Die Rolle der Serum- und Glukokortikoid-induzierbaren Kinase SGK1 in der Regulation des Glukosetransports

Role of Serum and Glucocoritcoid inducible Kinase SGK1 in the regulation of glucose transport

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Zusammenfassung

In hormonsensitiven Epithelien wird eine Steigerung des Glukosetransports hauptsächlich durch vermehrten Transport der Hexosetransporter-Isoformen GLUT1(SLC2A1) und GLUT4(SLC2A4) von intrazellulären Kompartimenten zur Plasmamembran erreicht. Es ist bereits bekannt, daß die Inhibition der PI3K die Stimulation der GLUT1 und GLUT4 Translokation durch Insulin und damit eine Erhöhung des Glukosetransports unterbindet. Die Serum- und Glukokortikod-induzierbare Kinase (SGK) 1 ist eine weitere Kinase abwärts der PI3K.

Die SGK1 ist eine Proteinkinase, welche die Funktion und Expression einer Reihe von Ionenkanälen und Transportern reguliert. Sie wurde als ein Gen identifiziert, welches akut in Brusttumorzellen und Fibroblasten durch die Applikation von Serum- und Glukokortikoiden stimuliert wird. Die Kinase wird durch Faktoren phosphoryliert, welche die PI3K aktivieren. Die Phosphorylierung erfolgt durch die 3-Phosphoinositid-abhängigen Kinasen (PDK) 1 und PDK2. In der phosphorylierter Form reguliert die Kinase die Aktivität ihrer Ziele durch Phosphorylierung an der SGK Konsensusstelle (Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr).

GLUT1 enthält eine Konsensusstelle (^{95}Ser) für die Phosphorylierung durch SGK1. während sich die Phosphorylierungsstelle auf dem GLUT4 an der Position ^{274}Ser befindet. Die vorliegende Studie untersucht die Hypothese, daß GLUT1 und GLUT4 durch die SGK1 reguliert werden. Zu diesem Zweck wurde die cRNA der Glukosetransporter und der Proteinkinase im heterologen Expressionssystem des *Xenopus laevis* Oocyten exprimiert. Die Quantifizierung der Transporteraktivität erfolgte durch die Bestimmung der Aufnahme von radioaktiver Deoxyglukose. In den betreffenden Studien in *Xenopus laevis* Oocyten und Adipocyten konnte nachgewiesen werden, dass die GLUT1 Aktivität durch die konstitutiv aktive SGK1 stimuliert wird.

Dieser Prozeß benötig eine intakte Katalysierungsstelle auf der SGK1, da die inaktive ^{K127N}SGK keinen Effekt auf den Transporter zeigt. Es konnte ebenfalls gezeigt werden, daß die Stimulierung des GLUT1 durch die ^{S422D}SGK1 nicht auf einer Erhöhung der GLUT1 *de novo* Synthese beruht sondern vielmehr auf einer Erhöhung der GLUT1-Abundanz an der Zelloberfläche. Die kinetische Analyse zeigte, daß die Stimulierung des GLUT1 zwar die maximale Transportrate, nicht aber dessen Affinität zum Substrat erhöht.

Durch Tracer-Flux-Studien in HEK-293 Zellen konnte gezeigt werden, dass GLUT1 durch die konstitutiv aktive SGK1 reguliert wird. Dieser Effekt benötigt eine intakte katalytische Aktivität der SGK1 da die inaktive SGK1 den Transporter nicht regulierte. Die kinetische Analyse in HEK-293 Zellen zeigte außerdem, daß die Aktivität des Transporters durch eine Erhöhung der maximalen Transportrate erreicht wird, die Substrataffinität aber unbeeinflusst bleibt. Außerdem konnte gezeigt werden, das der GLUT1-vermittelte Glukosetransport in Adipozyten von SGK1-knockout Mäusen verringert ist.

In der Zusammenfassung werden die Glukosetransporter GLUT1 und GLUT4 durch die Glukokortikod-induzierbare Kinase SGK1 moduliert. Die Kinase stimuliert den Transporter durch die Erhöhung seiner Zelloberflächenexpression. Die SGK1-abhängige Regulation der Glukosetransporter GLUT1 und GLUT4 könnte an der Anpassung der zellulären Glukoseaufnahme an den Bedarf der Zelle beteiligt sein.

SUMMARY

Insulin stimulates glucose transport in hormone responsive tissues mainly by inducing the redistribution of the facilitated hexose carrier isoforms GLUT1 (SLC2A1) and GLUT4 (SLC2A4) from intracellular compartments to the plasma membrane. Previous studies have shown that phosphatidylinositol 3-kinase (PI-3K) inhibition disrupts the ability of insulin to stimulate GLUT1 and GLUT4 translocation into the cell membrane and thus glucose transport. The Serum and Glucocorticoid inducible Kinase 1 SGK1 is a protein kinase which regulates the function and expression of several ion channels and transporters. It was initially recognized as an immediate early gene whose mRNA level is increased in mammary tumour cell and fibroblast cell lines upon serum or glucocorticoids. The kinase is activated by phosphorylation in response to signals that stimulate phosphatidylinositol 3-kinase. The phosphorylation is mediated by 3-phosphoinositide-dependent protein kinase1 (PDK1) and PDK2/H-motif kinase. Once phosphorylated, the kinase regulates its targets activity directly through phosphorylation at the SGK1 consensus site or indirectly through phosphorylation of the ubiquitin ligase or binding to the scaffolding protein NHERF2.

GLUT4 contains a putative SGK1 consensus site at ^{274}Ser for phosphorylation by SGK1 and GLUT1 at ^{95}Ser. Thus, in the present work it was investigated whether SGK1 regulates GLUT1 and GLUT4. To this end, membrane proteins encoding the glucose transporter and the kinases were expressed heterologously in *Xenopus laevis* oocytes. Tracer flux studies with 2 De-oxy glucose as a substrate and uptake was determined as a measure of GLUT4 activity. The concerned studies in oocytes demonstrated that GLUT4 activity is enhanced by constitutively active SGK1. The effect requires the kinase catalytical activity since the inactive mutant ^{K127N}SGK1 failed to modulate the facilitative GLUT4. Deleting the SGK1 phosphorylation site on GLUT4 (^{S274A}GLUT4) abrogated the kinase effects suggesting that the action of SGK1 occurs via direct phosphorylation. We also found that GLUT4 stimulation by ^{S422D}SGK1 is not due to *de novo* protein synthesis but rather to an increase of the transporter´s abundance in the plasma membrane. Kinetic analysis revealed that SGK1 enhances maximal transport rate without altering GLUT4 substrate affinity.

The effect of SGK1 on GLUT1 was examined in the mammalian renal cell line HEK 293. Cells were transfected with the empty vector or with SGK1 and 2-Deoxy-glucose uptake determined. Tracer flux studies in HEK-293 mammalian cells demonstrated that GLUT1 is regulated by the SGK1 kinase in its constitutively active form. The effect requires the catalytical activity since the

inactive mutant form of SGK1 failed to upregulate GLUT1. Kinetic analysis in HEK-293 cells revealed that SGK1 upregulates the transporter activity by enhancing the maximal transport rate without altering its substrate affinity. In order to pursue the physiological relevance of SGK1 mediated regulation of glucose transport adipocytes were isolated from mice lacking SGK1 kinase and glucose uptake was measured. Results demonstrated that glucose transport is reduced in isolated adipocytes from SGK1 knockout mice as compared to wild type littermates.

In conclusion, the facilitated glucose transporters GLUT1 and GLUT4 are modulated by the serum and glucocorticoid inducible kinase SGK1. The kinase stimulates GLUT1 and GLUT4 by enhancing the transporter plasma membrane abundance. The SGK1 dependent regulation of these two glucose transporters GLUT1 and GLUT4 may participate in the PI3 K-dependent adjustment of cellular glucose uptake to the demand.

Abbreviations

AMP	Adenosine Mono Phosphate
ATP	Adenosine Tri Phosphate
Bq	Bequerel
cAMP	Cyclic Adenosine Mono phosphate
^{o}C	Degree(s) Celsius (centigrade)
cDNA	complementary Deoxyribonucleic Acid
CNS	Central Nervous System
cRNA	complementary Ribonucleic Acid
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribo-Nucleotidetriphosphate
EDTA	Ethylene Diamine Tetra-Acetate
EGTA	Ethylene Glycol Tetraacetic Acid
ELISA	Enzyme-Linked Immunoabsorbent Assay
ENaC	Epithelial Sodium Channel
GLUT1	Glucose Transporter 1
GLUT4	Glucose Transporter 4
HEPES	N-(2-Hydroxyethyl)piperazine-N-(2-ethanesulfonic acid)
kDa	KiloDalton
µCi	Microcurie (1 Ci = 37 x 10^{9} Bq)
Nedd4-2	Neuronal cell expressed developmentally down regulated 4-2
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PKB (Akt)	Protein Kinase B; oncogene from Akt mouse
PKC	Protein Kinase C
Ser	Serine
SDS	Sodium Dodecyl Sulfate
SE	Standard Error
SGK	Serum and Glucocorticoid inducible protein Kinase
Thr	Threonine
TRIS	Tris (hydroxymethyl) aminomethane
V/v	Volume/volume

1. INTRODUCTION

1.1. Diabetes Mellitus

Human bodies need to maintain a glucose concentration level in a narrow range (70 - 109 mg/dl or 3.9 - 6.04 mmol/l). If the glucose concentration level is significantly out of the normal range (70 - 110 mg/dl), this person is considered to have hyperglycaemia (140 mg/dl or 7.8 mmol/l after an oral glucose tolerance test, or 100 mg/dl or 5.5 mmol/l after a fasting glucose tolerance test) or hypoglycaemia (less than 40 mg/dl or 2.2 mmol/l). Diabetes mellitus is a disease in the glucose-insulin endocrine metabolic regulatory system, in which the pancreas either does not release insulin or does not properly use insulin to uptake glucose in the plasma (1) (2) which is referred as hyperglycaemia. The consequences are that the body does not metabolize the glucose and builds up hyperglycaemia which eventually damages the regulatory system. Complications of diabetes mellitus include retinopathy, nephropathy, peripheral neuropathy and blindness (3). Diabetes mellitus is one of the worst diseases with respect to size of the affected population. The world wide diabetics affected population is much higher, especially in underdeveloped countries.

Diabetes mellitus is currently classified as type 1 or type 2 diabetes (2). Type 1 diabetes was previously called insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes. It develops when the body's immune system destroys pancreatic beta cells, the only cells in the body that synthesize the hormone insulin, which regulates blood glucose. This form of diabetes usually strikes children and young adults, although disease onset can occur at any age. Type 1 diabetes may account for 5% to 10% of all diagnosed cases of diabetes. Risk factors for type 1 diabetes include autoimmune, genetic, and environmental factors. Type 2 diabetes is adult onset or non-insulin-dependent diabetes mellitus (NIDDM) as this is due to a deficit in the mass of β cells, reduced insulin secretion (4), and resistance to the action of insulin. The relative contribution and interaction of these defects in the pathogenesis of this disease remains to be clarified (5). About 90% to 95% of all diabetics diagnose type 2 diabetes. Type 2 diabetes is associated with older age, obesity, family history of diabetes, prior history of gestational diabetes, impaired glucose tolerance, physical inactivity, and race/ethnicity. African Americans, Hispanic/Latino Americans, native Americans, some Asian Americans, native Hawaiian, and other Pacific Islanders are at particularly high risk for type 2 diabetes. Type 2 diabetes is increasingly being diagnosed

in children and adolescents. In addition to type 1 and type 2 diabetes, gestational diabetes is a form of glucose intolerance that is diagnosed in some women during pregnancy (1).

