes of the young type 2 (MODY2). Others are characterized by an in vitro thermal instability when expressed as recombinant glutathione S-transferase (GST) fusion proteins (9). A few activating mutations have also been identified, leading to a hypoglycemic hyperinsulinism of infancy (8).

In both hepatocytes and pancreatic β-cells GK is activated by the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (10). Little is known about covalent post-translational modifications of GK and their possible regulatory functions, and the molecular and cellular mechanisms involved in its degradation/turnover are also poorly understood. In this study, we have identified isoforms 1 and 2 of hGK as novel substrates for the ubiquitin (Ub)-conjugating enzyme system of the rabbit reticulocyte lysate. The functional implications of this posttranslational modification have been studied in vitro with reference to the major roles ubiquitination plays in regulating a broad array of basic cellular processes (11) and in particular in vitro and in vivo polyubiquitination; UIM, ubiquitin-interacting motif; WT, wild type; Chaps, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.
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ticular in relation to the described regulatory function on the Glc-induced insulin secretion and secretion in pancreatic β-cells (12–15).

EXPERIMENTAL PROCEDURES

Materials—Anti-GK (H-88, rabbit polyclonal), peroxidase-conjugated anti-rabbit IgG (SC-2313, donkey) and peroxidase-conjugated anti-mouse IgG (SC-2030, goat) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-Ub (13–1600, mouse) that recognizes conjugated mono- and poly-Ub and free Ub was from Zymed Laboratories Inc. (San Francisco, CA). The QuikChange site-directed XL mutagenesis kit was obtained from Stratagene (La Jolla, CA). MagicMark™ XP Western Protein Standard and expression vector pcDNA3.1/HisC were purchased from Invitrogen. TnT T7 Quick-coupled Transcription/Translation System and MagZ Protein Purification System were from Promega (Madison, WI). L-[35S]Met (code AG 1094) and 14C-methylated protein standards were purchased from GE Healthcare. Rabbit reticulocyte Fraction II, human recombinant Ub aldehyde and pen-tauubiquitin (Ub5, Lys-48-linked), ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme Ubch1 (E2–25K), and the proteasome inhibitor MG-132 were from Boston Biochemicals (Cambridge, MA). Polyubiquitin chains (Ub2–7-Lys-48) were from Affiniti Research Products (Mamhead, UK). Hexokinase was from Sigma-Aldrich. Creatine phosphokinase was from Sigma-Aldrich, factor Xa. was from Protein Engineering Technology ApS, and the Detergent Surfact-Amps™20 was from Pierce. Chaps, IPG ampholyte solution, pH 3–10 NL buffer, and glutathione-Sepharose 4B were from Amersham Biosciences. Other two-dimensional electrophoresis components were ZOOM IPGRunner cassette, ZOOM Strip, electrode wicks, and MOPS buffer from Invitrogen (Cambridge, MA). Polyubiquitin chains (Ub2–7-Lys-48) were from Affiniti Research Products (Mamhead, UK). Hexokinase was from Sigma-Aldrich. 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kDa. The enhanced chemiluminescence detection method was employed to develop the immunoblots.

INS-1 and HepG2 cells were lysed, GK immunoinosolated from the cytosolic fractions by Protein A-Sepharose, electrophoresed, and immunoblotted with anti-ubiquitin or anti-GK Ab. For details, see supplemental methods.

Assay of hGK Activity—In the standard assay the catalytic activity of purified recombinant hGK was measured in a reaction volume of 1 ml containing: 25 mm sodium Hepes, pH 7.4, 25 mm KCl, 2.5 mm MgCl₂, 1 mm dithiothreitol, 0.1% (w/v) bovine serum albumin, 2.5 mm ATP, 1 mm NAD⁺, 0.35 units of glucose-6-phosphatase, 0.5 µg of recombinant hGK, and 50 mm D-glucose. The time course of the NADH formation was followed at 340 nm in a thermostated cuvette (37 °C) of the Hewlett Packard photodiode array spectrophotometer (Agilent 8453), and the activity was calculated from the linear slope.

