

(Re-)Viewing Role of Intracellular Glucose Beyond Extracellular Regulation of Glucose-Stimulated Insulin Secretion by Pancreatic Cells

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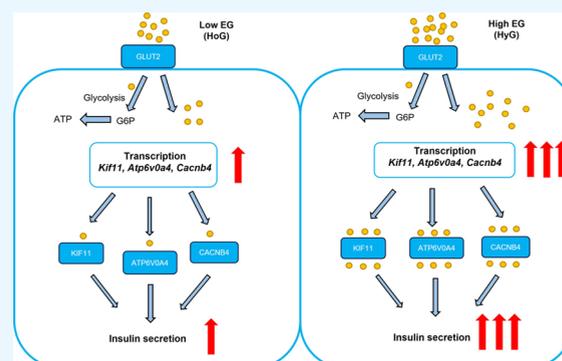
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ABSTRACT: For glucose-stimulated insulin secretion (GSIS) by pancreatic β -cells in animals, it is believed that ATP generated from glucose metabolism is primarily responsible. However, this ignores two well-established aspects in literature: (a) intracellular ATP generation from other sources resulting in an overall pool of ATP, regardless of the original source, and (b) that intracellular glucose transport is 10- to 100-fold higher than intracellular glucose phosphorylation in β -cells. The latter especially provides an earlier unaddressed, but highly appealing, observation pertaining to (at least transient) the presence of intracellular glucose molecules. Could these intracellular glucose molecules be responsible for the specificity of GSIS to glucose (instead of the widely believed ATP production from its metabolism)? In this work, we provide a comprehensive compilation of literature on glucose and GSIS using various cellular systems - all studies focus only on the extracellular role of glucose in GSIS. Further, we carried out a comprehensive analysis of differential gene expression in Mouse Insulinoma 6 (MIN6) cells, exposed to low and high extracellular glucose concentrations (EGC), from the existing whole transcriptome data. The expression of other genes involved in glycolysis, Krebs cycle, and electron transport chain was found to be unaffected by EGC, except *Gapdh*, *Atp6v0a4*, and *Cox20*. Remarkably, 3 upregulated genes (*Atp6v0a4*, *Cacnb4*, *Kif11*) in high EGC were identified to have an association with cellular secretion. Using glucose as a possible ligand for the 3 proteins, computational investigations were carried out (that will require future 'wet validation', both in vitro and in vivo, e.g., using primary islets and animal models). The glucose-affinity/binding scores (in kcal/mol) obtained were also compared with glucose binding scores for positive controls (GCK and GLUT2), along with negative controls (RPA1, KU70–80, POLA1, ACAA1A, POLR1A). The binding affinity scores of glucose molecules for the 3 proteins were found to be closer to positive controls. Therefore, we report the glucose binding ability of 3 secretion-related proteins and a possible direct role of intracellular glucose molecules in GSIS.



INTRODUCTION

Diabetes mellitus is a metabolic disease resulting from impaired glucose sensing, dampened insulin activity, or an absolute pancreatic β -cell destruction. As per the reports of the International Diabetes Federation (IDF), there were 537 million diabetics worldwide in 2021, which is expected to reach 643 million by 2030 and 783 million by 2045.¹ Diabetes mellitus (DM) can be classified as Type 1 and Type 2, respectively. Although accounting for only 5–10% of the total diabetic individuals, Type 1 DM is often fatal, characterized by an autoimmune pancreatic β -cell destruction, which leads to absolute insulin deficiency.² Type 2 DM is a chronic metabolic disease that happens due to genetic as well as acquired factors, resulting in impaired pancreatic β -cell functions as well as tissue insulin insensitivity.^{3,4} A decrease in both pancreatic β -cell mass and insulin secretory granules has been reported.⁵ In fact, a 60% decrease in pancreatic β -cell mass with an associated decrease in GSIS is also known.^{6,7} Impaired insulin

secretion leads to hyperglycemia with an adverse effect on internal organs and complications like retinopathy, nephropathy, neuropathy, cardiovascular disease, and sexual abnormalities.^{8,9} Inter- and intraislet cellular communication-regulated insulin secretion is required to maintain a basal level of insulin and stimulate GSIS response.¹⁰ Glucose metabolism through glycolysis is performed by glucokinase (GCK), which is an isoform of hexokinase, termed hexokinase IV- β . This enzyme has been known to be the “glucose sensor” in pancreatic β -cells, performing the rate-limiting step of glucose phosphorylation.¹¹ Glucose can regulate glucokinase activity, thereby

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Table 1. Different Insulin-Secreting Model Systems Obtained from Different Species Used to Study GSIS

source or species	cell type of cells	cells	doubling time	GSIS response	role of glucose behind GSIS (extracellular or intracellular)	refs
hamster	cell lines	HIT	34 h	For HIT cells, a low GSIS response is observed as compared to primary islets. The doubling time of INS-1, INS1 832/13, and INS-1E cells ranges from 82 to 166 h; thus, subculturing would be time-consuming, although they show GSIS at physiological glucose level. Insulin secreted per 10^6 cells per h for BRIN-BD11, RINr, RINm, and RINm5F cells is considerably low. CRI-G1 is not responsive toward physiological glucose level. MIN6 shows comparable GSIS to that of islets and insulin secreted per 10^6 cells per hr is considerably high. β TC-1, NIT-1, and IgSV195 cells are not responsive toward physiological glucose level. The doubling time of EndoC- β H1 cells is 120 h; thus, subculturing would be time-consuming. β loxS cells show a low insulin content. CM and TRM-1 do not show GSIS.	extracellular	44
rat		82–166 h	45–51			
mouse		MIN6, β TC-1, β TC-6, NIT-1, IgSV195, β HC9	48–58 h,			26,52–56
human		EndoC- β H1, β loxS, CM, TRM-1, NAKT-15	120 h			57–62
hamster	islets	primary islets	NA	Subculturing of primary islets is often limited by availability of endocrine tissue. The GSIS response seen in some cell lines was comparable to that of the islets. The observed fold change in GSIS varied between the islet species when treated with different EGC's.	extracellular	44
rat			63			
mouse						63,64
human						64

regulating glucose usage as well as GSIS in pancreatic β -cells.¹² Hence, GCK proteins are also well-established glucose interactors. The transport of glucose into the β -cells is not rate limiting, executed by the glucose transporters GLUT1 or GLUT2, depending on the species, human or rodent, respectively. This rapid transport of glucose results in an equilibrium between the extracellular and the intracellular glucose molecules. Hence, GLUT proteins are also well-established glucose interactors.^{13–15} As is known, insulin secreted from the β -cells of the pancreatic islets utilizes extracellular glucose, which is required for metabolic energy. Also, prevalent views on insulin secretion by pancreatic β -cells in animals are known to be dependent on the extracellular glucose concentration stimulus as the triggering signal behind GSIS. This is due to glucose metabolism that generates ATP, followed by closure of K_{ATP} channels and opening of Ca^{2+} channels that elevate cytosolic Ca^{2+} , accounting for exocytosis of insulin granules and, subsequently, the first phase of insulin secretion.^{16–18} However, ATP generation or a rise in the ATP/ADP ratio may happen from other intracellular pathways also. Thus, the specificity of glucose in GSIS is not attributable to ATP from glucose metabolism alone; in fact, the reason behind this specificity remains undiscovered to date. The amplifying signal of GSIS that accounts for the second phase of insulin secretion is tightly regulated by glucose and dependent on the metabolism of glucose, and glucose can independently enhance insulin secretion due to its effect on K_{ATP} channels.^{19–21} Both the phases of GSIS happen due to the movement of “restless newcomers” or the “readily releasable pool” of insulin granules as well as the “reserve pool” of insulin granules that engage upon glucose stimulus and fuse to the plasma membrane of pancreatic β -cells.²² It has also been reported that the rate of intracellular glucose transport by GLUT proteins surpasses glucose phosphorylation rate by 100 times.²³ There is limited knowledge about these transported but unphosphorylated glucose molecules. As a matter of fact, no direct link has been established until date between glucose and insulin secretion. In straightforward words, why is GSIS considered to be glucose specific only, i.e., what are the direct roles of glucose molecules in triggering as well as amplifying GSIS? To investigate these questions, we hypothesized that intracellular (unphosphorylated) glucose molecules are somehow involved in the regulation of insulin secretion. While there have been regular attempts to gain insights into the regulatory action of glucose and insulin secretion specifically pertaining to ‘extra-pancreatic-cell-homeostasis’ and signaling pertaining to the role of glucose amplification behind GSIS and as a “surface ligand” behind triggering of GSIS, the intracellular role of glucose has been attributed to generation of ATP primarily due to its metabolism.^{24,25} We move beyond the “convention”. Our work specifically addresses the possible role of intracellular glucose in the β -cells.