Gestational diabetes occurs more frequently among African Americans, Hispanic/Latino Americans, and native Americans. It is also more common among obese women and women with a family history of diabetes. During pregnancy, gestational diabetes requires treatment to normalize maternal blood glucose levels to avoid complications in the infant. After pregnancy, 5% to 10% of women with gestational diabetes are found to have type 2 diabetes. Women who have had gestational diabetes have a 20% to 50% chance of developing diabetes in the next 5-10 years. Other specific types of diabetes result from specific genetic conditions such as maturity-onset diabetes of youth, surgery, drugs, malnutrition, infections, and other illnesses. Such types of diabetes may account for 1% to 5% of all diagnosed cases of diabetes. The relative contribution and interaction of these defects in the pathogenesis of this disease remains to be clarified (5). Due to the large population of diabetes patients in the world and the big health expenses, many researchers are motivated to study the glucose-insulin endocrine metabolic regulatory system so that we can better understand how the mechanism functions (6;7) , what causes the dysfunctions of the system, how to detect the onset of the either type of diabetes including the so called prediabetes , (2;7) (8) and eventually provide more reasonable, more effective, more efficient and more economic treatments to diabetics.

1.2 Glucose-insulin endocrine metabolic regulatory system

Metabolism is the process of extracting useful energy from chemical bounds. A metabolic pathway is a sequence of enzymatic reactions that take place in order to transfer chemical energy from one form to another. The chemical adenosine-triphosphate (ATP) is a common carrier of energy in a cell. There are two different ways to form ATP by adding one inorganic phosphate group to the adenosine-diphosphate (ADP), or adding two inorganic phosphate groups to the adenosine-monophosphate (AMP). The process of inorganic phosphate group addition is referred to phosphorylation. Due to the fact that the three phosphate groups in ATP carry negative charges, it requires lots of energy to overcome the natural repulsion of like-charged phosphates when additional groups are added to AMP. So considerable amount of energy is released during the hydrolysis of ATP to ADP.

In the glucose-insulin endocrine metabolic regulatory system, the two pancreatic endocrine hormones, insulin and glucagon, are the primary dynamic factors that regulate the system. Glucose stimulates insulin secretion from β-cells by activating two pathways that require

metabolism of the sugar: the triggering and the amplifying pathway (9) . In the triggering pathway, the facilitative glucose transporter GLUT2 transports the glucose into the β cell. It causes the rise in the ratio of ATP/ADP which leads ATP-sensitive K^+ channels (KATP channels) in the plasma membrane to close. The decreased K^+ permeability leads to membrane depolarization, opening of voltage-dependent Ca^{2+} channels, Ca^{2+} influx, and the eventual rise of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]c$) that triggers exocytosis of insulin containing vesicles. This pathway is also called KATP channel-dependent pathway. Please see Figure 1 for an illustration. The amplifying pathway which is a KATP channel-independent pathway, simply increases the efficiency of the Ca^{2+} on exocytosis when the concentration of Ca^{2+} has been elevated. Once insulin has been secreted, the hormone exerts its effect by binding to insulin receptors.

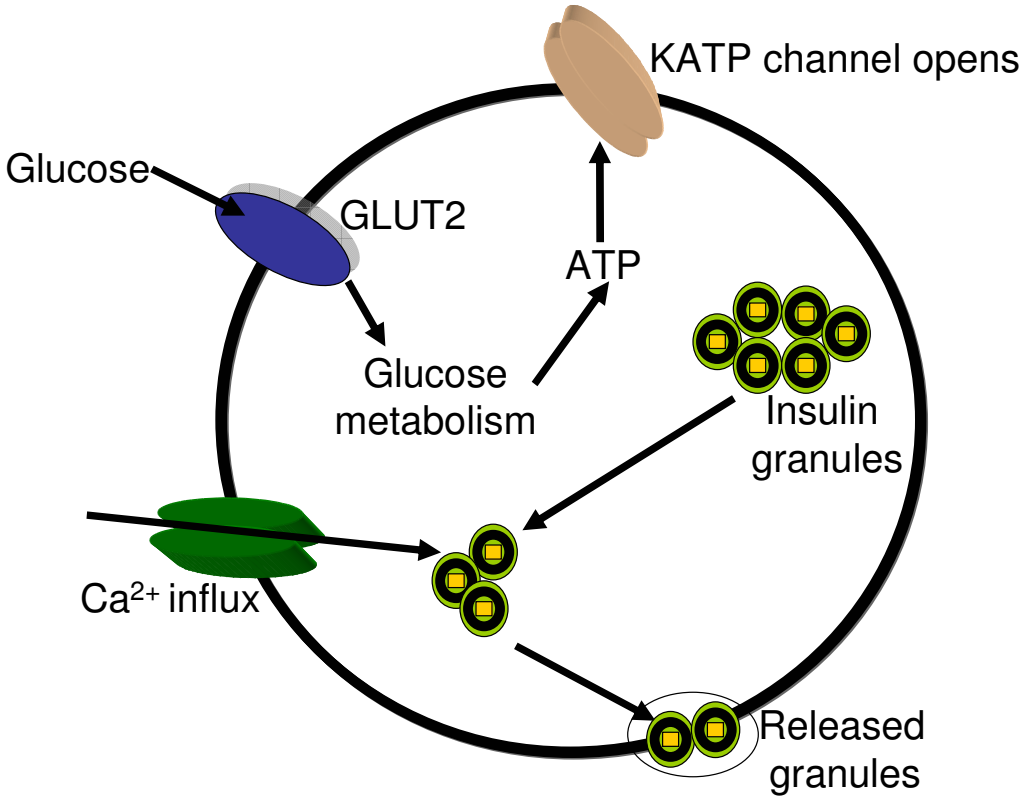

Figure 1. Pancreatic beta cells secrete insulin when glucose concentration levels are elevated. The facilitated glucose transporter GLUT2 transports the glucose into the cell where it is phosphorylated by glucokinase. The glucose metabolism causes ATP-sensitive K^+channels to close, the membrane to depolarize and the Ca^{2+} channels to open. This triggers a cascade of protein phosphorylations leading to insulin exocytosis.

The insulin receptor is a transmembrane glycoprotein that belongs to the large class of tyrosine kinase receptors. Two α subunits and two β subunits make up the insulin receptor. The β subunits pass through the cellular membrane and are linked by disulfide bonds (10) .

The insulin receptors are embedded in the plasma membrane of myocytes and adipocytes (11). The binding of insulin to the receptors is the initial step in a signal transduction pathway, triggering the consumption and metabolism of glucose. Bound by insulin , the insulin receptor phosphorylates several proteins in the cytoplasm, including insulin receptor substrates (IRS-1 and IRS-2) that activate Phosphatidylinositol 3-kinase (PI-3-K) leading to an increase in the facilitative glucose transporters (GLUT4 and GLUT1) molecules in the outer membrane of muscle cells and adipocytes, and therefore to an increase in the uptake of glucose from blood into muscle and adipose tissue. Figure 2 elucidates this signaling pathway (12).

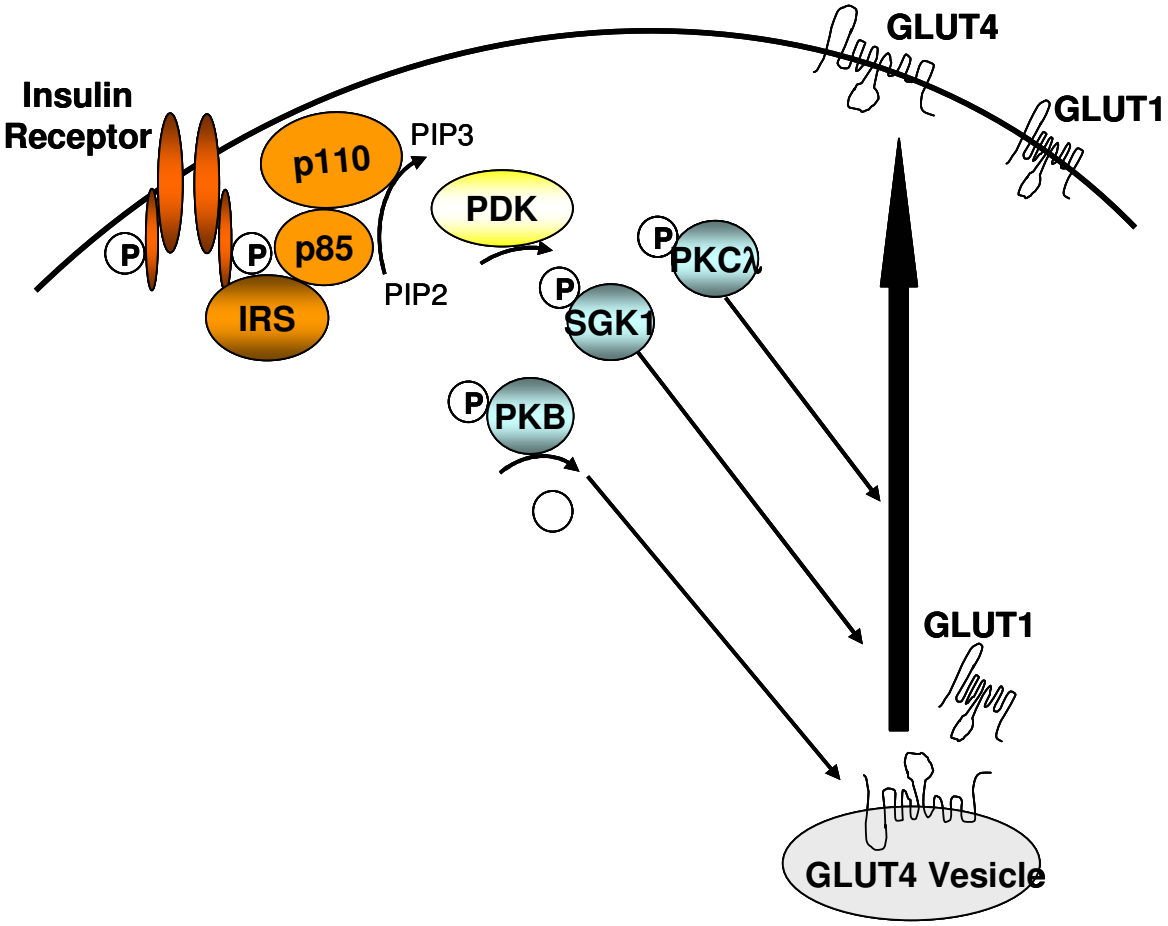

Figure 2. Insulin signals cells to utilize glucose. Insulin binds to its receptors on the membrane of the cells and induces phosphorylation of several proteins in the cytoplasm, including insulin receptor substrates (IRS-1 and IRS-2) which activate Phosphatidylinositol 3-kinase (PI-3-K) thereby leading to an increase in glucose transporter (GLUT1 and GLUT4) molecules in the plasma membrane. GLUT1 and GLUT4 transport the glucose into the cells efficiently.

The kinetics of insulin receptor binding is complex. The number of insulin receptors of each cell changes opposite to the circulating insulin concentration level. Increased insulin circulating level reduces the number of insulin receptors per cell and the decreased

circulating level of insulin triggers the number of receptors to increase (13). The number of receptors is increased during starvation and decreased in obesity and acromegaly (14). The affinity of the receptor for the second insulin molecule is significantly lower than for the first bound molecule. This may explain the negative cooperative interactions observed at high insulin concentrations. That is, as the concentration of insulin increases and more receptors become occupied, the affinity of the receptors for insulin decreases (15). Conversely, at low insulin concentrations, positive cooperation has been recorded. That is, the binding of insulin to its receptor at low insulin concentrations seems to enhance further binding.

The alpha cells in the pancreas release glucagon, a protein hormone that has important effects in the regulation of carbohydrate metabolism. Glucagon mobilizes glucose, fatty acids and amino acids from storage into the blood. When the glucose concentration level in the plasma is low, the liver converts glucagon to glucose. Both insulin and glucagon are important in the regulation of carbohydrate, protein and lipid metabolism. So when the plasma glucose concentration level is high, the pancreas initiates the release of insulin from beta cells. Insulin then leads to the translocation of GLUT1 and GLUT4 to the plasma membrane so that the glucose is transported into the cells where it is converted to energy molecules. These processes lower the concentration of glucose in blood. When a human`s plasma glucose concentration becomes low, glucogen is released from alpha cells of the pancreas. In the liver glucagon converts glycogen into glucose molecules thereby increasing the glucose concentration level in human plasma. Figure 3 illustrates the glucose-insulin endocrine metabolic regulatory system.