Intrinsic Tryptophan Fluorescence—Intrinsic tryptophan fluorescence was performed on a PerkinElmer LS-50B instrument at 25 °C in a buffer containing 20 mm Hepes, 100 mm NaCl, and 1 mm dithiothreitol, pH 7.0, and a protein concentration of 0.03 mg/ml. The excitation and emission wavelengths were 295 and 340 nm, respectively, with slit widths of 4 and 7 nm.

Equilibrium Binding of Polyubiquitin Chains to hGK and hGKΔC24—The chromatographic holdup assay was performed essentially as described by Charbonnier et al. (16). For details, see supplemental methods.

RESULTS

Structural Analysis—An analysis of the three-dimensional structure of hGK revealed the sequence EEESGGAAALYSAVA at positions 442–456 at the C-terminal charged residue, details, see supplemental methods.

The sequence is homologous to the core ubiquitin-interacting motif (UIM) sequence eeexdxXXXSSXxe, where e is a negatively charged residue, φ is most often the hydrophobic residues Leu, Ile, or Ala, and X is any amino acid (17, 18). Interestingly, the helix length, and thus the conformation of this site, changes upon binding of Glc and an allosteric activator (Fig. 1B), and its orientation relative to the specifically interacting helix 6 (residues 204–217) changes as well (Fig. 1C). In general, UIM sites promote ubiquitination as well as binding of polyubiquitin chains (17, 18), and both possibilities were addressed in the present study.

In Vitro Synthesis and Posttranslational Ubiquitination of hGK—As seen from Fig. 2, lane 1, [35S]methionine (Met)-labeled His₆-hGK synthesized in the coupled in vitro transcription-translation system of the RRL is a substrate for the Ub-conjugating enzyme system of the lysate. Several high molecular mass bands were observed in addition to the full-length His₆-hGK (56 kDa). Some minor low molecular mass bands were regularly observed, presumably representing incomplete chains (19) because their presence was not affected by protease or proteasome inhibitors. An identical electrophoretic pattern was observed with a construct of WT-hGK (Fig. 2, lane 2), but of slightly lower molecular mass. The apparent molecular masses of the individual radioactive bands were 56, 67.5, 74, and 79 kDa for His₆-hGK and 53, 64.5, 71, and 76 kDa for WT-hGK, respectively, and the high molecular mass bands amounted to ~17% of the total. The [35S]Met-labeled His₆-hGK was purified by affinity binding to and gradient elution from Ni²⁺-chelated magnetic beads. The 56-kDa His₆-hGK was preferentially eluted at 1 M imidazole (Fig. 2, lane 3). Using the purified His₆-hGK as the substrate in the in vitro RRL system the level of Ub conjugates was found to increase in proportion to the concentration of the RRL lysate used in the incubation (supplemental Fig. S1A, lanes 1–6, and S1B).

To further characterize the Ub conjugates of hGK, purified recombinant hGK (isoforms 1 and 2) was used as substrate for the Ub-conjugating enzyme system of the RRL. When analyzed by two-dimensional electrophoresis in a high-resolution polyacrylamide gradient gel, immunodetection with anti-GK revealed that the high molecular mass forms were positioned in a diagonal pattern at a progressively higher pl than the unmodified hGK, as expected for a polyubiquitinated protein (Fig. 3, A and B). Immunodetection with anti-ubiquitin Ab revealed a similar pattern (Fig. 3A, inset). The conjugates in these experiments represented ~50% of the total immunoreactive protein in the gels for both isoforms. Interestingly, the molecular mass position of hGK-Ub₁ and hGK-Ub₂ revealed a double spot. The respective spots demonstrated the same pl but different mobilities as SDS denatured proteins, presumably due to different conformations, thus indicating two putative ubiquitin acceptor sites in hGK.