To test the hypothesis as explained above, and as mentioned in Table 1, we used an experimental system whose GSIS was comparable to that of mouse islets (275 mU/10⁶ cells/24 h at 25 mM glucose stimulus) and has provided significant insights into GSIS over the years, i.e., the cell culture model system of Mouse Insulinoma 6 (MIN6) cells.^{26,27} From whole transcriptome studies of Min6 cells exposed to extracellular hyperglycemic conditions, we identified the upregulation of 3 secretion-specific genes corresponding to the proteins KIF11, ATP6V0A4, and CACNB4. KIF11, also known as Kinesin-related motor protein Eg5, mediates secretory protein

transport to the cell membrane from the Golgi complex through the transport of Rab6 containing CARTS,²⁸ expanded as carriers of trans-Golgi to the cell surface toward the cell membrane through the course of protein secretion.²⁹ CACNB4, one of the $Ca_v\beta4$ auxiliary subunits, remains attached to the transmembrane $Ca_v\alpha1$ subunit, thereby making up the functional Ca_v voltage-dependent calcium channel staged on the pancreatic β -cell membrane. These channels regulate β -cell insulin secretion.^{30–33} Mutation at the $\alpha3$ locus or loss of function of the $\alpha3$ isoform of V-type ATPase dampened insulin secretion in the isolated islets of oc/oc mice, indicating that the protein participates in the exocytosis of insulin secretory vesicles.³⁴ Also, the $\alpha4$ isoform of V-type ATPase has been found to be expressed in kidneys regulating acid–base equilibrium.³⁵ Based on our transcriptome data that showed upregulation of the $\alpha4$ isoform of V-type ATPase, we assumed that this protein might be involved in insulin secretion along with the $\alpha3$ isoform. The question that we aimed to address is whether these proteins sequester glucose or not. Using AutoDock Tools 4.2.6 (<http://autodock.scripps.edu>; ADT), glucose was investigated as a possible ligand for the above 3 proteins; the docked conformations were subsequently visualized using Discovery Studio 2021 (<https://discover.3ds.com/discovery-studio-visualizer-download>; DS). ADT is a docking program based on the Lamarckian genetic algorithm (LA). A free energy force field creates or predicts conformations of 10 docked ligand poses with their corresponding binding energies. The force field includes six pairwise evaluations (V), including respective sets of bound and unbound ligand (L), protein (P), and protein–ligand (P–L) conformations. The pairwise evaluations consider parameters consisting of repulsion, hydrogen bonding, electrostatics, and desolvation.^{36–40} For proteins not resolved entirely by X-ray, NMR, or EM, AlphaFold-predicted protein structures were used from AlphaFold⁴¹ protein structure Database v2.0 (<https://alphafold.ebi.ac.uk/>).

RESULTS AND DISCUSSION

Model Systems for Insulin Secretion that Mimic Pancreatic β -Cell Physiology. To emphasize the role of internalized and nonphosphorylated glucose molecules behind GSIS, we compiled a list of different insulin-secreting model systems that are known to perform GSIS and also looked into the role of glucose behind the same, as shown in Table 1. This includes cell lines and primary islets. In Table S14, we have shown Table 1 in detail. This includes the name (source or species) of the model systems and the procedure by which they were isolated, the stimulus method behind GSIS, extent of maximum insulin content or secretion, type of insulin assay, extent of fold change observed as a response to GSIS, and the role of glucose behind GSIS, with appropriate references. Each of the model systems has its own unique properties, which relate to pancreatic β -cell physiology or function. Starting with primary rat, hamster, mouse, human islets, or well-established cell lines, we also review nonislet cells engineered to produce insulin.^{42,43}

Compared to MIN6 cells, some of the cell lines secreted lower insulin (in terms of mU/10⁶ cells/h); some were unresponsive to glucose stimulation beyond a certain stimulus; and some did not exhibit GSIS at physiological glucose concentration, or did not show a considerable fold change in GSIS at increasing glucose concentrations. Since MIN6 cells have a doubling time of 48 h as opposed to 82–166 h of rat

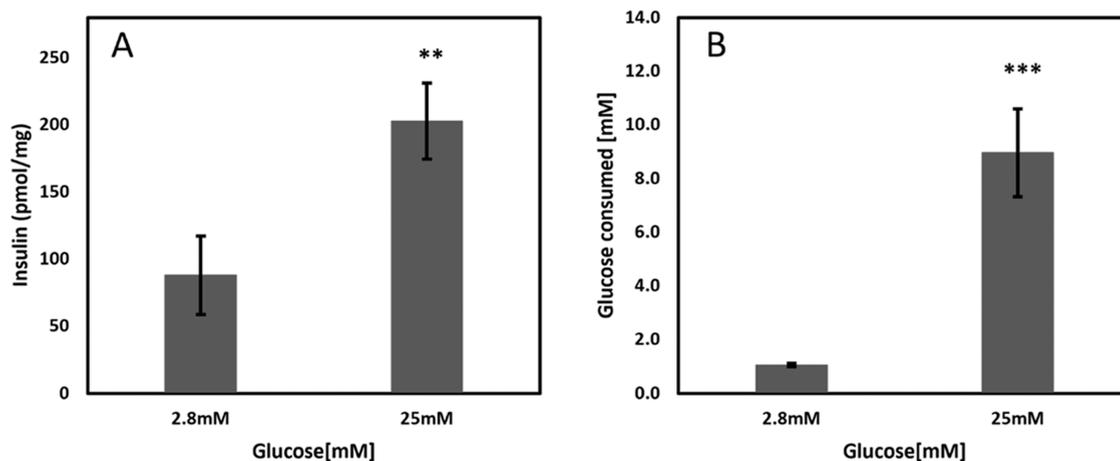


Figure 1. Insulin secretion and glucose consumption by MIN6 cells under HoG and HyG conditions. (A) Fold change in insulin secretion after treatment with 2.8 and 25 mM extracellular glucose. (B) Glucose consumption by cells, calculated by measuring the residual glucose concentration in the culture medium after 1 h of incubation with the initially supplied glucose concentrations of 2.8 and 25 mM extracellular glucose.