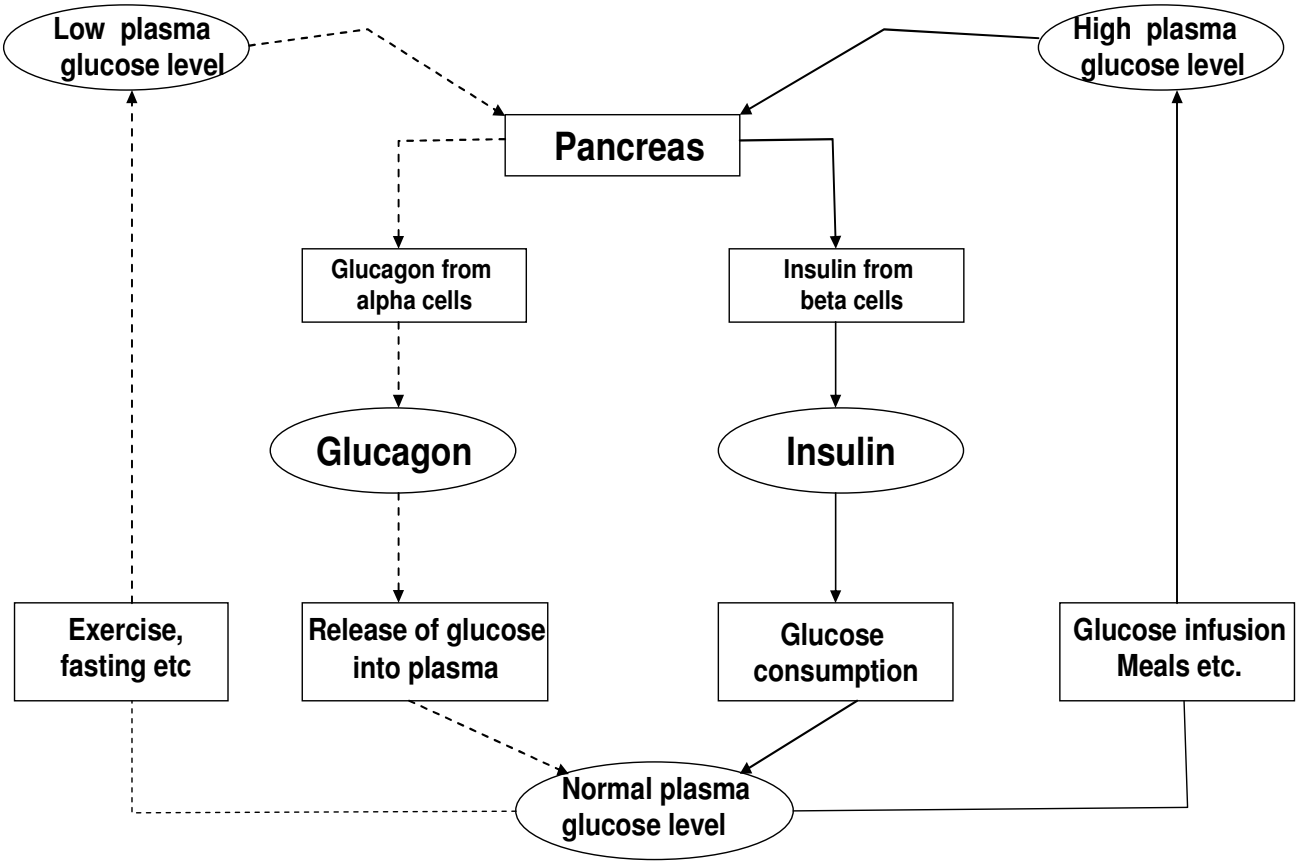

Figure 3. Glucose- insulin endocrine metabolic regulatory system. The dashed lines indicate that exercises and fasting consume glucose and lower the glucose concentration, which signals the pancreas to release glucagon in the liver converts glycogen to glucose. The solid lines indicate that the glucose infusion elevates the plasma glucose concentration level which signals the pancreas to secrete insulin and decrease plasma glucose.

The glucose transport through facilitative glucose transporters into the beta cells, myocytes and adipocytes plays a key role in the maintenance of glucose homeostasis.

1.3 The family of facilitative glucose transporters

Facilitated diffusion of glucose and related hexoses across biological membranes is catalysed by members of the SLC2 family, referred to as glucose transporters or GLUTs. These transporters function as simple carriers and the movement of hexose across the plasma membrane proceeds in the direction imposed by its electrochemical gradient. A common structural feature of the SLC2 family members is the presence of 12 transmembrane domains (TM) with both the amino and carboxy-terminal ends present on the cytosolic side and a unique *N*-linked oligosaccharide side-chain present either in the first or the fourth extracellular loop (16). Signature sequences conserved between the

different members of the SLC2 family are present at distinct locations in the primary structure. The presence of these sequences, however, does not predict the substrate specificity of these transporters.

Glucose transporters are expressed in every cell of the body, as might be anticipated from the key role of glucose in providing metabolic energy and building blocks for the synthesis of biomolecules. The specific physiological role of the isoforms expressed in tissues involved in the control of glucose homeostasis, i.e. muscle, adipose tissue, liver, pancreatic beta-cells and brain, has been studied in greatest detail. Indeed, in these tissues glucose transporters play important roles in the control of glucose utilization, glucose production and glucose sensing and their dysregulated expression may underlie pathogenetic mechanisms leading to development of diabetes mellitus, but also other specific monogenic diseases (17).

Facilitated diffusion of glucose across plasma membranes has been studied for several decades (18). The recognition that human erythrocytes have a high density of glucose transporters allowed the initial biochemical purification of this transporter and the preparation of specific antibodies. These were then used for initial cloning of a human glucose transporter by screening an expression library prepared from a human hepatoma cell line (HepG2) (4). This glucose transporter, named GLUT1 (SLC2A1) , was then used for subsequent cloning, by low-stringency screening, of GLUT2–5 (SLC2A2, SLC2A3, SLC2A4, SLC2A5) Class I is comprised of the extensively characterized glucose transporters GLUT1 to GLUT4, which can be distinguished on the basis of their distinct tissue distributions (GLUT1, erythrocytes, brain micro vessels; GLUT2 , liver, pancreatic islets; GLUT3, neuronal cells; GLUT4, muscle, adipose tissue) and their hormonal regulation (e.g., insulin sensitivity of GLUT4) (19). Class II is comprised of the fructose-specific transporter GLUT5 (20) and three related proteins, GLUT7 (SLC1A7), GLUT9 (SLC1A9), and GLUT11 (SLC1A11) (21) For GLUT11, fructose-inhibitable glucose transport activity has been demonstrated in a system of reconstituted vesicles. Class III is characterized by the lack of a glycosylation site in the first extra cellular linker domain and by the presence of such a site in loop 9. HMIT1 (SLC1A13), can be included in the class III GLUTs . Glucose transport activity has been demonstrated for GLUT6 and GLUT8. It should be emphasized, however, that the designation of the family does not necessarily reflect the substrate specificity of its members, which may transport sugars or polyols other than glucose (e.g. GLUT5-fructose, MIT1-myoinositol) (20).

1.3.1 The facilitative glucose transporters in the CNS and blood brain barrier

Glucose is the preferred energy substrate of the brain. Due to its expression in the endothelial cells forming the blood brain barrier, the glucose transporter GLUT1 is essential for glucose delivery to the brain (22).Given the fact that the abluminal surface of brain capillaries is covered by specialized astrocytic end-feet that also express GLUT1, the astrocytes probably constitute a major site of glucose uptake (23) In astrocytes, glucose is catabolized by glycolysis to lactate, which may be delivered to neurons through a glial-specific monocarboxylate transporter (MCT1) and a neuron-specific one (MCT2). In neurons, lactate is converted to pyruvate, which enters the tricarboxylic acid cycle to generate ATP. Glucose can also be taken up directly by neurons, which express the GLUT3 isoform (24). GLUT2 is also expressed in the brain in specific regions such as the hypothalamus and the brain stem where it may participate in the mechanisms of glucose sensing involved in the control of glucose homeostasis.

The role of GLUT8 in some specific neurons remains unclear. It is localized to intracellular vesicles and may possibly move to the cell surface upon as yet unidentified stimuli (25) . Finally, HMIT is expressed in astrocytes and in neurons. In astrocytes, HMIT is both intracellular and at the plasma membrane, whereas its subcellular localization in neurons is under investigation (26).

1.3.2 The facilitative glucose transporters as pharmaceutical targets

Elevation of blood glucose is the main symptom of types 1 and 2 diabetes. The GLUT isoforms that transport glucose represent therefore a potential therapeutic target for normalizing glycaemia. A compound that increases the V_{max} maximal velocity of GLUT1 would increase whole-body glucose utilization. Given the fact that this isoform is almost ubiquitous, such activation could, however, also lead to severe hypoglycaemia. Another possible site of action for limiting the blood glucose level would be inhibition of glucose absorption in the intestine or reabsorption in the kidney. In the intestine, this could be possible by blocking both GLUT2 and the alternative membrane-traffic-based pathway of basolateral glucose release. In the kidney, GLUT2 deficiency results in glucose excretion in the urine, which decreases glycaemia (27). Inhibition of GLUT2 specifically in the kidney could thus treat hyperglycaemia. However, the sodium dependent glucose transporter

SGLT2 (SLC5A1) seems to be a more interesting target in the kidney for this purpose since its expression is more limited.

Type 2 diabetes is characterized by the loss of insulin sensitivity that leads to a decrease in GLUT1 translocation to the plasma membrane in response to a high blood glucose. To compensate the resulting reduced flux of glucose into muscle and adipocytes, it would be useful to find a pharmacological compound that increases the V_{max} of GLUT1 for glucose, or stimulate its translocation to the cell surface.

An impaired brain inositol metabolism has been linked to psychiatric diseases, in particular bipolar disorders (28). Indeed, current treatments of these mood disorders relies on the use of Li^+ salts, valproic acid and carbamazepine, drugs whose action may interfere with inositol metabolism.(28) It is well established that one mechanism of action of Li^+ is the inhibition of inositol monophosphate phosphatase and polyphosphoinositide 1-phosphate phosphatase (29), which blocks recycling of inositol phosphate and reduces the availability of inositol for subsequent cycles of intracellular signal transduction. Inhibition of HMIT could also lead to such beneficial effects for bipolar disorders by decreasing the intracellular inositol concentration.

Some members of the GLUT family (GLUT1, 2 and 4) can transport glucosamine, which is important in the biosynthesis of glycoproteins and, in particular, glycosaminoglycan synthesis in cartilage (30). In association with collagen fibres, these molecules are responsible for the resilience of the cartilage to deformation. Destruction of joint cartilage occurs in osteoarthritis, and several studies have shown that glucosamine is beneficial for this disease . Given the fact that GLUT1 is expressed in chondrocytes, the cells that synthesize cartilage, glucosamine has favourable effects for osteoarthritis are probably mediated by transport across GLUT1 into these cells. Furthermore, glucosamine absorption seems to be mediated in part by GLUT2. This provides an example of the use of GLUT isoforms to deliver therapeutic molecules to their site of action.

1.3.3 The facilitative glucose transporter GLUT1

GLUT1 cDNA was isolated from an expression library using antibodies against the human´s erythrocyte glucose transporter(4). Although cloned from a hepatoma cDNA library, GLUT1 is not expressed in normal hepatocytes. It is, however, induced during oncogenic transformation of most cell types and its expression correlates with the increase in glucose

metabolism observed in tumour cells (31). GLUT1 is found in almost every tissue with different levels of expression in different cell types. The expression level usually correlates with the rate of cellular glucose metabolism. As mentioned above, it is also expressed highly in blood-tissue barriers, in particular in the endothelial cells forming the blood-brain barrier. Several heterozygous mutations resulting in GLUT1 haploinsufficiency have been identified. These cause hypoglycorrachia, a condition characterized by seizures, developmental delay, acquired microcephaly, and hypotonia, and which is due to a decrease rate of glucose transport from the blood into cerebrospinal fluid.