To possibly identify the target residue(s) for ubiquitination, the 22 lysine residues common to the isoforms were individually mutated to arginine (Arg) (supplemental Table S1) and the mutant forms were expressed in vitro as His₆-tagged and [35S]Met-labeled proteins in the RRL system. For all mutant forms, identical electrophoretic patterns of Ub conjugates were observed, as shown for WT-hGK in Fig. 2. The lysine residues are distributed rather uniformly along the protein, with some residues closely spaced in the linear sequence of flexible loop structures or closely spaced in the three-dimensional structure (20). Hence, multiple lysine residues of hGK presumably function as redundant acceptor sites. When the hGKΔC24-truncated form, devoid of the putative UIM site (Fig. 1), was expressed in the RRL system the double bands observed for His₆-hGK-Ub₁ and His₆-hGK-Ub₂ of the WT-hGK were replaced by single bands (Fig. 4, A and B). This finding indicates that the ubiquitination of one of the target sites is coupled to the UIM site. Interestingly, the C-terminal-truncated form revealed a total loss of catalytic activity (data not shown).

Proteasomal Degradation of Newly Synthesized and Ubiquitinated hGK—Proteasome-dependent degradation of hGK was studied co-translationally by expressing [35S]Met-labeled His₆-hGK for only 30 min in the standard RRL system. Then its stability was followed in a proteasome- and ATP-enriched RRL system, in the absence and presence of the proteasome inhibi-
tor MG-132. As seen from Fig. 5, the total intensity of the hGK signal (radioactivity), including His6-hGK and its ubiquitinated forms, increased 1.8-fold (n = 5 and p = 0.008) in the presence of MG-132. Thus, inhibition of the proteasomal activity resulted in a stabilization of the newly synthesized enzyme. The degree of inhibition observed may to some extent have been

FIGURE 1. The C-terminal domain of hGK with the putative UIM site and the conformational changes induced upon binding of D-glucose and allosteric activator (Compound A). A, close-up view into the putative UIM site in the three-dimensional structure of the nonliganded (super-open) conformation (Protein Data Bank 1v4t) of hGK with the key interacting residues indicated. B, schematic representation of the change in the backbone dihedral torsion angles (\(\Delta \phi + \Delta \psi\)) for residues 442–464 upon binding of D-glucose and allosteric activator as calculated from the structures of the unliganded (super-open) form (PDB 1v4t) and the liganded (closed) form (PDB 1v4s). The boxes represent the difference in the length of the C-terminal \(\alpha\)-helix in the super-open conformation (helix 17, residues 448–459) and the closed conformation (helix 19, residues 444–459). C, the figure demonstrates the change in relative orientation of the interacting C-terminal \(\alpha\)-helix (helix 17/19) and helix 6 (residues 204–217) upon transition from the super-open to the closed conformation. D, schematic representation of the static solvent accessibility of Glu-442, Glu-443, Glu-445, Ala-449, and Ser-453 as calculated by the CUPSAT algorithm (available at cupsat.uni-koeln.de/) in the super-open conformation (open bars) and closed conformation (filled bars) in which the allosteric activator (Compound A) interacts with Val-452 and Val-455. The three-dimensional images were generated with PyMol (www.pymol.org).

FIGURE 2. Expression of hGK cDNA in the in vitro transcription-translation system. His6-hGK (plasmid pcDNA3.1/HisC) and WT-hGK (plasmid pcDNA3.1+) were translated in a transcription-linked RRL translation system at 30 °C for 90 min as described under "Experimental Procedures." Lanes 1 and 2 represent total [\(^{35}\)S]Met-labeled translation products analyzed by SDS-PAGE (10%) with His6-hGK (lane 1) and WT-hGK (lane 2). Lane 3 represents His6-hGK purified by affinity chromatography on a Ni\(^{2+}\)-chelate resin (elution with a concentration gradient of imidazole) with partial recovery of the high molecular mass forms (indicated by arrows) at 1 M imidazole; the high molecular mass forms preferentially eluted at lower imidazole concentrations. Lane 4, the recombinant GST-WT-hGK (76-kDa), and lane 5, the recombinant WT-hGK (50 kDa) and the GST fusion partner (26 kDa) after cleavage of the fusion protein with factor Xa.