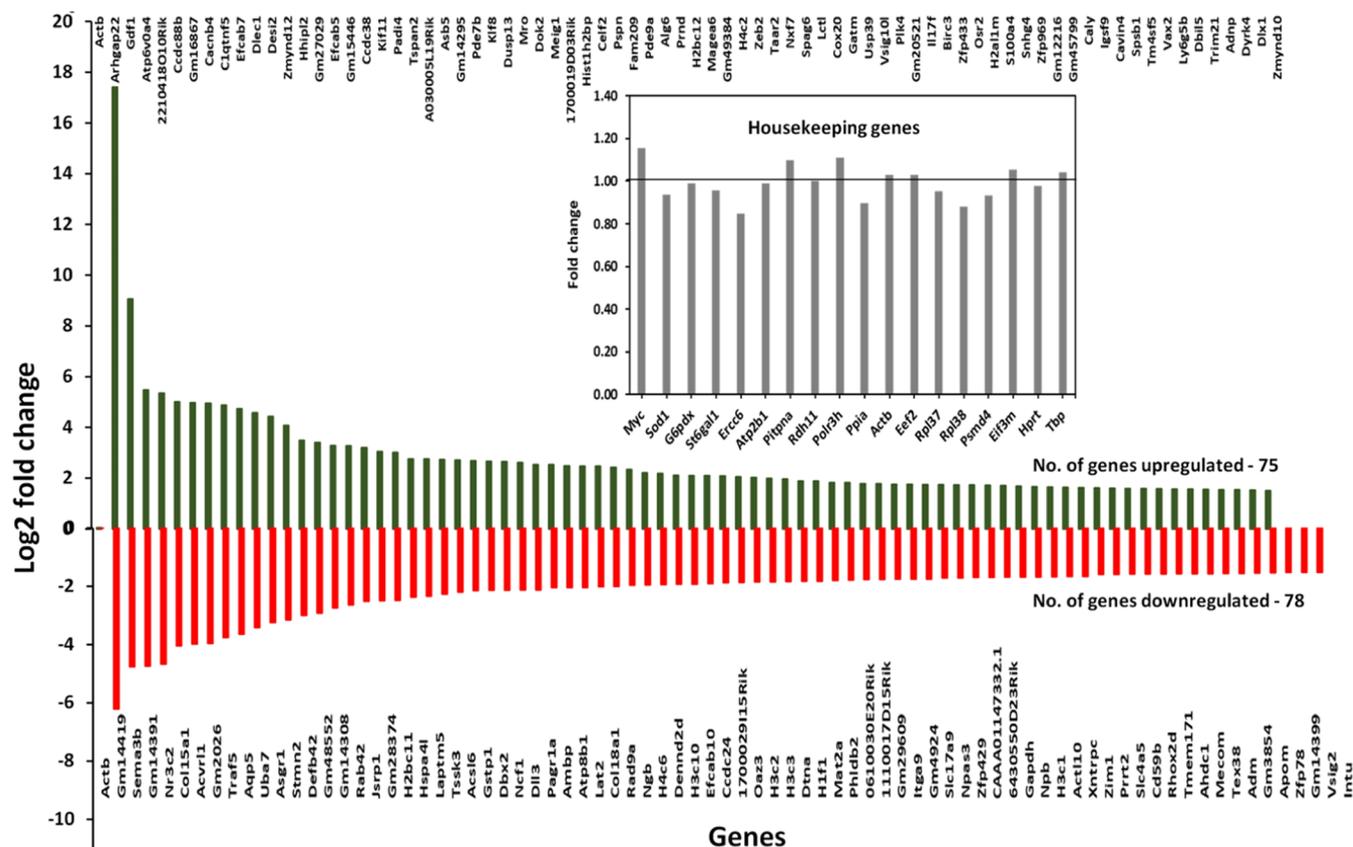


Figure 2. Transcriptome analyses of MIN6 cells under HoG and HyG conditions. The log₂ fold change (log₂ fc) was set at a threshold of ± 1.5 ; 75 protein-coding genes were upregulated and 78 protein-coding genes were downregulated at high EGC, in terms of FPKM. The inset shows control transcriptome analyses - housekeeping genes are relatively unaffected in HoG and HyG conditions (only fold changes are shown to highlight the very minor variabilities observed).

insulinoma cells or 120 h of human insulinoma cells, they are convenient for subculturing. As mentioned earlier, MIN6 cells secrete a considerable amount of insulin (275 mU/10⁶ cells/24 h at 25 mM EGC), which is comparable to that of mouse islets. Not only do they respond to physiological glucose levels but they also show enhanced GSIS with a significant fold change at incremental EGCs. Thus, all of these factors consolidate MIN6

cells as a robust *in vitro* model system for studying pancreatic β -cell physiology.

Insulin Secretion. MIN6 cells have been triggered by 2.8 and 25 mM extracellular glucose concentrations. Figure 1A shows increased insulin secretion when treated with 25 mM extracellular glucose, as opposed to 2.8 mM extracellular glucose. Glucose consumption was calculated by measuring the residual glucose concentration in the culture medium after 1 h

Table 2. Selection of Protein Structures: The Protein Structures Used for Glucose Binding Analyses through ADT

gene	protein	residues	organism	PDB/UniProt accession
KIF11	kinesin-like protein	1052	<i>Mus musculus</i>	Q6P9P6
KIF11	kinesin-like protein	1056	<i>Homo sapiens</i>	P52732
CACNB4	voltage-dependent L-type calcium channel subunit β -4	519	<i>M. musculus</i>	Q8R0S4
CACNB4	voltage-dependent L-type calcium channel subunit β -4	520	<i>H. sapiens</i>	O00305
ATP6V0A4	V-type proton ATPase116 kDa subunit a isoform 4	833	<i>M. musculus</i>	Q920R6
ATP6V0A4	V-type proton ATPase116 kDa subunit a isoform 4	840	<i>H. sapiens</i>	Q9H8G4
GCK	hexokinase 4	465	<i>M. musculus</i>	P52792
GCK	hexokinase 4	465	<i>H. sapiens</i>	1V4S/P35557
SLC2A2	solute carrier family 2, facilitated glucose transporter member 2	523	<i>M. musculus</i>	P14246
SLC2A2	solute carrier family 2, facilitated glucose transporter member 2	524	<i>H. sapiens</i>	P11168
RPA1	replication protein A 70 kDa DNA-binding subunit	623	<i>M. musculus</i>	Q8VEE4
RPA1	replication protein A 70 kDa DNA-binding subunit	616	<i>H. sapiens</i>	P27694
XRCC6/XRCC5	Ku70–80 heterodimer	A chain –609 B chain –565	<i>H. sapiens</i>	1JEQ
XRCC6	Ku70	608	<i>M. musculus</i>	P23475
XRCC6	Ku70	609	<i>H. sapiens</i>	P12956
XRCC5	Ku80	732	<i>M. musculus</i>	P27641
XRCC5	Ku80	732	<i>H. sapiens</i>	P13010
POLA1	DNA polymerase α catalytic subunit	1465	<i>M. musculus</i>	P33609
POLA1	DNA polymerase α catalytic subunit	1462	<i>H. sapiens</i>	P09884
ACAA1A	3-ketoacyl-CoA thiolase A, peroxisomal	424	<i>M. musculus</i>	Q921H8
ACAA1A	3-ketoacyl-CoA thiolase A, peroxisomal	424	<i>H. sapiens</i>	P09110
POLR1A	DNA-directed RNA polymerase I subunit RPA1	1717	<i>M. musculus</i>	O35134
POLR1A	DNA-directed RNA polymerase I subunit RPA1	1720	<i>H. sapiens</i>	O95602

of incubation with the initially supplied glucose concentration, as shown in Figure 1B. Insulin secretion increased up to 2.3-fold as compared to basal glucose level secretion. Next, we go through transcriptome studies to estimate the gene expression profile between HoG (2.8 mM) and HyG (25 mM) glucose concentrations, respectively.

Transcriptome Studies. In this work, we analyzed differential gene expression in MIN6 cells exposed to low and high extracellular glucose concentrations (EGC), from their whole transcriptome data (55,467 distinct transcripts, of which 14,427 were identified as successfully expressed genes).⁶⁵ The study of differentially expressed genes (DEGs) can be obtained with high precision when the true number of DEGs ranges from 15 to 30%, even in the absence of biological replicates.⁶⁶ We obtained a value of 26%; thus, our study of DEGs was precise enough. The data were submitted to the NCBI-GEO (National Center for Biotechnology Information – Gene Expression Omnibus) database repository with an assigned accession number record of GSE226652. The entire differential gene expression analyses study can be viewed through the link <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE226652> and reference⁶⁵. As shown in Figure 2 (inset), the fold change of housekeeping genes is unaffected under both HoG and HyG conditions. We have taken these genes as controls to check for differentially expressed genes at different glucose treatments. The log₂ fold change (log₂ fc) was set at a threshold of ± 1.5 . In terms of FPKM, we found 75 protein-coding genes to be upregulated and 78 protein-coding genes to be downregulated, respectively, at high EGC, as shown in Figure 2 as well as Table S1a,b, respectively. Of these, we identified that only 3 out of the upregulated genes (*Atp6v0a4*, *Cacnb4*, *Kif11*) are involved in secretion. These 3 genes were upregulated in terms of both FPKM and TPM. So, we went forward with FPKM values based on our results. Remarkably, other than 3 (*Gapdh*, *Atp6v0a4*, and *Cox20*), the

expression of genes associated with glycolysis, the TCA cycle, and ETC was found to be unaffected by EGC, as shown in Table S2. Thus, we report that glucose consumption as a nutrient is independent of EGC in GSIS.