The topological arrangement of GLUT1 within the plasma membrane has been confirmed using several experimental approaches. Recently, two models have been proposed for the tertiary structure of GLUT1. The first is based on data obtained from cysteine scanning mutagenesis of five of the α-helices of GLUT1 together with information from site-directed mutagenesis (32). The second is based primarily on the proposed helical bundle arrangement of the Lac permease and has been refined using energy minimization algorithm (33) These two models describe a key role for helix 7 in the formation of a water-filled channel which may form the path for glucose across the plasma membrane.

The transport of glucose may be described as an alternating confirmation model in which the transporter has mutually exclusive binding sites located on the extracellular (import site) and on the intracellular face (export site) of the transporter (Figure 4). Binding of glucose to one site induces the transporter to switch to the opposite conformation, a process that is accompanied by a movement of the substrate across the plasma membrane (34). In human erythrocytes, GLUT1 is thought to be present as homodimers or homotetramers, with the conversion between both oligomeric forms being dependent on the redox state, . GLUT1 transports glucose with an affinity constant (K_m) of ~3 mM. Other transported substrates are galactose (30mM) (34), mannose (35) (20mM) and glucosamine 2.1$\pm$0.5mM (36). Glucose transport by GLUT1 is sensitive to several inhibitors that also block transport by other isoforms. Many of them are competitive inhibitors of sugar binding, either to the extracellular or the cytosolic sugar binding sites. Cytochalasin B binds to the inner surface of GLUT1 and inhibits its glucose transport activity with an IC_{50} of 0.44 μM. Binding of cytochalasin B is to a site which contains tryptophan 388 and 412. Also acting on the same intracellular site is the diterpene toxin forskolin. Forskolin has been used as a photoaffinity label with some specificity for the glucose transporter and its affinity is increased in the 3-iodo4-azidophenethylamido-7-*O*-succinyldeacetyl (IAPS) derivative. An iodinated derivative

of forskolin (7-aminoalkylcarbamate) with a very high affinity (IC_{50} 200 nM) has also been described (37).

Glucose transport activity of GLUT1 is inhibited by $HgCl_2$ (IC_{50} 3.5 µM), phloretin (IC_{50} 49 µM), phlorizin (IC_{50} 355 µM) and 4,6-*O*-ethylidene-D-glucose (IC_{50} 12 mM), which bind

to the external glucose binding site where glutamine 161 appears to be critical for inhibitor binding (16).

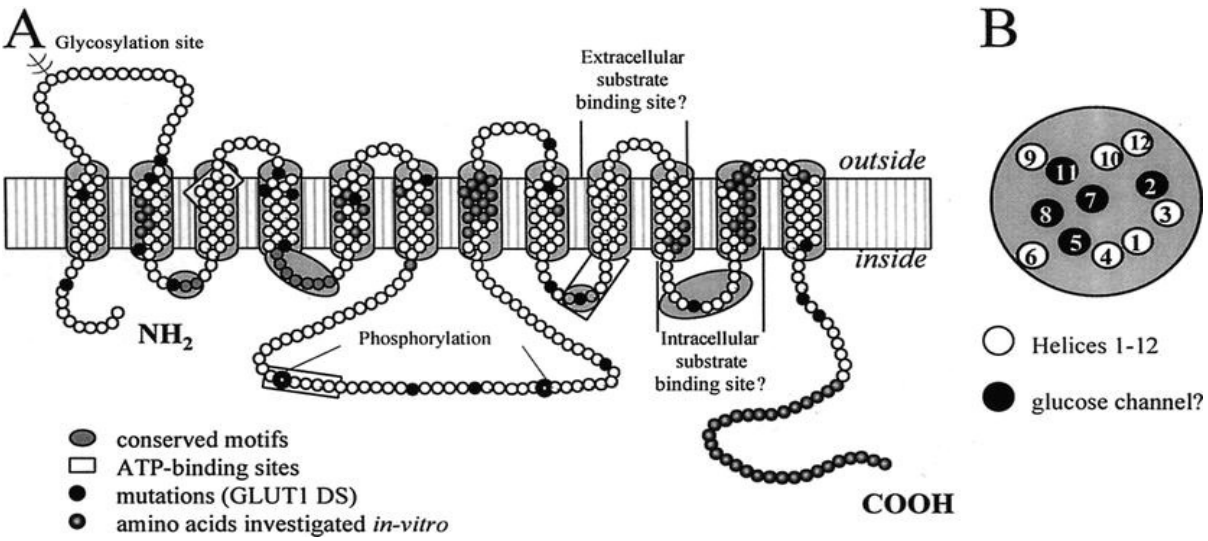

Figure 4. Conformational model of GLUT1 in the membrane. **A** The side view of the GLUT1 protein shows the 12 transmembrane helices with a large intracellular loop connecting helix 6 and 7, the N-glycosylation site in the first extracellular loop, and the amino- and carboxy-termini located in the cytosol. Conserved motifs within the GLUT family and functional domains such as ATP binding sites, phosphorylation sites, and substrate import and export sites are indicated. Also shown are mutations (filled circles) identified in patients with GLUT1 deficiency syndrome (GLUT1DS) and amino acids (filled grey circles with white centre) analyzed by in-vitro studies]. **B** End-on view from the extracellular surface. Amphipathic helices thought to participate in the glucose channel are shown in black.

1.3.4 Glucose transporter 4

After nutrient intake, the beta cells of the pancreas response by releasing insulin into the blood. The insulin controls many post prandial events in which the localization of the glucose transporter in the cytoplasm versus plasma membrane plays an important role. Insulin resistance is associated with insufficient recruitment of GLUT4 to the membrane despite the protein is expressed in a normal manner. This leads to study the mechanisms involved in the insulin regulated GLUT4 translocation and glucose uptake so that effective treatment and preventive measures can be achieved. Although there is an improvemnt

within the last two decades, the whole mechanism linking insulin to GLUT4 translocation have yet to be fully resolved.

Earlier it was found that, in rat adipocytes insulin triggers the movement of the sugar transporter that is found in these cells from cytoplam to the plasma membrane. That was later confirmed when GLUT4 was identified as the main glucose transporter in these cells. GLUT4 primarily found in muscle and fat cells, is found in a complex intracellular tubulo–vesicular network that is connected to the endosomal–*trans*-Golgi network (TGN) system. It has 12 membrane spanning domains with amino and carboxyl termini in the intracellular region. In the basal state, GLUT4 cycles continuously between the plasma membrane and one or more intracellular compartments, with 90-95 percent of the transporter residing within the cell interior, tightly packaged into vesicles. The overall insulin-induced GLUT4 vesicle trafficking results in a >10-fold increase of GLUT4 protein at the cell surface (38). GLUT4 is found within large tubulo-vesicular structures in the perinuclear region of the cell and within small vesicles dispersed throughout the cytoplasm, also known as GLUT4 storage vesicles (GSVs) (39) (40). Perinuclear GLUT4 is likely localized in endosomes. GSVs on the other hand co-localize with insulin-responsive aminopeptidase (IRAP) and vesicle-associated membrane protein 2 (VAMP2). Upon insulin stimulation, it appears that GSVs are translocated to the cell surface, as there is an increase of GLUT4 at the plasma membrane that is proportional to the reduction in GLUT4-containing vesicles from the cytosolic compartment (41) (42), while the level of perinuclear GLUT4-containing vesicles remains relatively the same. Furthermore, total internal reflection fluorescence microscopy has revealed that in basal adipocytes, GLUT4-containing vesicles are located near the plasma membrane and are recruited to the cell surface with insulin stimulation (43) (Figure 2).

1.3.5 Role of insulin signalling in GLUT4 regulation

Activation of the insulin receptor triggers a cascade of phosphorylation events that ultimately promote GLUT4 vesicle exocytosis. The classical insulin signaling pathway (Figure 2) involves docking of the insulin receptor substrate (IRS) to the insulin receptor (IR), activation of phosphatidylinositol 3-kinase (PI3K) which leads to formation of plasma membrane phosphatidylinositol 3,4,5-trisphosphate (PI -P3), subsequent PI -P3-mediated activation of Akt and atypical protein kinase C (aPKC). Additionally, a PI3K-independent pathway involving c-Cbl, c-Cbl associated protein (CAP), and the GTPase TC10 may also regulate GLUT4 translocation. Insulin action is initiated when this peptide hormone binds to its receptor. The insulin receptor is composed of two disulphide-linked heterodimers, each of

which has an α and β subunit. Insulin binding to the two high-affinity extracellular α subunits leads to activation of intrinsic tyrosine kinase activity of the transmembrane β subunits and autophosphorylation of specific tyrosine residues (44), (45). Phosphorylation enhances tyrosine kinase activity of the b subunits towards a host of proteins including members of the insulin receptor substrate family (IRS-1, -2, -3, -4, -5, and -6), Cbl, SIRP (signal regulatory protein) family members, and APS [adapter protein containing a pleckstrin homology (PH) and Src-homology 2 (SH2) domain , (46). IRS-1 is found to be expressed in the adipose tissues and muscles and IRS-2 in the pancreas and liver. Studies with knockout mouse and siRNA silencing revealed the importance of these substrates in the concerned tissues.

1.3.6 PI3K dependent pathway

Insulin-dependent tyrosine phosphorylation of IRS-1/2 creates docking sites for downstream effector molecules including Class IA PI3K. Activated PI3K catalyzes the phosphorylation of PI-P2 on the 3 position of the inositol ring, forming PI-P3. Increased membrane PI-P3 is crucial for insulin-stimulated GLUT4 translocation, as this phospholipid provides docking sites for downstream molecules via their pleckstrin homology (PH) domains. Inhibition of PI-P3 formation with wortmannin or LY29004 effectively blocks insulin-stimulated GLUT4 translocation and glucose transport (47), (48) . Phosphatase regulators of PI3K generated lipids include PTEN and SHIP2. The PTEN catalyzes the dephosphorylation of phosphatidylinositols at the D3 position, while the latter catalyzes dephosphorylation of PI -P3 to yield PI -P2 (49). PI -P3 formation mediates the plasma membrane translocation of two PH domain containing proteins which is important for insulin-regulated glucose uptake: Akt (protein kinase B, PKB) and phosphoinositide-dependent-kinase-1 (PDK1). PDK2 is recently identified as the protein kinase mTOR (mammalian target of rapamycin) complexed to the regulatory protein rictor (50). There are three isoforms of protein kinase AKT (1-3) (51) in which GLUT4 translocation is dependent on AKT2 (52). AKT2 acts through it downstream molecule AS160 (53). AS160 contains a GTPase activating domain for Rabs, small G proteins which is involved in the vesicle trafficking (54), (55). Along with recruiting and activating Akt, PI 3,4,5-P3 formation in concert with PDK1 also leads to the activation of atypical protein kinase C (aPKC), and both aPKC isoforms z and l have been implicated in GLUT4 translocation (56). PKC knockout models and expression of kinase-inactive PKC-z/l inhibits GLUT4 translocation and glucose uptake in a variety of cell types, and this phenotype can be reversed in PKC-l–/– cells by expressing wild-type aPKC. Furthermore, expression of

constitutively active aPKC recapitulates the effects of insulin on GLUT4 translocation and glucose transport.

1.3.7 PI3-K independent pathway

It is well established that activation of GLUT4 translocation by insulin requires a PI3K signal involving the upstream IR and IRS activators and the downstream Akt and PKC target enzymes and AS160 protein. Some studies over the past decade have also suggested that a second pathway occurs as a consequence of Cbl tyrosine phosphorylation (57), (58). Cbl and the adaptor protein CAP are recruited to the insulin receptor by APS (59). Once tyrosine phosphorylated by the receptor, Cbl can recruit the adaptor protein CrkII to lipid rafts, along with the guanyl nucleotide exchange factor C3G (60). C3G can then activate the GTP-binding protein TC10, which resides in lipid rafts . The correct spatial compartmentalization of these signaling molecules in the lipid raft microdomain appears to be essential or insulin-stimulated GLUT4 translocation and glucose transport, as these insulin-mediated events are abolished by dominant-interfering mutants of CAP that prevent the localization of Cbl to lipid rafts (61). Nevertheless, investigation suggests a role of TC10 in the regulation of actin dynamics and phosphoinositides. Cellular cortical actin exists in two forms: monomeric globular actin (G-actin) and filamentous actin (F-actin). In the case of cytosketal fusion, microtubules and cortical actin plays the important role. When the actin network (62) in skeletal muscle is treated with actin depolymerizing agent cytochalasin D or the actin monomer binding red sea sponge toxins Latrunculin A or B, it leads to the inhibition of glucose uptake and GLUT4 translocation (63). Two potentially overlapping models hypothesizing the role of actin in glucose uptake. According to the first one, insulin causes cortical actin remodeling, such that incoming vesicles can travel through the peripheral actin mesh to fuse with the plasma membrane (64) . The second suggests that actin filaments function as “highways,” upon which vesicles travel to reach the plasma membrane. Regardless of the exact actin function, it is apparent that insulin signaling to rearrange cortical actin represents a required pathway for optimal movement or fusion of GLUT4-containing vesicles and plasma membranes. GLUT4 exocytosis and endocytosis are also regulated by microtubule network (65). Dynein and kinesins motor proteins are involved in these machanisms (66).