FIGURE 3. Immunodetection of mono- and poly-/multiubiquitinated recombinant WT-hGK in a reconstituted RRL system. A, hGK pancreatic isoform 1. B, hGK liver isoform 2. Two-dimensional electrophoresis (4–12% (w/v) gradient gel) and Western blot analysis using the anti-GK Ab demonstrated the high molecular mass Ub conjugates as a ladder of diagonal spots characteristic of mono/poly-Ub conjugates of the two isoforms. Inset in panel A, two-dimensional electrophoresis of hGK pancreatic isoform 1 and Western blot analysis using anti-ubiquitin Ab. The arrows indicate hGK and Ub-related spots; asterisks indicate nonspecific ubiquitinated proteins in the reticulocyte lysate.
Glucokinase Activation and Proteasomal Degradation

Equilibrium Binding of Polyubiquitin Chains and Catalytic Activation of WT-hGK—To further characterize the C-terminal UIM site (Fig. 1), we studied the equilibrium binding of polyubiquitin chains (mainly Ub<sub>5</sub>-Lys-48-linked) to GST-WT-hGK or GST-hGKΔC24 immobilized on glutathione-Sepharose 4B using a chromatographic holdup assay. The amount of bound and free analyte was measured by SDS-PAGE (Fig. 4C), which revealed that polyubiquitin chains bind to GST-WT-hGK in the absence of Glc (Fig. 4D, columns 1 and 2), but not to GST-hGKΔC24 lacking the UIM site (Fig. 4D, columns 3 and 4). Moreover, in the presence of 50 mM Glc, the binding of the analyte to GST-WT-hGK was markedly reduced (columns 5 and 6) as expected from the lower accessibility of the key interacting residues of the UIM site in the ligand-bound conformation of WT-hGK (Fig. 1D).

As seen from Fig. 6A and 6B, column 2, free polyubiquitin chains also stimulated the catalytic activity of recombinant hGK in a concentration-dependent manner and in the low nanomolar concentration range. An ~1.4-fold stimulation (n = 4) was observed at 97 nM Ub<sub>5</sub>-Lys-48, and the $\left[S\right]_{0.5}$ value for Glc was slightly reduced, i.e. from 8.62 ± 0.22 to 8.18 ± 0.27 mM (data not shown). A similar small reduction was observed in the $K_d$ value for the equilibrium binding of Glc at 25 °C, i.e. from 4.8 ± 0.06 to 4.3 ± 0.15 mM, as determined by intrinsic trypto-

affected by the relatively high concentration of nonspecific Ub conjugates in the RRL representing competitive substrates in terms of proteasomal degradation (21).
Glucokinase Activation and Proteasomal Degradation

In pancreatic β-cells, the ubiquitin-proteasome pathway has been found to have a regulatory role in the Glc-stimulated insulin release (15), Glc-stimulated (pro)insulin synthesis (13), and in the biogenesis and surface expression of the ATP-sensitive K⁺ (K_ATP) channels (12) as well as in maintaining a normal function of the voltage-dependent calcium channel (14). In the present study, we demonstrate that hGK of the pancreatic β-cell (isoform 1) and the hepatocytes (isoform 2) are posttranslationally modified by multiple moieties of Ub. Two-dimensional electrophoresis (Fig. 3, A and B) demonstrate the formation of hGK-Ub1−3 on at least two putative target lysine residues where the double spot of SDS-denatured hGK-Ub1 and hGK-Ub2 in the second dimension represent two ubiquitinated species with presumably different conformations having the same pI. A similar heterogeneity (double bands) has been reported for in vitro ubiquitinated luciferase (23) as well as for Lys-48-Ub2 and Lys-48-Ub3 synthesized in vitro (24). However, on site-directed mutagenesis, in which 22 of the lysine residues in hGK were individually mutated to Arg and expressed in vitro, no specific acceptor site could be identified. Multiple lysine residues in hGK, therefore, seem to function as redundant acceptor sites as previously exemplified by cyclin B (25) and cyclin A (26). It should be noted that the mutant forms tested were catalytically active with a specific activity in the range of 65–99% of WT-hGK (data not shown). Deletion of the 24 C-terminal amino acids, with the UIM site, resulted in polyubiquitination at an apparently single site (Fig. 4A), indicating that the ubiquitination of one site is promoted by the UIM site as previously shown for UIM-containing proteins (27).