Selection of Protein Structures, Glucose Binding Studies through ADT, and Subsequent Visualization of the Docked Conformations through DS. The protein structures used for glucose binding analyses were downloaded from RCSB PDB (<https://www.rcsb.org/>) and UniProt database (<https://www.uniprot.org/>). AlphaFold-predicted protein structures were used from AlphaFold protein structure Database v2.0 (<https://alphafold.ebi.ac.uk/>). The details are shown in Table 2. Truncated mouse KIF11 (residue 90–400) and truncated mouse CACNB4 (residue 60–450) were also used for glucose binding analyses.

The binding energies (kcal/mol) and the total number of interactions for the respective 10 conformations within a single protein as well as for every other protein molecule used are shown in Tables S6a,b, S7, and S8a,b, respectively. They have been categorized into 3 distinct protein sets: Test molecules identified by transcriptome analyses under hyperglycemic condition—KIF11 mouse, CACNB4 mouse, and ATP6V0A4 mouse as shown in Table S6a, and for truncated KIF11 (90–400) and truncated CACNB4 (60–450) as shown in Table S6b; positive controls, known interactors with glucose—GCK (human and mouse) and GLUT2 (human and mouse), as shown in Table S7; and negative controls, not known to interact with glucose—RPA1 human, KU70–80 human, POLA1 human, POLR1A human, and ACAA1A mouse, as shown in Table S8a,b, respectively. The highest binding energy values (kcal/mol) corresponding to a particular protein conformation have been shown in Table 3 for test molecules, positive controls, and negative controls. Accordingly, the two-dimensional (2D) diagram of the ligand-bound protein conformations that exhibited the highest binding energy values

Table 3. Summary of the Highest Glucose Binding Scores (kcal/mol) Obtained for the Respective Conformations of the Test Molecules, Positive Controls, and Negative Controls, Respectively

protein	conformation with the highest binding energy	binding energy (kcal/mol)
KIF11 mouse	10	-4.62
truncated KIF11 mouse (90–400 residues)	9	-4.56
CACNB4 mouse	6	-4.19
truncated CACNB4 mouse (60–450 residues)	3	-4.46
ATP6V0A4 mouse	1	-4.09
GCK mouse	4	-5.70
GCK human	1	-7.16
GLUT2 mouse	9	-5.35
GLUT2 human	3 and 4	-4.64
RPA1 human	4	-3.81
KU70–80 human	9	-3.75
POLA1 human	4	-3.58
POLR1A human	7	-2.66
ACAA1A mouse	1 and 3	-2.82

along with the different interactions as obtained through DS have been shown in Figure 3A–E, corresponding to KIF11, CACNB4, ATP6V0A4, truncated KIF11 and truncated CACNB4 respectively; Figure 3F–J corresponding to human GCK, mouse GCK, third docked conformation of human

GLUT2, fourth docked conformation of human GLUT2, and mouse GLUT2, respectively, and in Figure 3K–O corresponding to RPA1, KU70–80, POLR1A, ACAA1A, and POLA1, respectively. The highest binding energy corresponding to the respective conformation of the protein molecule along with the orientation or the binding propensity of the docked ligand (D-glucose) was chosen and is shown in Table S10.

Calculation of Pearson Correlation Coefficient (r). Pearson correlation coefficient (r) was calculated between the number of interactions for the respective conformations within a single protein molecule and the corresponding binding energies (kcal/mol) obtained. This was done for every other protein molecule used, as shown in Table S9. A scatter plot was drawn, as shown in Figure 4, which showed the number of interactions for every conformation within a single protein on the X-axis and the corresponding binding energy scores (kcal/mol) obtained on the Y-axis. This was done for all of the 14 protein molecules. Since binding energy should decrease and tend toward negative with increasing number of interactions as a stable ligand-protein interaction is achieved, one would expect a negative correlation between the 2 parameters for all of the protein molecules.

Binding Studies with Other Ligands. As described in the methodology for glucose binding analyses, binding studies were further done with hexophosphate ligands involved in glycolysis like G6P, F6P, and F16BP; epimers of glucose such as mannose and galactose; aldohexoses such as altrose, allose, gulose, idose, and talose, and ketohexoses such as fructose,

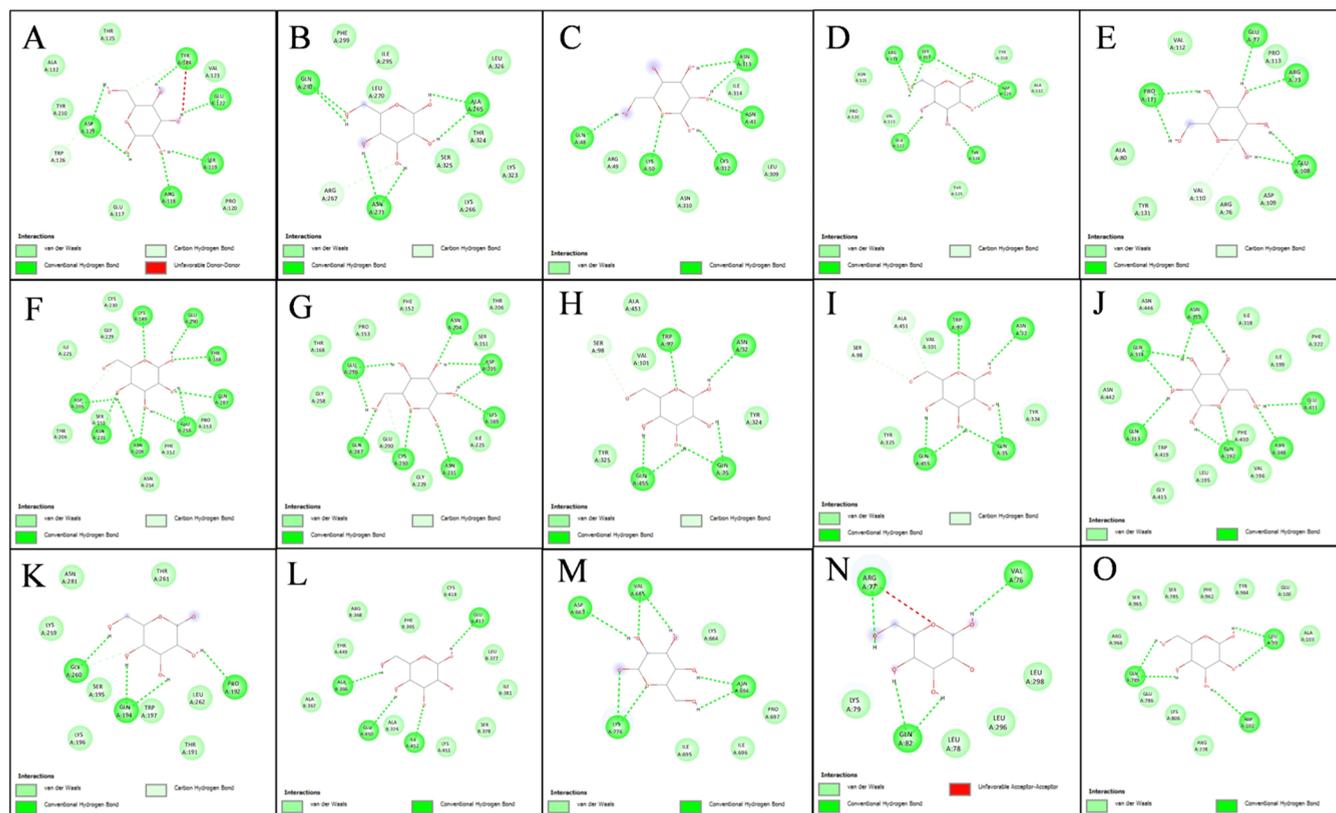


Figure 3. 2D diagrams of the ligand-bound protein conformations. The ligand is D-glucose and the proteins in (A–E) are KIF11, CACNB4, ATP6V0A4, tKIF11, and tCACNB4; these complexes were found to have the highest binding energy value scores (in kcal/mol). 2D diagrams of positive controls for the ligand-bound protein conformations; the proteins in (F–J) are human GCK, mouse GCK, 3rd conformation of human GLUT2, 4th conformation of human GLUT2, and mouse GLUT2, respectively. 2D diagrams of negative controls for the ligand-bound protein conformations; the proteins in (K–O) are RPA1, KU70–80, POLR1A, ACAA1A, and POLA1, respectively.