1.3.8 Role of SNARE proteins in GLUT4 regulation

After the insulin-mediated arrival of GLUT4-containing vesicles from intracellular storage sites to the plasma membrane, regulated fusion of these vesicles ensues. Exocytosis of GLUT4-containing vesicles is mediated by interactions between specific vesicular and plasma membrane protein complexes known as SNAREs. Vesicle SNAREs (v-SNARES, vesicle soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) bind target membrane SNAREs (t-SNAREs) in company with numerous accessory proteins. Syntaxin4 and SNAP23 (23 kDa synaptosomal-associated protein) are the t-SNARES and VAMP2 is the v-SNARE involved in GLUT4 vesicle fusion (67) While SNAREs are essential in GLUT4 exocytosis, they themselves do not appear to be the direct targets of insulin action. Rather, studies suggest that the accessory proteins Munc18 and Synip may be regulated by insulin to accomplish fusion events. Three Munc18 isoforms (Munc18ac) have been identified in mammalian cells: Munc18a (67) is a neuronal isoform and Munc18b and Munc18c are expressed in muscle and adipose tissues. In addition to Munc18, the accessory protein Synip may also play a role in insulin stimulated GLUT4 vesicle fusion (68), although data are conflicting. Synip was first identified by Min and colleagues, who determined that this protein dissociates from syntaxin4 in an insulin-dependent manner and is directly involved in GLUT4 translocation. Recently, it was reported that Akt2 phosphorylates Synip on serine 99 and this phosphorylation mediates the Synip-syntaxin4 dissociation necessary for GLUT4 vesicle exocytosis (69). However, recent studies argue against this possibility and show that a serine-to-alanine Synip mutant (S99A) does not impair GLUT4 translocation.

1.3.9 Role of GLUT4 dysfunctions in obesity and type 2 diabetes

Insulin resistance is significantly caused by both genetic and environmental components. Mutations in the insulin receptor are rare but result in extremely severe insulin resistance. These include Leprechaunism, Rabson-Mendenhall Syndrome, and the type A syndrome of insulin resistance (70). Type 2 diabetes is polygenic, probably involving defects at numerous points in the glucose regulatory system. For example, skeletal muscle analyzed from type 2 diabetic subjects displays diminished insulin-stimulated IRS-1 tyrosine phosphorylation and decreased PI3K activity coupled to impaired glucose transport. These defects could not be explained by alterations in protein expression. Likewise, skeletal muscle and adipocytes from obese, Type 2 diabetic patients demonstrate impaired insulin-triggered IRS-1 associated

PI3K activity (71), (44). Impaired insulin signaling through downstream Akt2 and AS160 proteins has also been reported in skeletal muscle. Furthermore, the fatty acid metabolite ceramide causes insulin resistance that is coupled to impaired membrane recruitment and phosphorylation of Akt (72). Knowledge on mechanisms of such defects has remained underdeveloped. Increased IRS-1 serine phosphorylation may also help explain insulin resistance, as phosphorylated serine residues are thought to sterically hinder interactions with downstream PI3K. Dysregulated PKC activity in insulin resistance could increase serine phosphorylation (73) and PKC knockout mice are protected from insulin resistance (74).

Membrane and cytoskeletal defects are also a possible basis of insulin resistance. We now know that moderate increase in plasma membrane fluidity increase glucose transport . Furthermore, it has been shown that basal glucose transport is not fully active in fat cells and that it can be increased further by augmenting membrane fluidity. Consistent with membrane fluidity influencing insulin responsiveness, insulin-stimulated glucose transport is decreased when fluidity diminishes (75). Recent data suggest that the anti-diabetic drug metformin enhances insulin action by increasing membrane fluidity (76). Interestingly, the beneficial effects of chromium supplementation on insulin responsiveness may also be linked to membrane fluidity (77). With regards to cytoskeletal defects, recent study of various cell culture models of insulin resistance suggests that an underlying basis of reduced cellular insulin sensitivity may be perturbations in phosphoinositide-regulated cortical F-actin structure. In particular, PI -P2 control of cortical F-actin is disturbed by hyperinsulinaemic (78) and hyperendothelinaemic insulin-resistant conditions (79). Furthermore, isolated adipocytes from ethanol-induced insulin resistant Wistar rats (80) and skeletal muscle from obese insulin-resistant Zucker rats display altered actin polymerization. These findings agree with the necessity of an intact cytoskeleton for proper glucose regulation and suggest a membrane/cytoskeletal component of insulin resistance. Finally, some study has also revealed that insulin-resistant conditions are associated with defects in the SNARE pathway. As future research continues to expand our understanding of the signaling pathways of insulinregulated GLUT4 translocation and glucose transport, our ability to develop interventions to prevent, reverse, and ameliorate insulin resistance in obesity and type 2 diabetes will be favourably reached.

1.4 The Serum and Glucocorticoid inducible Kinase SGK1

The Serum and Glucocorticoid inducible protein Kinase 1 (SGK1) was originally cloned in 1993 as an immediate early gene transcriptionally stimulated by serum or glucocorticoids in rat and mammary tumor cells (81;82). Transcription of SGK1 was also shown to occur rapidly in response to many agonists like mineralocorticoids(83) , follicle stimulating hormone (FSH) (84), transforming growth factor (TGF- β) (85;86), thrombin (87), hypertonicity (88-90), high glucose (85;88) and neuronal injury or excitotoxicity (88;91;92)
SGK1 belongs to the 'AGC' subfamily of serine/threonine protein kinases, which include protein kinase A (PKA) or adenosine 3', 5' monophophate (cAMP)-dependent protein kinase, protein kinase G (PKG) or guanosine 3', 5' monophosphate (cGMP)-dependent protein kinase and isoforms of protein kinase C (PKC). SGK1 is present in the genomes of all eukaryotic organisms examined so far, including *Caenorhabditis elegans*, *Drosphila*, fish and mammals. Structure of SGK1 has been highly conserved through evolution like many other protein kinases (90;93;94).

Two other isoforms of SGK1 that have been identified in mammals and are named as SGK2 and SGK3. The catalytic domains of SGK2 and SGK3 isoforms share 80% amino acid sequence identity with one another and with SGK1 (89). The human gene encoding SGK1 was found in chromosome 6q23 (94) whereas the gene encoding SGK2 was identified in chromosome 20q12. SGK-like gene which encodes a protein having predicted amino acid sequence identical to that of human SGK3 (95) was found in chromosome 8q12.2.

SGK1 is expressed in humans that have been studied including the pancreas, liver, heart, lung, skeletal muscle, placenta, kidney and brain (90) but SGK1 is not expressed in all cell types within those tissues. For example, SGK1 transcript levels are found high in acinar cells in the pancreas (96). High transcript levels of SGK1 are also found in the distal tubule and collecting duct of the kidney and in thick ascending limb epithethial cells (85). The expression of SGK2 mRNA is restricted in human tissues. It expresses most abundantly in liver, kidney and pancreas (89). As like SGK1, SGK3 mRNA is present in all human and murine tissues examined but expression is particularly high in the mouse, heart and spleen and in the embryo (89;97).

SGK1 has been observed as cytosolic in differentiated cells such as luteal cells (84;93) or in tumour cells arrested in the G_1 phase of the cell division cycle by glucocorticoids (84;98). It has also been observed as nuclear in proliferating glomerulosa cells (84;93;98) or mammary tumour cells during the S and G_2-M phases of the cell cycle{Gonzalez-Robayna, 1999 514 /id}. However, the localization of SGK1 in any given cell is regulated by extracellular signals. Thus, in serum-stimulated mammary epithelial cells, the endogenously expressed SGK1 is nuclear, but becomes cytosolic after the inhibition of phophatidylinositol (PI) 3-kinase. Translocation from the cytosol to the nucleus also occurs in response to serum stimulation of HEK293 or COS cells transfected with SGK1(99).

To become functional, SGK1 is activated by phosphorylation through a signaling cascade including phosphatidylinositol (PI) 3-kinase and phosphoinositide dependent kinase PDK1 and PDK2/H-motif kinase. While PDK1 phosphorylates SGK1 at ^{256}Thr, PDK2/H-motif kinase phosphorylates the kinase at ^{422}Ser. SGK2 and SGK3 may similarly be activated by PDK1 and PDK2/H-motif kinase. The equivalent phosphorylation sites for SGK2 and SGK3 are found at ^{193}Thr/^{356}Ser and ^{253}Thr/^{419}Ser, respectively (89)

Replacement of the serine at position 422 by aspartate in the human SGK1 leads to the constitutively active ^{S422D}SGK1 whereas replacement of lysine at position 127 with asparagine, within the ATP binding region required for the enzymatic activity, leads to the constitutively inactive ^{K127N}SGK1. Analogous mutations in SGK2 and SGK3 lead to the constitutively active ^{S356D}SGK2 and ^{S419D}SGK3, and the constitutively inactive ^{K64N}SGK2 and ^{K191N}SGK3 (89).

SGK isoforms resemble PKB in the substrate specificity, recognizing a serine or threonine residue lying in Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr sequence (where Xaa is a variable amino acid) and thereby phosphorylating it (89). SGK1 has a considerable physiological role through the regulation of transporters and ion channels. Sodium channel conductance stimulated by SGK1 may result in cell volume regulation (88;90;100). SGK1 mediated activation of sodium channels leads to Na^+ entry which in turn depolarizes the cell membrane. The depolarized cell membrane allows the entry of chloride ions and the accumulation of NaCl that further increases the intracellular osmolarity. The osmotic gradient makes water to enter the cell by which the volume of cell increases (94). SGK1 was found to stimulate Na^+, K^+ and $2Cl^-$ cotransporter activity in the thick ascending limb of the

kidney, a key nephron segment in urinary concentration, which is of importance in renal Na^+ reabsorption (85). Abundant SGK1 gene transcription has been observed in diabetic nephropathy (85-87), fibrosing pancreatitis (90) and inflammatory bowel disease (96) but SGK1 involvement in the formation of abnormal fibrosis tissues remains to be established.

Moreover SGK1 and its isoforms are well proved in stimulating the activity and the cell membrane abundance of several transporters and ion channels. For instance, SGK isoforms regulate the epithelial Na^+ channel, ENaC (101) , the voltage-gated Na^+ channel, SCN5A (101;102), the K^+ channels ROMK1 (103), KCNE1/KCNQ1 (104) and $K_v1.3$ (105), the Na^+/H^+ exchanger NHE3 (103), the dicarboxylate transporter NaDCT (103), the glutamate transporters EAAT1 (106), EAAT3 (107), EAAT4 (108) and EAAT5 (109) and the Na^+/K^+-ATPase. The regulatory activity of SGK1 plays a diverse role in essential cell functions such as epithelial transport, excitability, cell proliferation and apoptosis.

Individuals carrying a certain variant of SGK1 [the combined presence of distinct polymorphisms in intron 6(I6CC) and in exon 8 (E866) have been shown to associated with increased blood pressure. It occurs due to enhanced stimulation of ENaC by SGK1. Moreover, the latter study revealed the role of SGK1 in the hypertension paralleling insulinemia. The outlined SGK1 gene variant may further accelerate intestinal glucose absorption by stimulation of SGLT1 and glucose accumulation in peripheral tissues including fat. Increased SGLT1 activity leads to accelerated intestinal glucose absorption, excessive insulin release, fat deposition, a subsequent decrease of plasma glucose concentration, and triggering of repeated glucose uptake and thus obesity.