Polyubiquitination often serves as a signal for targeting cytoplasmic and nuclear proteins to the proteasome for subsequent degradation (reviewed in Ref. 11). We found that the 30-min in vitro translated [35S]Met-labeled His₆-hGK and its ubiquitinated forms were unstable in a proteasome- and ATP-enriched reticulocyte lysate degradation assay that was sensitive to the proteasome inhibitor MG-132 (Fig. 5). It has recently been proposed (28) that the key targeting step for proteasome-mediated degradation is the conjugation of multiple short ubiquitin chains, independent of linkage type. This finding may explain the proteasomal degradation of newly synthesized hGK in our cell-free system, suggesting that its degradation may function as a regulatory mechanism in the homeostatic control of the cellular GK protein expression as demonstrated for several long-lived proteins (29). In hepatocytes (rat) expressing most of the total GK the sequestration and degradation of cytoplasmic GK occurs by the autophagosomal-lysosomal pathway at a rate of 3.5%/h and a half-life of 12.7 h (30). So far, we have no experimental data to support that ubiquitinated hGK is preferentially targeted to the autophagosomal-lysosomal pathway. An active autophagocytosis has been demonstrated in mouse β-cells (31, 32) and in NIT insulinoma cells (33), including autophagy of insulin secretory granules, and may in this way be involved in the turnover of the membrane-bound form of GK. In certain diabetes-associated mutations in hGK (MODY 2) a reduced stability of the enzyme has been considered as a possible mechanistic explanation for the hyperglycemia and reduced Glc-stimulated insulin secretion (9). Because ubiquitination of misfolded proteins associated with cytoplasmic chaperones may be degraded predominantly through the ubiquitin-proteasome system (34), further studies are in progress to investigate this possibility for selected loss-of-function mutant forms of hGK.

Ubiquitin-binding domains are found in proteins that function in a vast range of cellular events, including the activation of kinases in the nuclear factor-κB signaling pathway (35). Here we demonstrate that free poly-Ub chains (Ub₅-Lys-48-linked) allosterically activate hGK at low nanomolar concentrations (Fig. 6, A and B), i.e. at ten times lower concentrations than for monoubiquitin. From Fig. 6A it is seen that the catalytic activity of hGK is increasingly stimulated by Ub₅ in the concentration range of 20–100 nM, with an apparent EC₅₀ value of 93 nM (Fig. 6A). It is shown that the polyubiquitinated chains act by an equilibrium binding to the UIM site (Fig. 1), as demonstrated by the holdup binding assay (Fig. 4, C and D). The UIM site is located in the highly mobile C-terminal part including helix 17/19, which specifically interacts with helix 6 (Fig. 1C) both in the “super-open” (helix 17) and in the closed (helix 19) form of hGK. Interestingly, seven activating mutations of hGK have presently been characterized, all clustered in this defined area, and have been considered to represent an allosteric activator site (36). Two of the mutations (Y214C and Y215A) are located in helix 6 and three (V455M, A456V, and A460R) in helix 17/19. Moreover, several synthetic organic compounds, which activate the enzyme by a V_max and [S]₀.₅ effect, have been found to interact with the same site (i.e. Val-452 and Val-455) (20, 37) and thus considered to represent potential antidiabetic drugs (20, 37). The putative physiological endogenous activator interacting at this site has, however, still to be discovered.