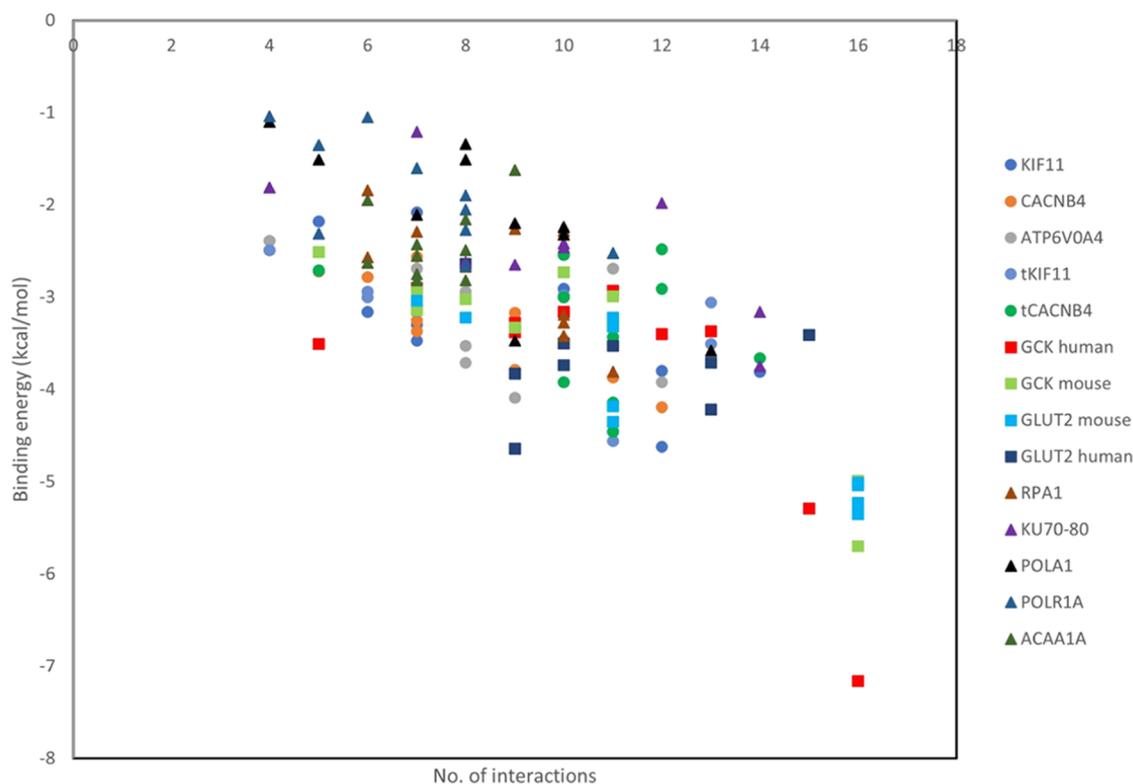


Figure 4. Decrease in value of binding energy with increasing number of interactions. The scatter plot shows the number of interactions for the respective 10 conformations of each protein molecule docked with glucose in the X-axis with their corresponding binding scores (kcal/mol) obtained in the Y-axis.

psicose, tagatose, and sorbose. The three-dimensional (3D) conformers of various ligands downloaded as .sdf files and used for binding studies have been shown in Table S11, with their respective PubChem CIDs (<https://pubchem.ncbi.nlm.nih.gov/>). The highest binding score obtained is indicated in parentheses alongside the corresponding conformation and is shown in Table S3. The binding scores obtained corresponding to the 10 docked conformations using the ligands as mentioned above have been shown in Table S3a–o, respectively, and the respective grid box parameters have been shown in Table S4a–o, respectively.

Comparison between the Binding Scores Obtained for Different Ligand-Protein Interactions. The highest binding score was shown by GCK (human -7.16 kcal/mol and mouse -5.7 kcal/mol, respectively) when docked with glucose, substantiating the fact that they are well-known glucose interactors. GLUT2 proteins (human -4.64 kcal/mol and mouse -5.35 kcal/mol, respectively) also showed a high binding score when docked with glucose, thus being well-known glucose interactors. Interestingly, the test proteins KIF11, CACNB4, and ATP6V0A4 showed binding scores of -4.62 , -4.19 , and -4.09 kcal/mol, respectively, when docked with glucose. These binding scores are closer to the glucose binding scores obtained for the positive controls. The negative controls RPA1, KU70–80, POLA1, ACAA1A, and POLR1A showed binding scores of -3.81 , -3.75 , -3.58 , -2.62 , and -2.66 kcal/mol, respectively, when docked with glucose. Surprisingly, mannose showed a binding score of -4 kcal/mol with GLUT2 mouse, and galactose showed a binding score of -4.05 kcal/mol with GLUT2 human, respectively. Psicose showed a binding score of -4.03 kcal/mol with GLUT2 human. Also, allose and talose showed a binding score of

-4.13 and -4.09 kcal/mol, respectively, when docked with GCK human, and gulose showed a binding score of -4.18 kcal/mol with GCK mouse.

Relevance of Grid Box Size Dimensions with Key Glucose Binding Residues of Human Glucokinase and Glucose Binding Score (kcal/mol). For glucose binding analysis with human GCK, the grid box size or the axes dimension points were first set at $x = 40$, $y = 40$, and $z = 40$, respectively. The highest binding score thus obtained with glucose as the ligand was -4 kcal/mol, and subsequent analysis through DS, as shown in Figure 5D, showed Lys¹⁶⁹ and Asp²⁰⁵ residues to be present in the glucose binding cavity. The grid box size was then increased to $x = 126$, $y = 126$, and $z = 126$, respectively. The highest binding score obtained with glucose as the ligand was -7.16 kcal/mol, and subsequent analysis through DS as shown in Figure 5A revealed Thr¹⁶⁸, Asn²⁰⁴, Asn²³¹, Glu²⁵⁶, and Glu²⁹⁰ to be present in the binding cavity residue along with Lys¹⁶⁹ and Asp²⁰⁵, participating in hydrogen bond formation with glucose. Interestingly, Glu²⁵⁶ is a key glucose binding residue in the active site of GCK. The natural GCK mutation at 256 E-K has been found in maturity-onset diabetes of the young (MODY2), and site-directed mutagenesis resulted in loss of glucose binding ability, rendering the enzyme catalytically inactive.^{67–70}

Validating AutoDock's Computational Output. Human GCK complexed with glucose, PDB – 1V4S, was taken, as shown in Figure 5B. The 2D diagram of the number of interactions obtained with glucose as analyzed through DS is shown in Figure 5C. Now, glucose was docked with human GCK protein through ADT and the 2D diagram of the conformation exhibiting the highest binding score of -7.16 kcal/mol was analyzed through DS, as shown in Figure 5E,F.