To date, two modes of SGK1 action in regulating transporters and ion channels have been identified. It either regulates transporters by phosphorylating them at the putative consensus site (Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr) or by inhibiting the downregulating effect of protein ubiquitin ligase Nedd4-2. These two modes of regulation of SGK1 were observed in epithelial Na^+ channel, ENaC (110) (Figure 5).

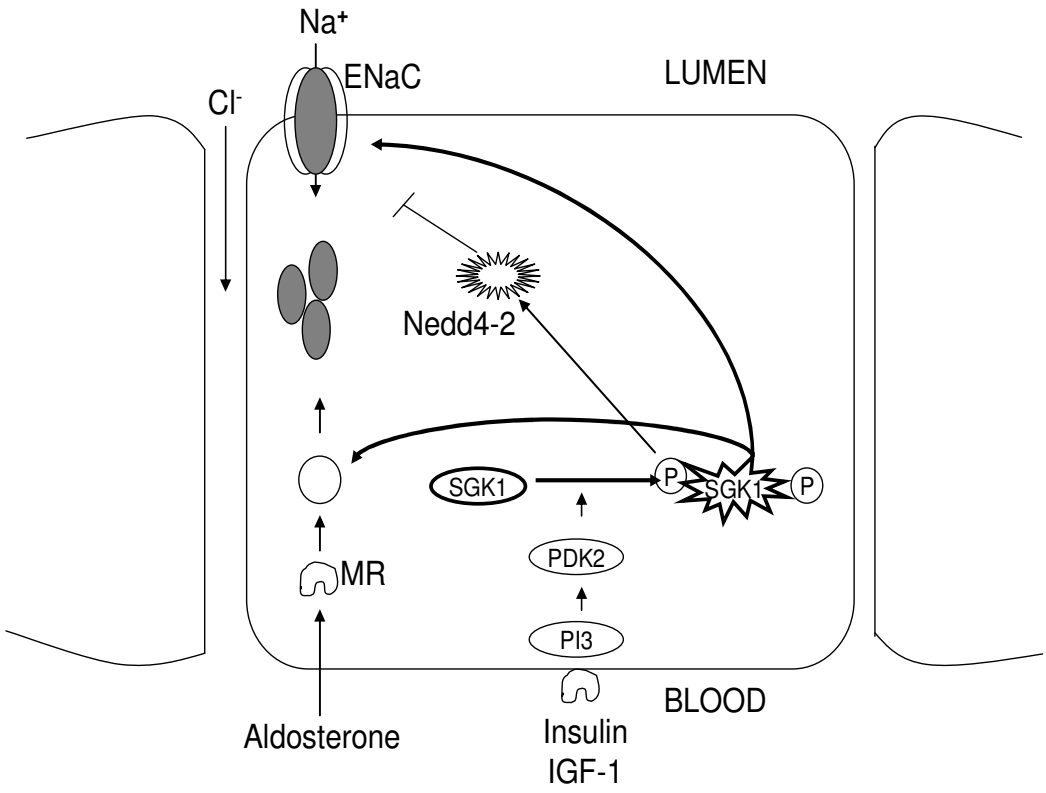

Figure 5. Schematic model showing molecular mechanisms of ENaC regulation by SGK1. Aldosterone binding to the mineralocorticoid receptor (MR) can stimulates the transcription of SGK1 as well as ENaC. Insulin or Insulin-like growth factor (IGF-1) phosphorylates SGK1 at Ser422 through PI3 kinase and PDK2/H-motify kinase signaling cascade. Activated (phosphorylated) SGK1 enhances ENaC plasma membrane abundance either directly by phosphorylating the channel or indirectly by inhibiting the downregulating effect of the ubiquitin ligase Nedd4-2.

2. AIM OF THE STUDY

Insulin stimulates glucose transport in hormone responsive tissues mainly by inducing the redistribution of the facilitated hexose carrier isoforms GLUT1 and GLUT4 from intracellular compartments to the plasma membrane. The cascade of signalling events involved in glucose transporter trafficking to the cell surface in response to insulin is triggered by an increase in insulin receptor tyrosine kinase activity followed by tyrosine phosphorylation of the insulin receptor substrates (IRS1, IRS2) and activation of the phosphatidylinositol 3-kinase (PI-3 kinase). Downstream elements of PI-3 kinase include the phosphoinositide dependent kinase PDK-1 which in turn phosphorylates and thus activates the serine/threonine kinase Akt/protein kinase B (PKB) (111) (81).

The role of PI-3 kinase in insulin-dependent and insulin-independent stimulation of GLUT1 and GLUT4 translocation has been confirmed by several studies using pharmacological (Wortmannin) inhibition and genetic (PI-3 kinase dominant-negative mutants) knockout of the kinase . The effect of PI-3 kinase on GLUT4 trafficking is mediated by PKB. PKB is, however, at least in some cells, not required for the PI-3 dependent trafficking of GLUT1. Also results from cell culture experiments support a role of PKB and the atypical PKC isoforms (aPKCs) in the insulin-stimulated GLUT4 translocation. Coexpression of constitutively active PKB or aPKCs promotes glucose transport and persistent GLUT4 localization to the plasma membrane, whereas expression of dominant-interfering PKB mutants or activation-resistant aPKCs inhibit glucose transport. Even though these inactive mutants reduced glucose transport, the insulin-mediated effect on glucose transport was not abrogated completely. Additionally, the ablation of PKBα or aPKCζ resulted in mice with no obvious defects in glucose homeostasis, indicating that additional PI3K dependent protein kinases are involved in the regulation of GLUT4.

The role of PI-3 kinase in insulin-dependent and insulin-independent stimulation of GLUT1 and GLUT4 translocation has been confirmed by several studies using pharmacological (Wortmannin) inhibition and genetic (PI-3 kinase dominant-negative mutants) knockout of the kinase . The effect of PI-3 kinase on GLUT4 trafficking is mediated by PKB. PKB is, however, at least in some cells, not required for the PI-3 dependent trafficking of GLUT1.
Thus, some other PI-3 kinase dependent protein kinases are presumably involved in the regulation of GLUT1.

A further downstream molecule in PI-3 kinase signalling cascade is the serum-and glucocorticoid-inducible kinase SGK1. The serum and glucorticoid inducible kinase SGK1 regulates several transporters by enhancing their plasma membrane abundance. Since GLUT1 contains a putative consensus site for phosphorylation by SGK1 at 95Ser, and GLUT4 at 274Ser, the present study aimed at investigating whether these transporters are regulated by the kinase and whether the modulation occurs through changes in the transporter plasma membrane expression.

3. MATERIALS AND METHODS

3.1 Chemicals and reagents

Agar Agar	Roth, Karlsruhe, Germany
Agarose electrophoresis grade,	Life Technologies, Paisley, Scotland
Bovine serum albumin	Sigma-Aldrich, Deisenhofen, Germany
Bromphenol blue	Sigma-Aldrich Chemie, Germany
Chemoluminescence ECL kit	Amersham, Freiburg, Germany
Collagenase D	Roche, Mannheim, Germany
cRNA synthesis and DNAse I	
mMessage-mMachine kit,	Ambion, Austin,USA
Developer and replenisher Biomax MR,	Eastman Kodak Company,NY, USA
Diethylpyrocarbonate (DEPC)	Sigma-Aldrich, Deisenhofen, Germany
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, Deisenhofen, Germany
DNA-Ladder, 1 kb and 100bp	Life Technologies, Germany
DNAase (RNAase free)	Roche, Mannheim, Germany
dNTPs	Life Technologies, Germany
Ethidium bromide	Sigma-Aldrich Chemie, Germany
Fixer and replenisher	Biomax MR, Eastman Kodak Company, Rochester NY, USA
QuickChangeTM Site-directed Mutagenesis kit	Stratagene, Heidelberg, Germany
Restriction enzymes	Boehringer, Mannheim, Germany
Scintillation fluid Ultima Gold,	Packard, Groningen, the Netherlands

3.2 Constructs and site-directed mutagenesis

The constitutively active human ^{S422D}SGK1, ^{T308DS473D}PKB and inactive human ^{K127N}SGK1 were kindly provided by Sir Philip Cohen (University of Dundee, Dundee, Scotland). The mutatated glucose transporter 4 ^{S274A}GLUT4 was generated by site-directed mutagenesis (QuikChange site-directed mutagenesis kit; Stratagene, Heidelberg, Germany) according to the manufacturer's instructions.

5´ CGTGAGCGGCCACTGGCCCTGCTCCAGCTCCTG 3´

5´ CAGGAGCTGGAGCAGGGCCAGTGGCCGCTCACG 3´.

The mutant was sequenced to verify the presence of the desired mutation.

3.3 Xenopus laevis oocytes

One of the first and still most widely used assay system for quantifying an authentic protein biosynthetic process is the fully grown oocyte of the South African clawed frog, *Xenopus laevis*. The value of *Xenopus laevis* first became apparent in 1971, when Gurdon and co-workers discovered that the oocyte constitutes an efficient system for translating foreign messenger RNA.

The *Xenopus* oocyte is a cell specialized for the production and storage of proteins for later use during embryogenesis and developmentally divided into 6 stages (112). In addition, the complex architecture of the frog oocyte includes the subcellular systems involved in the export and import of proteins. Therefore, the mRNA-microinjected oocyte is an appropriate system to study the synthesis of specific polypeptides, as well as the storage of particular proteins in various subcellular organelles and the export of others into the extracellular space. Moreover, the subcellular compartmentalization, as well as the structure and biochemical, physiological, and biological properties of the synthesized protein, may be examined from exogenous proteins in the injected oocyte. For experimental studies oocytes of stages V-VI are used with a diameter of some 1.3 mm allowing easy preparation. The developmental stages V-VI are characterized by the occurence of 2 poles i.e. the vegetable (light) and the animal (dark) poles.

The main ion conductance in *Xenopus* oocytes is a Ca^{2+}-dependent Cl- conductance governing the resting membrane potential close to the Cl- reversal potential of -40 mV. Despite their advantages, several precautions should be taken into consideration. First, the expression of endogenous carriers may interfere with the exogenously expressed proteins in various ways. For instance, it has been observed that injection of heterologous membrane proteins at high levels can induce endogenous channels (113). Second, due to the fact that *Xenopus laevis* is a poikilothermic animal, its oocytes are best kept at lower temperature and most experiments are carried out at room temperature. Hence, temperature sensitive processes i.e. protein trafficking or kinetics may be altered. Finally, since *Xenopus* oocytes may have different signaling pathways, precaution should be taken when studying the regulation of expressed proteins. It has been revealed that the PTH receptor regulates the internalization of the sodium-phosphate transporter NaPi, mediated by the PKA and PKC pathway. However, in NaPi-3 expressing *Xenopus* oocytes PKC-mediated PTH regulation

can not be observed. Instead, coupling to the PKA pathway leads to the alteration of PKA-regulated ion channels (114). Exposing the *Xenopus* oocytes to the regulators of intracellular signaling such as PKC activator phorbol esters may unspecifically lead to internalization of the plasma membrane and the expressed proteins (115;116). In summary, the *Xenopus* oocyte system has the advantage that channels, receptors and transporters can rapidly be expressed and identified by their electrophysiological properties. Once cDNA clones have been isolated, oocytes are an excellent system for correlating structure with function using a combination of molecular biological and electrophysiological techniques and analyzed both biochemically and electro physiologically in an *in vivo* situation.

In this study we used *Xenopus* oocytes to express the facilitative glucose transporter GLUT1 and to determine its putative modulation by the insulin-sensitive protein kinase SGK1. Advantages of the oocytes over other expression systems are that GLUT1 activity can be tested individually and that endogenous glucose transport is nearly detectable. Furthermore, the differential targeting of mammalian GLUT1 and GLUT4 is maintained in the *Xenopus* oocytes which suggests that the cellular apparatus controlling the intracellular targeting of GLUTs is conserved and that oocytes are a good expression system to elucidate the mechanism of GLUT regulation.