Our data support the conclusion that polyubiquitin chains, either free or conjugated to proteins (Fig. 6), may represent such a physiological allosteric activator. Interestingly, the maximum stimulation of catalytic activity by Ub₅-Lys-48 (V_max) was similar to that recently reported for the synthetic allosteric activator RO-28-1675 (~1.5-fold) (9). Within an in vivo context, free polyubiquitin chains or polyubiquitinated proteins in GK-expressing cells (supplemental Fig. S2) may have a similar function. Thus, the level of Ub is reported to be 10–20 μM in a variety of cultured cell lines in and rabbit reticulocytes (38–40), where the Ub conjugates represent ~80% of the total Ub level (38). Furthermore, free polyubiquitin chains represent a substantial portion of the total Ub pool in vivo (41), as in the reticulocyte lysates used in the present study (supplemental Fig. S2A).
The bulk concentration of Ub conjugates was estimated by SDS-PAGE and immunoblots to be $\sim 9 \mu M$ (Ub$_{2-7}$ polyubiquitin chains served as a standard of reference), and on two-dimensional electrophoresis polyubiquitin chains represented a major fraction, $\sim 4 \mu M$. Estimation of Ub conjugates in GK-expressing cells, i.e. MIN-6 and HepG2 cell lines, revealed even higher levels of immunoreactive Ub conjugates than in the reticulocyte lysate (supplemental Fig. S2A). Moreover, immunoprecipitated GK from INS-1 and HepG2 cells demonstrated on SDS-PAGE and immunoblotting with anti-ubiquitin Ab several high molecular mass bands (supplemental Fig. S2B, lanes 2 and 4, respectively) distributed over a larger $M_r$ range than that observed in the in vitro RRL system. Several potential mechanisms exist for the homeostatic regulation of the fractional level of Ub conjugates in the cell (39), including the ATP-dependent ligation of Ub (42) and the expression and activity of multiple deubiquitinating enzymes (15, 43). Both the ligation and the degradation system are energy dependent, and the cellular level of conjugated Ub has been found to be regulated by the ATP level (44). In the context of the pancreatic β-cell the glucose-stimulated increase in ATP level, which triggers insulin secretion by closing the $K_{ATP}$ channels, depolarization of the plasma membrane, and Ca$^{2+}$ influx may also promote the formation of polyubiquitin conjugates and thus amplify the glucose response by activating the glucokinase (positive feedback). However, the Ub-conjugating/deconjugating enzymes of the β-cells (12–15) remain to be further characterized.

In general, an unanswered query in the biology of ubiquitin interaction is how ubiquitin/polyubiquitin dissociates from ubiquitin-binding domains. In the case of hGK the affinity of Ub$_b$ binding is higher in the absence of Glc (super-open conformation) than in the presence of Glc (closed conformation) (Fig. 4, C and D). It should be noted that the static solvent accessibility calculated for key interacting residues of the UIM site in the ternary hGK-Glc-GKA complex (Fig. 1D) may not be completely representative for the binary hGK-Glc complex. Thus, it is not clear to what extent the binding of the GK activator (Compound A) perturbs the structure of the binary complex.

Our study supports the conclusion that poly-/multimerization of hGK in vitro serves as a signal for proteasomal degradation of the newly synthesized protein. Moreover, recombinant hGK interacts with and is allosterically activated up to $\sim 1.4$-fold by purified free polyubiquitin chains at low nanomolar concentrations assigned to their equilibrium binding to an UIM site at the C-terminal. Both ubiquitin-mediated processes represent potential physiological regulatory mechanisms for GK as a glucose sensor in pancreatic β-cells and hepatocytes.

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