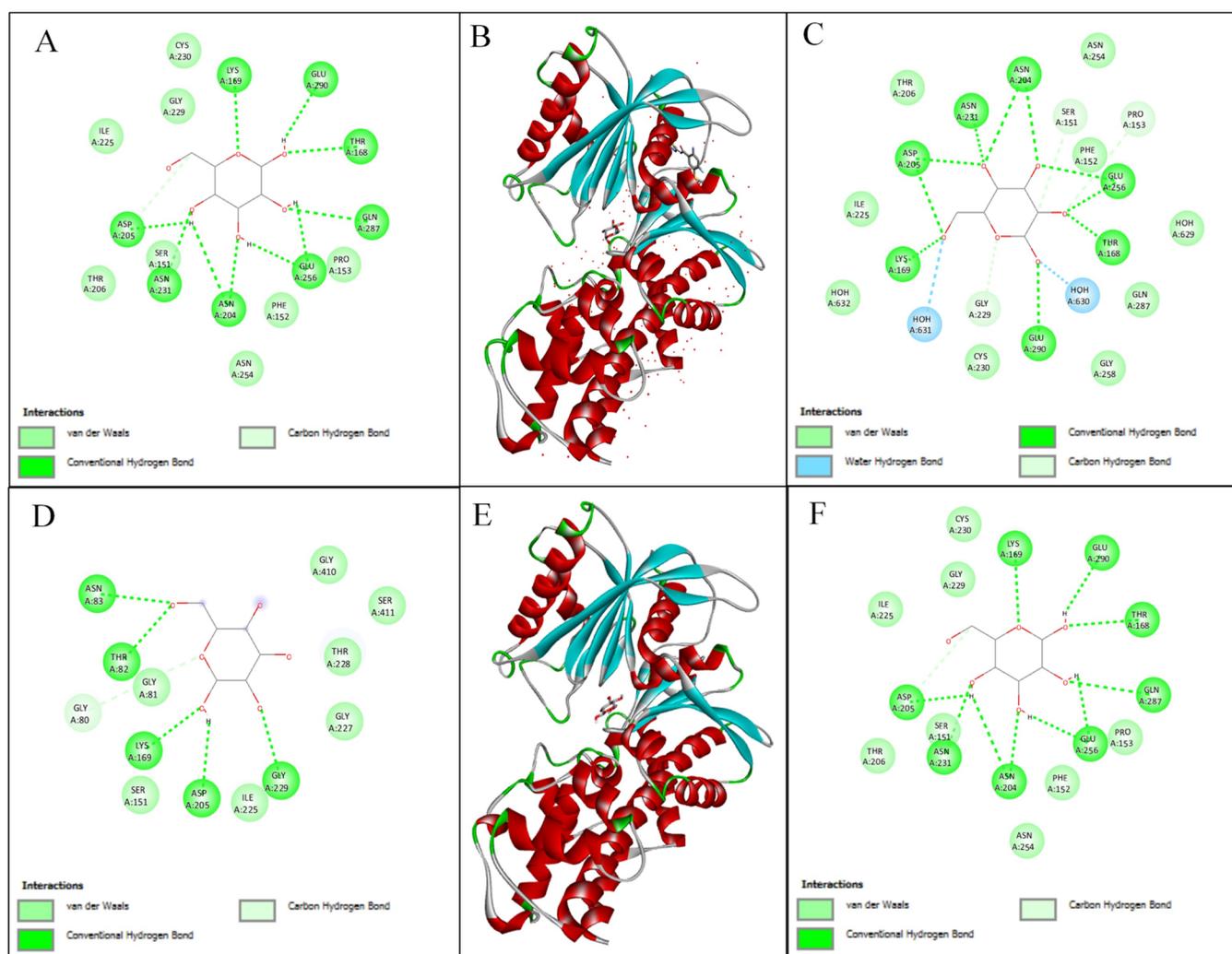


Figure 5. Relevance of the grid box size dimensions with the key glucose binding residues of human GCK protein and computation of glucose binding score (kcal/mol) along with validation of AutoDock's computational output. Human GCK protein interacting with glucose as analyzed through ADT and DS – Conformation 1 on the left (A) exhibiting the highest binding energy of -7.16 kcal/mol with all the 7 catalytic residues present, whereas only 2 catalytic residues are present (or 5 absent) in Conformation 4 at the bottom (D) exhibiting the highest binding energy of -4 kcal/mol. (B) shows the PDB structure (1V4S) of human GCK and (C) shows the interactions analyzed through DS. (E) shows conformation 1 of glucose-bound-human-GCK as obtained through ADT and (F) shows the interactions analyzed through DS.

The glucose binding pockets, as mentioned earlier, were remarkably similar in both Figure 5C and F involving the glucose-interacting residues Thr¹⁶⁸, Lys¹⁶⁹, Asn²⁰⁴, Asp²⁰⁵, Asn²³¹, Glu²⁵⁶, and Glu²⁹⁰.

Biological Validation of the Computational Results. To further validate AutoDock's computational output, experimentally obtained binding affinities between a particular protein and ligand in terms of K_m (mM) were categorized into positive and negative controls, and then were compared with the obtained binding scores in terms of kcal/mol of the same protein–ligand set. The protein–ligand sets are shown in Table 4, with appropriate references.^{71–76} A linear regression scatter plot was drawn between the binding affinity values in terms of K_m (mM) in the X-axis and AutoDock's computational output (binding score values) in terms of kcal/mol in the Y-axis, as shown in Figure 6. A strong/greater binding affinity is characterized by a lower/lesser K_m , and this should correspond to a lower (more negative) binding score in terms of kcal/mol. This was seen from the scatter plot, which showed

Table 4. Biological Validation of Computational Results

control	protein–ligand	binding score obtained from AutoDock (kcal/mol)	binding affinity obtained from literature, K_m (mM)	refs
positive control	GCK–glucose, close	-7.16	6	73
	GCK–glucose, close	-5.7	6	73
	islet GLUT2–glucose	-5.35	17	74
	liver GLUT2–glucose	-4.64	19	72
negative control	GCK without glucose, super open	NA	30	76
	soybean β -amylase	NA	45	75
	GLUT2 fructose	-3.66	76	71
	GLUT2 galactose	-4.05	92	71
	GLUT2 mannose	-2.4	125	71

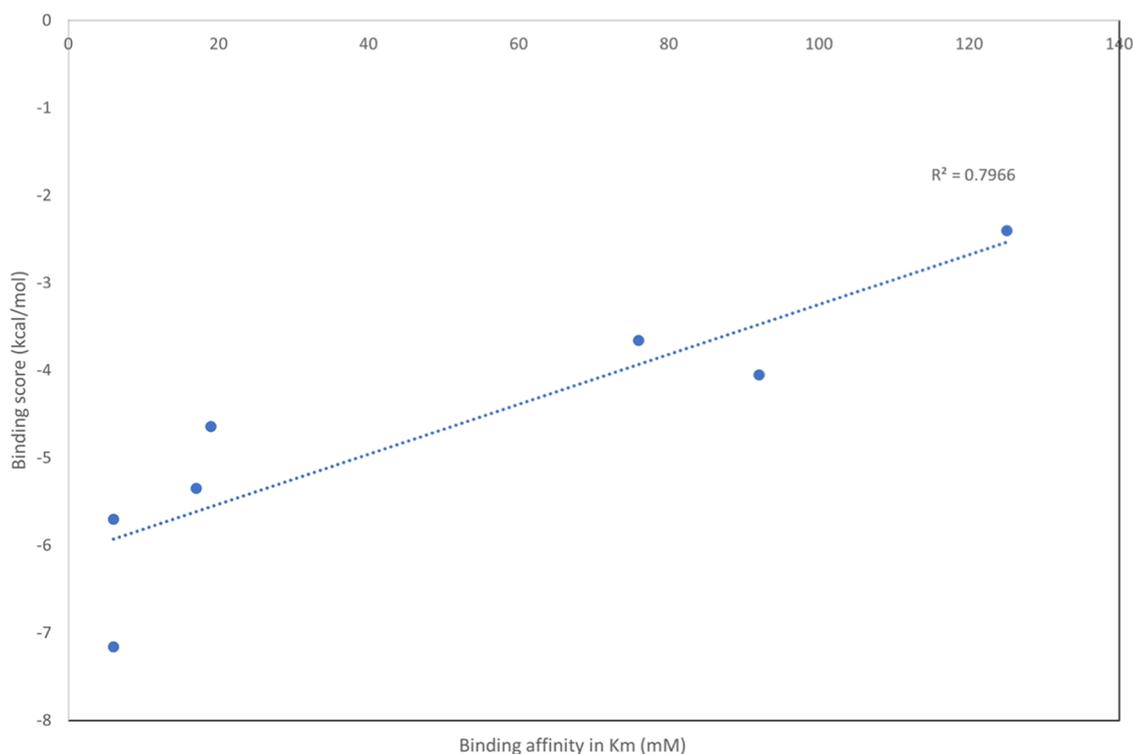


Figure 6. Biological validation of computational results. A linear regression scatter plot ($R^2 = 0.79$) between the experimental binding affinity values in terms of K_m in the X-axis and AutoDock-obtained binding scores in terms of kcal/mol in the Y-axis.

an R^2 value of 0.79 or 0.88 (data not shown), thus indicating a linear relationship between the two parameters.