3.3.1 In vitro RNA transcription

In-vitro cRNA transcription involves 2 consecutive steps i.e. linearization of the plasmid DNA containing the inserted cDNA of interest by the corresponding restriction enzyme and the synthesis of RNA.

a. The inserted DNA should be cut at the 3′ end yielding a 5′ protruding or a blunt end by restriction enzyme. Plasmid DNA (10 µg) was incubated with 20 U restriction enzyme and an 10x buffer (5 µl) in a final volume of 50 µl at 37°C for 2 h or overnight.

b. To ascertain the linearization process, a 5 µl aliquot was taken out and analysed on a 1% agarose.

c. 1 volume isopropanol (50 µl) and 1/10 volume 3 M sodium acetate (5 µl) pH 5.2 was then added and incubated at room temperature for 10 min to precipitate the DNA.

d. The precipitated DNA was recovered by centrifugation at 17,000 rpm for 15 min at 4°C. The DNA pellet was washed by adding 100 µl of cold 70% ethanol to the pellet followed by centrifugation at 17,000 rpm for 5 min at 4°C. This washing stage was repeated. The DNA pellet was air dried and then resuspended in 10 µl of DNase free H_2O. The concentration of DNA was determined spectrophotometrically by measuring the absorbance at 260 nm.

e. 1 µg of linearised DNA was added to 1 µl rNTPS (20 nM), 2.5 µl Cap analogue (to prevent the degradation of the 5′ end of the synthesized RNA), 1 µl RNAase inhibitor (to protect the RNA from degradation by RNAase) and 2.4 µl 10 x transcription buffer(s). After mixing, 1 µl of T7 polymerase was added and the n incubated at 37°C for 1 hr. 1 µl DNase was added and the mixture was subsequently shaken for 15 min at 37°C. After addition of 100 µl DEPC-water and 125 µl phenol chloroform, the mixture was centrifuged at 13,000 rpm for 2 min.
f. To purify the generated RNA, 100 µl DEPC water and 125 µl of phenol-chloroform mixture was added and centrifuged at maximum speed for 2 minutes. The upper inorganic phase was carefully taken into a new eppendorf tube and 12.5 µl of 3 M sodium acetate (pH 5.2) and 375 µl of 100% ethanol was added and mixed by pulse vortex and further incubated at -70ºCovernight.
g. After incubation, the mixture was centrifuged at 17000 rpm for 15 minutes at 4 ºC. The supernatant was removed and the pellet was washed twice with 200 µl of 70% ethanol. Finally the pellet was dried at room temperature and reconstituted in 25 µl of DEPC water and vortexed. Then concentration of RNA was measured by taking 1 µl of RNA in 69 µl water using an Eppendorf Biophotometer (Hamburg, Germany). The quality of the RNA generated was checked by gel electrophoresis.

3.3.2 Preparation of Xenopus oocytes

An adult female *Xenopus laevis* frog was submersed in one liter of 3- aminobenzoic acid ethyl ester (0.1%) for about 15-30 min (Figure 6A). After the frog was fully anesthetized it was placed on ice for surgery.

A small abdominal incision (1 cm) was carried out and a segment of ovary was removed (Figure 6B,C). Subsequently the wound was closed with a reabsorbable suture (Figure 6D). The frog was then kept wet and warm by placing it in a cavity filled by a small amount of warm water to avoid drowning and hypothermia.

The ovarial sacs were manually separated into groups of 10-20 oocytes, put into a 15 ml tube and then enzymatically defolliculated by treatment with an OR- 2 (Oocytes-Ringer) (Table 1) solution containing 1-2 mg/ml collagenase A for 2-2.5 h at room temperature (Figure 6E) with gentle agitation. Defolliculation of the oocytes was stopped by washing several times with ND96 (Table 1).This step also removes all detritus permitting oocyte sorting. Oocytes were then sorted using a self-made apparatus (Figure 6F). Only large oocytes (stage V or VI) were selected and stored overnight in a ND96 storage solution at 16°C.

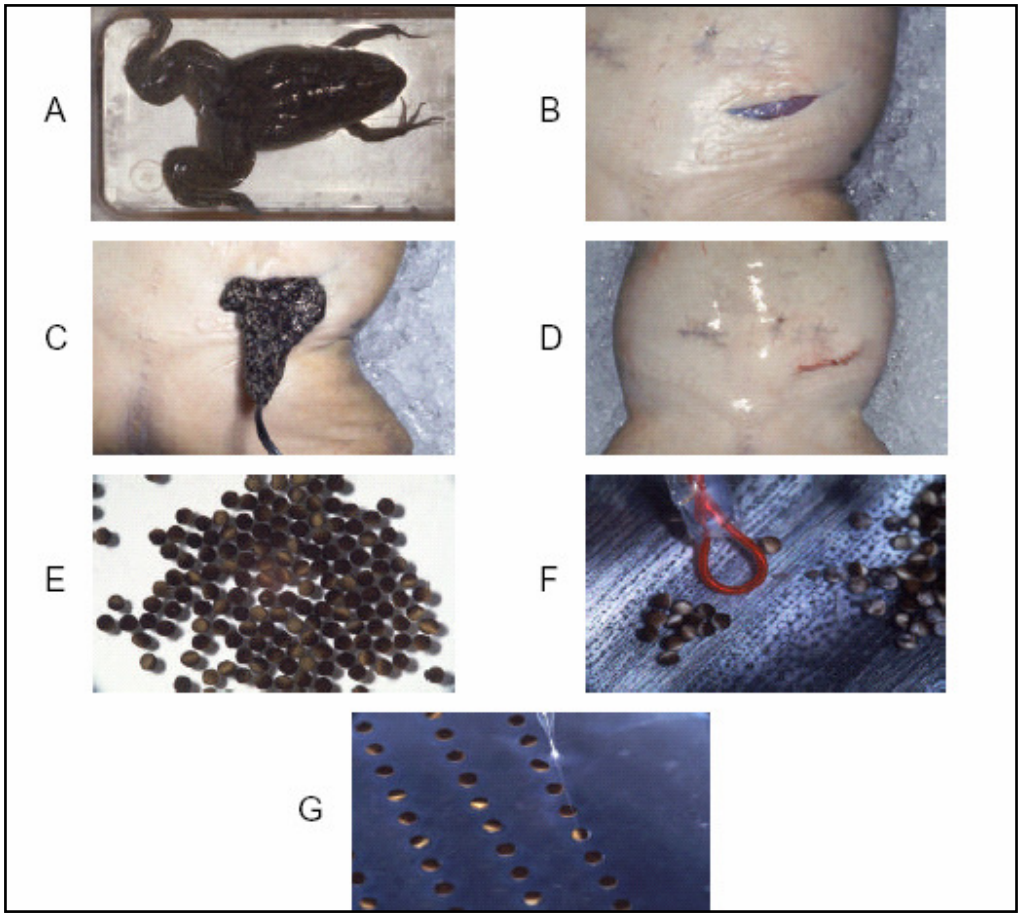

Figure 6. Oocytes preparation and injection. A The frog is anesthetized in 1 liter of 3-aminobenzoic acid ethyl ester (1%) in tap water at near room temperature. **B** The frog is placed on its back during operation. An incision about 1 cm long is made in the skin. **C** A small portion of the ovary is pulled out with forceps and removed with a pair of scissors. **D** The peritoneum and the muscle tissue are sewn up and then the skin closed off using cat gut. **E** The clump of oocytes is immediately transferred to a petri dish containing modified Barth medium with antibiotic. **F** Oocytes of stage V and VI are separated with a platinum wire loop. **G** For injection, the oocytes are aligned relative to the tip of the needle.

	ND96	ND96 Storage solution	OR-2 (Oocytes Ringer)
NaCl (mmol/l)	96	96	82.5
KCl (mmol/l)	2	2	2
$CaCl_2$ (mmol/l)	1.8	1.8	-
$MgCl_2$ (mmol/l)	1	1	1
Tris-HEPES (mmol/l)	5	5	5
Na-Pyruvate (mmol/l)	-	2.5	2.5
Theophylline(mmol/l)	-	0.5	0.5
Gentamycin (mmol/l)	-	50	-
pH	7.4	7.4	7.4

Table 1. Composition of buffers used in *Xenopus* oocytes experiments

3.3.3 cRNA injection

After storing overnight, oocytes were injected using glass microcapillaries (filled with the required cRNA) mounted in a micromanipulator-controlled microinjector (Figure 7). Precaution was taken so that cRNA was not degraded by RNAases and that the injection capillary was not clogged with small particles. To avoid those problems several procedures were carried out such as using only sterile pipettes, gloves and DEPC treated water for dilution of cRNA. Glass capillaries were pulled using a normal puller. The tip was manually broken under the microscope (diameter of about 10-20 µm), backfilled with paraffin oil to seal the pipette from air and loaded with cRNA by suction (usually 1-2 µl). Oocytes were

then placed into a 35 mm petri dish with a polypropylene mesh glued to the bottom to fix the oocytes and injected with a given volume of cRNA (usually 27.6 nl). The oocytes injected are listed in the Table 2.

After injection, oocytes were kept in storage solution at 15°C. To avoid sticking of oocytes to the petri dish or to other oocytes, the dish was gently shaken. At least every two days the storage solution was exchanged and damaged oocytes were removed to maximise the survival of the oocytes.

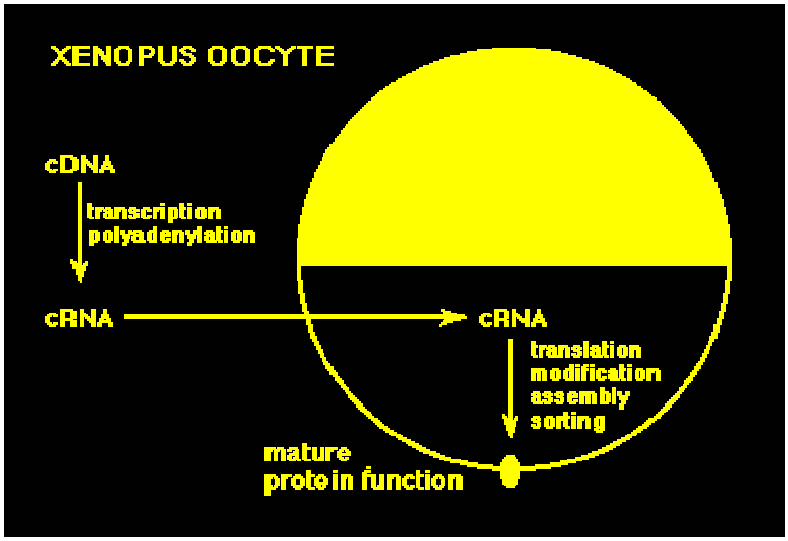

Figure 7. cRNA injection into the oocytes. *In vitro* transcribed poly (A)-cRNA is microinjected into the cytoplasm of the oocyte and in the following days the function of the encoded protein can be measured.

Protein	RNA polymerase	cRNA injected (ng/nl)
GLUT4	SP6	15
GLUT4(SA)	SP6	15
^{S422D}SGK1	T7	7.5
^{K127N}SGK1	T7	7.5
PKB	T7	7.5

Table 2. Enzymes used for invitro transcription and amount of cRNA injected per oocyte.

Isolation of mouse adipocytes

Adipocytes were isolated from epididymal fat pads of 129/SvJ mice by collagenase digestion as described previously (117). Epididymal fat pads were minced and digested with 2 mg/ml type II collagenase for 1h at 37°C in Krebs Ringer bicarbonate Hepes buffer (KRBH: 120 mmol/l NaCl, 4 mmol/l KH_2PO_4, 1 mmol/l $MgSO_4$, 1 mmol/l $CaCl_2$, 10 mmol/l $NaHCO_3$, 200 nmol/l adenosine, 30 mmol/l Hepes, pH 7.4) containing 1 % fraction V BSA. The resulting cell suspension was filtered through a nylon mesh (250 µm) and washed 3 times with KRBH buffer containing 3 % BSA. Then adipocytes were resuspended in KRBH buffer with 3 % BSA. An aliquot of the final cellular suspension was taken to measure lipocrit and cell number.