Comparison between the Glucose Binding Scores Obtained with the Test Proteins and the Binding Scores Obtained with Other Ligands. The highest binding scores (kcal/mol) obtained with the test proteins using glucose as the ligand were statistically compared with the highest binding scores (kcal/mol) obtained with the test proteins for every other ligand, using two-tailed Student's t test assuming equal variances. The p-values thus obtained are shown in Table S12. Interestingly, none of the glycolytic metabolites or hexose sugars exhibited a similarity with respect to the glucose binding scores.

Comparison of Glucose Binding Scores between Homologous Proteins. Homologous proteins belonging to the same protein sets were chosen that were previously not used for binding studies, and then, binding studies were done specifically using glucose as the ligand for comparison. The glucose binding scores as obtained are shown in Table S13a. The glucose binding scores for the 10 docked conformations obtained for the homologous proteins are shown in Table S5. The binding scores of the test proteins of a particular species were statistically compared with the binding scores of positive controls, negative controls, and in-between positive and negative controls, respectively, of the same species. This was repeated for the other species too. Then, binding scores of similar proteins from two different species but belonging to the same set were statistically compared. Statistical analyses were done through two-tailed Student's t test assuming equal variances. The p-values thus obtained are shown in Table S13b.

CONCLUSIONS

Glucose functions to stimulate and, consequently, increase insulin secretion. This process involves glucose-mediated glycolysis and ATP utilization as an energy source. However, within biological systems, cells can generate energy to a certain extent, prompting questions about the conservation of cellular energy. Recent research studies provide insights into how cells conserve energy and secrete specific proteins, often at the expense of other secretory proteins.⁶⁵ Notably, glucose molecules play a dual role: they not only contribute to energy generation through GLUT2-glucokinase-mediated phosphorylation in glycolysis but also enhance insulin secretion by binding to other proteins that facilitate glucose-mediated insulin secretion. Transcriptomic analyses of hyperglycemic-exposed-MIN6 cells identified upregulation of 3 secretion-specific genes corresponding to proteins KIF11, ATP6V0A4, and CACNB4. We have correlated the relevance of these 3 proteins with respect to cellular secretion and/or insulin secretion. Briefly, KIF11, also known as Kinesin-related motor protein Eg5, mediates secretory protein transport to the cell membrane from the Golgi complex through the transport of RAB6 containing CARTS²⁸ expanded as carriers of the trans-Golgi to the cell surface toward the cell membrane.²⁹ RAB6 is a marker for secretory vesicles and can regulate post-Golgi secretion.⁷⁷ RAB6 mediates insulin vesicle exocytosis and secretion.⁷⁸ RAB6 also controls targeting of constitutive secretory vesicles through microtubule protein motor Kinesin-1 to ELKS-rich sites in the cell membrane.⁷⁹ ELKS is present in pancreatic β -cells near insulin granules docked on the PM and silencing of ELKS expression reduced GSIS.⁸⁰ CACNB4, one of the $Ca_v\beta$ 4 auxiliary subunits, remains attached to the transmembrane $Ca_v\alpha$ 1 subunit, thereby making up the functional Ca_v voltage-dependent calcium

channel staged on the pancreatic β -cell membrane. These channels regulate β -cell insulin secretion.^{30–33} Mutation at the $\alpha 3$ locus or loss of function of the $\alpha 3$ isoform of V-type ATPase dampened insulin secretion in the isolated islets of *oc/oc* mice, indicating that the protein participates in the exocytosis of insulin secretory vesicles.³⁴ Also, the $\alpha 4$ isoform of V-type ATPase has been found to be expressed in kidneys regulating acid–base equilibrium.³⁵ Thus, based on our transcriptome data, we proceeded with these 3 proteins only, which are also known to be involved in cellular secretion. These proteins might sequester intracellular but nonphosphorylated glucose and may take the center stage behind GSIS. Hence, using ADT, glucose was investigated as a possible ligand for the above 3 proteins. Glucose showed a higher binding score (in kcal/mol) among all metabolites and other hexoses ($C_6H_{12}O_6$'s) with all the 3 proteins. Further, the glucose-affinity or binding scores with the 3 proteins were also compared with the scores (in kcal/mol) for glucose as a ligand for positive controls, GCK and GLUT2, and along with negative controls, RPA1, KU70–80, POLA1, ACAA1A, and POLR1A. The binding scores of glucose molecules for KIF11, ATP6V0A4, and CACNB4 were found to be closer to positive controls with statistically significant scores compared to negative controls. Of course, future wet-laboratory work on the binding of glucose to purified KIF11, CACNB4, and ATP6V0A4 molecules would provide further validation to the results obtained here. In this context, “wet validation” may explicitly require a careful dissection of the results depending on the technique (e.g., in vitro (i) single-cell-based vs population cell cultures^{85,86}, (ii) types of cells, e.g., MIN6 vs primary islets etc., or in vivo) while considering specific intracellular conditions (e.g., compartmental/local pH⁸⁶ etc.) encountered in specific wet-experimental systems. Furthermore, intracellular energy transactions,^{65,87} especially viewing the role of glucose molecules as intracellular ligands rather than as a source of metabolic energy, will also need to be carefully analyzed. The computational approaches utilized here do not consider such variables arising out of wet-experimental set ups. The above said, we report glucose sequestration ability OR regulation-by-glucose-binding of the three cellular secretion-related proteins for the first time, thereby presenting strong evidence relating to a possibly direct role of intracellular but unphosphorylated glucose molecules in GSIS. This opens a unique and novel perspective toward understanding the pathology of clinical conditions pertaining to exogenous-insulin-requiring diabetes, including (onset of) Type 1 Diabetes, at a cellular level.

MATERIALS AND METHODOLOGY

Chemicals. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Penstrep, and trypsin-EDTA were obtained from Gibco. Monosodium phosphate (NaH_2PO_4), Rat/Mouse (EZRMI-13K) Insulin ELISA Assay Kit, and ethanol were purchased from Merck. Magnesium chloride ($MgCl_2$), glucose, calcium chloride ($CaCl_2$), potassium chloride (KCl), HEPES, monopotassium phosphate (KH_2PO_4), sodium chloride (NaCl), dipotassium phosphate (K_2HPO_4), sodium bicarbonate ($NaHCO_3$), formaldehyde, 2-propanol, BSA, and Bradford were purchased from Himedia, India. Glucose oxidase, 4-aminoantipyrine *N*-ethyl-*N*-sulfo-propyl-*M*-toluidine, horseradish peroxidase, disodium phosphate (Na_2HPO_4), and chloroform were purchased from

Sigma, India. Trizol was obtained from Ambion Life Technology.

Cell Maintenance. MIN6 (Mouse Insulinoma 6) cells were obtained from NCCS (National Center for Cell Sciences, Pune). Cells were fed with DMEM containing 25 mM of glucose and 10% FBS with 1% Penstrep at 37 °C and 5% CO_2 as per literature.^{26,27} For long-term experiments and maintenance of healthy cells, cells were supplemented with media every second day and split when necessary. Cells with passages below 30 were used for the experiments.

Glucose-Stimulated Insulin Secretion (GSIS) Assays on MIN6 Cells. Cells were seeded at a density of 1×10^6 cells per well and used at 75% confluency for the experiments. Cells were washed with Krebs Ringer Bicarbonate Buffer (KCl 4.7 mM, NaCl 119 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.19 mM, $CaCl_2$ 2.5 mM, HEPES 10 mM, and $NaHCO_3$ 20 mM with 0.1% BSA) at pH 7.4 without glucose. Cells were treated with 2.8 mM glucose for 0.5 h and then stimulated at 25 mM glucose in KRBH for 1 h at 37 °C and 5% CO_2 as per protocol.^{27,81} The supernatant was removed and centrifuged at 2500 rpm for 5 min and stored at -20 °C for insulin estimation using the Insulin ELISA EZRMI-13K kit. Normalization of secreted insulin was done using total cellular protein, which was estimated by Bradford assay using BSA protein as a standard. Experiments were performed in triplicates.

RNA Isolation and Transcriptome Study. Cells were treated as described above for transcriptome studies. RNA was isolated by the Trizol method according to the manufacturer's protocol. Cells were treated with 1 mL of Trizol and the cell pellet was homogenized two to three times. Next, 0.2 mL of chloroform was added and incubated for 5 min on ice. The microcentrifuge tube was inverted and mixed 10 times, and the samples were incubated again for 2–3 min on ice. The sample was centrifuged in microcentrifuge tubes at 14,000 rpm for 15 min at 4 °C. After centrifugation, 3 distinct layers were formed. The aqueous layer containing RNA was transferred to a new tube and 0.5 mL of 2-propanol was added. The tube was inverted and mixed 10 times, and the samples were incubated for 10 min at -20 °C. The tube was then centrifuged at 14,000 rpm for 15 min to pellet down the total RNA. Next, the supernatant was discarded and 1 mL of 75% ethanol was added. The sample was then air-dried and dissolved in RNase-free water. It was then stored at -80 °C for the RNA sequencing studies. The purity of RNA was checked by a Multiskan GO Spectrophotometer.

Transcriptome Study and Data Analysis. Transcriptome studies were performed by Agrigenome Lab Private Limited, Kerala, India. The quality and quantity of isolated RNA were checked using an Agilent TapeStation 2200 and Qubit 3.0 fluorimeter. After preparation of the sample library, the quality of library was checked using Agilent TapeStation 2200 and quantified using a Qubit 3.0 fluorimeter, and the validated libraries were sequenced on Illumina HiSeq X10 Platform for 2×150 bp length with 50 million reads. Since 30–40 million reads are sufficient to be technically precise in measuring differential gene expression,^{82,83} we sequenced our cDNA libraries with 50 million reads to achieve sufficient technical precision. Differential expression analysis was done using Cuffdiff, and gene ontology analysis (biological process, molecular function, and cellular component) was done through the Uniprot database. Mapping to a reference genome is a crucial factor in evaluating the sample quality. The mapping

was done on HISAT2 taking reference genome GRCm38 of *M. musculus*.

Transcriptome results were analyzed and presented in terms of Fragments Per Kilobase of transcript per million mapped reads (FPKM). However, it was recently suggested that Transcripts Per Million (TPM) may be a more accurate way to compare transcriptomes across samples.⁸⁴ Conversion of TPM was done using the equation $TPM = 10^6 \times \frac{FPKM}{\sum(FPKM)}$. Differential gene expression was analyzed in terms of both FPKM and TPM, but the genes of interest were found to be upregulated in both cases. So, we chose FPKM to present our transcriptome results.

Selection of Protein Structures, Glucose Binding Analysis Using AutoDock Tools 4.2.6 (ADT), and Subsequent Visualization of the Docked Conformations through Discovery Studio 2021 (DS). The protein structures used for glucose binding studies have been mentioned in Table 2 under “Results and Discussion section”. The exact methodology, various file formats, and pipeline used for glucose binding analyses through ADT and subsequent visualization through DS have been mentioned elaborately in “Docking pipeline” in Supporting Information. Briefly, the protein file (.pdb format) was opened in ADT and water molecules were deleted, followed by addition of polar hydrogens and Kollman charges. The ligand file (.pdb format) was then opened in ADT and after proper initialization, the respective ligand-protein complex was saved (.pdbqt format). The grid box size dimensions were set to size_x = 126, size_y = 126, and size_z = 126. The respective Autogrid parameter file was then saved (.gpf format) and after a successful run, an Autogrid log file (.glg format) was generated. Then, the docking parameter file (.dpf format) was generated using Lamarckian Genetic Algorithm. After a successful run, a docking log file (.dlg format) was generated that contained information on 10 binding scores (kcal/mol) of the corresponding 10 docked ligand binding poses with the respective protein. The .dlg file was saved (.pdbqt format) for further analyses in DS as a 2D protein–ligand interaction.

Calculation of Pearson’s Correlation Coefficient. The binding energies (kcal/mol) and the number of interactions for the respective conformations within a single protein were noted. This was done for every other protein molecule used. Pearson’s correlation coefficient (*r*) was calculated between the obtained binding energies (kcal/mol) and the number of interactions for the respective conformations within a single protein, and for every other protein molecule used.

Statistical Analyses. Two-tailed Student’s *t* test assuming equal variances was performed using Microsoft Excel 2016 to calculate the statistical significance between the groups. The *p*-values <0.05 were considered as statistically significant.

■ ASSOCIATED CONTENT

Data Availability Statement

The data sets generated and/or analyzed during the current study are available in the Gene Expression Omnibus (GEO) repository (NCBI-GEO), Accession ID: GSE226652 (Token: sbcxuogopdmpin, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE226652>).

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.3c09171>.

Fold change in log₂ FPKM at HyG condition set at a threshold of +1.5 for upregulated protein-coding genes. *Kif11*, *Cacnb4* and *Atp6v0a4* is marked in bold, and Fold change in log₂ FPKM at HyG condition set at a threshold of −1.5 for downregulated protein-coding genes, respectively (Table S1a,b); expression of 154 genes involved in glycolysis, TCA cycle, and electron transport chain were found to be unaffected by EGC as seen by FPKM log₂FC, corresponding numbers are indicated in parentheses (Table S2); highest binding scores (kcal/mol) obtained for every ligand used, the corresponding conformation is indicated in parentheses alongside highest binding scores obtained (Table S3); binding scores of the 10 docked conformations for every protein–ligand used (Table S3a–o); respective Ligand–Protein coordinates with X, Y, Z axes dimension points, spacing and X, Y, Z axes points of center grid box as chosen in AutoDock Tools 4.2.6 (Table S4a–o); glucose binding scores (kcal/mol) of 10 docked conformations of the homologous proteins (Table S5); respective conformations, number of interactions and glucose binding scores (kcal/mol) obtained for the test molecules (KIF11, CACNB4, ATP6V0A4) as shown in Table S6a,b; positive controls (human and mouse Glucokinase, human and mouse GLUT2) in Table S7; and negative controls (RPA1, KU70–80, POLR1A, ACAA1A) in Table S8a,b respectively, using AutoDock Tools (ADT) (Tables S6a,b, S7, and S8a,b); calculation of Pearson’s correlation coefficient (Table S9); visualization of the binding propensity of D-glucose (Table S10); all the ligands used for binding studies have been shown with their respective PubChem CID’s (Table S11); statistical analyses between binding scores (kcal/mol) showing *p*-values as obtained (Table S12); glucose binding scores of homologous proteins previously not used for binding analyses and subsequent statistical analyses between binding scores (kcal/mol) showing *p*-values as obtained (Table S13a,b); different insulin-secreting model systems obtained from different species used to study GSIS (Table S14); docking pipeline: Glucose binding analyses using AutoDock Tools 4.2.6 (ADT) and subsequent visualization of the docked conformations through Discovery Studio 2021 (DS). The exact methodology and pipeline used has been shown (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

GSIS:glucose-stimulated insulin secretion; EGC:extracellular glucose concentrations; MIN6:mouse insulinoma 6; ADT:AutoDock Tools; DS:Discovery Studio 2021; DM:diabetes mellitus

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