3.5 Detection of cell surface expression by chemiluminescence

To quantify the abundance of the transporters expressed on the plasma membrane of oocytes, chemiluminescence assay was employed. The protein expressed in the cell membrane is recognized first by the primary antibody which binds to an extracellular loop of the membrane protein. Then the bound primary antibody forms a complex with the secondary antibody that is coupled with horse radish peroxidase, an enzyme that catalyzes a reaction giving a product and light. The light is then detected by the luminometer.

Defolliculated oocytes were first injected with constitutively active SGK1 (15 ng/oocyte) and 1 day later with GLUT4 (10ng/oocyte). Initially the oocytes were incubated in a blocking solution of ND96 with 1% BSA solution for 30 minutes kept in ice. The oocytes were incubated with 2 µg/ml primary goat polyclonal anti-GLUT4 antibody which recognizes an N-terminal extracellular domain of GLUT4 (Santa Cruz Biotechnology, Heidelberg, Germany). This primary antibody treatment is done for 60 minutes kept in ice. After one

hour of incubation, oocytes were washed with ND96/1%BSA solution for five times in 30 minutes. Futher incubation is done in a solution 4 µg/ml secondary, peroxidase-conjugated donkey anti-goat IgG antibody (Santa Cruz Biotechnology, Heidelberg, Germany) for one hour. Oocytes were again washed in ND96/1%BSA solution for 10 times in one hour. Finally oocytes were washed with ND96 buffer without BSA for 3 times in 15 mintues. Individual oocytes were placed in 96 well plates with 100µl of ND96 and 20 µl of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL), and chemiluminescence was quantified by luminometry by integrating the signal over a period of 1 s.

3.6 Tracer flux Measurements

Tritium-labeled 2-deoxy-D-glucose (2-DOG) was used as the glucose analogue for uptake determination. Groups of 10-15 oocytes injected with GLUT4 were incubated for 2 h with 1 µM insulin. Thereafter oocytes were placed in 0.25 ml of ND96 (96 mmol/l NaCl, 2 mmol/l KCl, 1.8 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$ and 5 mmol/l HEPES, pH 7.4) containing 37 KBq ^{3}H 2-DOG and 50 µmol/l (or the indicated amount for kinetic analysis) of unlabeled 2-DOG. After incubation for 10 min at room temperature with 2-DOG (linear range of uptake), uptake was terminated by washing the oocytes four times with 3 ml of ice-cold phosphate-buffered saline (PBS) containing 100 mmol/l 2-DOG. Oocytes were individually transferred into scintillation vials and dissolved by adding 200 µl of 10% SDS before radioactivity was determined.

In HEK-293 cells, 2-DOG uptake was measured 2 days after transfection by incubating cells at 37°C for 5 min (linear range of uptake) in glucose free krebs Ringer HEPES buffer containing ^{3}H 2-DOG 0.1 µCi/well and 0.3 mmol/l cold 2-DOG with or without 0.1 mmol/l phloretein. Uptake was terminated by rapid aspiration of uptake solution and washing four times with ice-cold PBS containing 50 mmol/l unlabeled 2-DOG. Thereafter, cells were lyses with 10mmol/l NaOH/0.1%/Triton-X-100, and radioactivity incorporated into the cells was measured with a liquid scintillation counter. Protein concentrations were determined by the Bradford method. For kinetic analyis, 2-DOG uptake was measured by incubating cells with ^{3}H 2-DOG 0.1 µCi/well and various concentrations of unlabeled 2-DOG at 37°C for 5 minutes.

In isolated adipocytes, 2-DOG uptake was measured by incubating the cells with 0.1 µCi ^{3}H 2-DOG and 0.1 mmol/l cold 2-DOG with or without 0.1 mmol/l phloretin. Uptake was

terminated after 5 min by centrifugation of cells through dinonylphthalate. The separated cells were removed from the top of the oil layer, and cell-associated radioactivity was quantified.

3.7 Western blotting

To determine whole-cell GLUT1 and SGK1 expression in HEK-293 and adipocytes, cells were homogenized in lysis buffer and 30 µg protein were separated on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking with 5% non fat dry milk in PBS/0.15% Tween-20 for 1 h at room temperature, blots were incubated overnight at 4°C with a goat anti-GLUT1 antibody (diluted 1:100 in PBS/0.15% Tween 20/5% nonfat dry milk; Santa Cruz Biotechnology), a rabbit anti-SGK1 antibody (diluted 1:1,000 in PBS/0.15% Tween 20/5% nonfat dry milk; Upstate, Dundee, U.K.), or a rabbit anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) horseradish peroxidase conjugated antibody (diluted 1:1,000 in PBS/0.15% Tween 20/5% nonfat dry milk). GAPDH was used to demonstrate equal protein loading. Secondary peroxidase-conjugated donkey anti-goat IgG antibody (diluted 1:2000 in PBS/0.15% Tween 20/5% nonfat dry milk; Santa Cruz Biotechnology) or sheep anti-rabbit IgG antibody (diluted 1:1,000 in PBS/0.15% Tween 20/5% nonfat dry milk; Amersham, Freiburg, Germany) was used for chemiluminescent detection of GLUT1 or SGK1 with an enhanced chemiluminescence kit (Amersham), respectively. Band intensities were quantified using Quantity One Analysis software (Biorad, Munich, Germany).

To determine whole cell GLUT4 and SGK1 expression in oocytes and adipocytes, cells were homogenized in lysis buffer and 30 µg protein was separated on a 10 % polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking with 5 % non-fat dry milk in PBS / 0.15 % Tween 20 for 1h at room temperature, blots were incubated overnight at 4 °C with a goat anti-GLUT4 antibody (Santa Cruz Biotechnology, Heidelberg, Germany, diluted 1:100 in PBS / 0.15 % Tween 20 / 5 % non-fat dry milk), a rabbit anti-SGK1 antibody (Upstate, Dundee, UK) diluted 1:1000 in PBS / 0.15 % Tween 20 / 5 % non-fat dry milk or a rabbit anti-GAPDH HRP conjugated antibody (diluted 1:1000 in PBS / 0.15 % Tween 20 / 5 % non-fat dry milk). GAPDH was used to demonstrate equal protein loading. Secondary peroxidase-conjugated donkey anti-goat IgG antibody (Santa Cruz Biotechnology, Heidelberg, Germany), diluted 1:2000 in PBS / 0.15 % Tween 20 / 5 % non-fat dry milk or sheep anti-rabbit IgG antibody (Amersham, Freiburg, Germany), diluted 1:1000 in PBS / 0.15 % Tween 20 / 5 % non-fat dry milk was used for chemiluminescent detection of GLUT4 or

SGK1 with enhanced chemiluminescent ECL kit (Amersham, Freiburg, Germany) respectively. Band intensities were quantified using Quantity One® analysis software (Biorad, Munich, Germany).

Calculations

Data are provided as means ± SEM, n represents the number of oocytes investigated.

All experiments were repeated with atleast three batches of oocytes. Uptake values were normalized in each batch of oocytes to the mean value obtained in oocytes expressing GLUT4 alone. GLUT4/GAPDH band intensities from three independent experiments were normalized to the mean value of GLUT4/GAPDH band intensity in oocytes expressing GLUT4 alone. For western blotting analysis of isolated adipocytes, GLUT4 band intensities were normalized to the value of GLUT4 intensity in adipocytes isolated from *sgk1*$^{+/+}$ mice. Cell surface expression was normalized in each batch of oocytes to the mean relative light units value obtained in oocytes expressing GLUT4 alone.

4. RESULTS

4.1 ^{S422D}SGK1 enhances GLUT4 mediated glucose transport in Xenopus oocytes

To investigate the role of SGK1 in the modulation of insulin mediated GLUT4 activity, GLUT4 was expressed in *Xenopus laevis* oocytes and labelled 2 deoxy-glucose was measured in the presence of SGK1 and PKB protein kinase as the positive control.

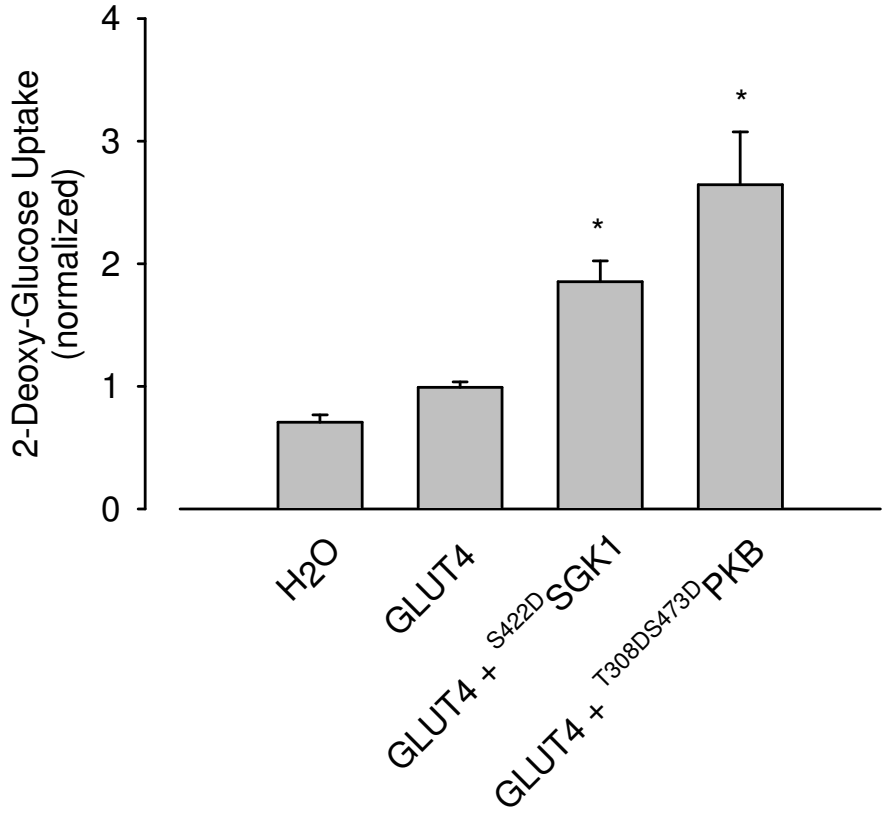

Figure 8. ^{S422D}SGK1 enhances GLUT4 mediated glucose transport. *Xenopus* oocytes were injected with GLUT4 alone or along with constitutively active ^{S422D}SGK1 or constitutively active ^{T308DS473D}PKB. H_2O injected oocytes served as control. 4 days after cRNA injection, labeled 2-DOG uptake was studied as a measure of GLUT4 activity. Arithmetic means ± SEM. * indicates statistically significant difference to uptake in *Xenopus* oocytes expressing GLUT4 alone. Uptake values were normalized in each batch of oocytes to the mean value obtained in oocytes expressing GLUT4 alone.

Coexpression of the constitutively active ^{S422D}SGK1 in GLUT4-expressing oocytes resulted in an increase of radiolabeled 2-deoxy-glucose uptake (Figure 8, n = 24) while coinjection of ^{S422D}SGK1 in water-injected oocytes did not have any significant effect on intrinsic glucose transport (0.13 ± 0.01 pmol/min/oocyte, n = 26, in oocytes injected with ^{S422D}SGK1 alone vs. 0.14 ± 0.01 pmol/min/oocyte, n = 26 in water-injected oocytes) demonstrating that the kinase does not affect intrinsic transporters. Coexpression of the constitutively active ^{T308DS473D}PKB,

used as a positive control, strongly activated glucose uptake as expected (Figure 8, n = 22). Expression of ^{T308DS473D}PKB alone did not significantly increase glucose uptake (0.15 ± 0.01 pmol/min/oocyte, n = 24, in oocytes injected only with ^{T308DS473D}PKB vs. 0.14 ± 0.01 pmol/min/oocyte, n = 26 in water-injected oocytes).

4.2 SGK1 catalytical activity is involved in the upregulation of GLUT4 activity

GLUT4 stimulation by ^{S422D}SGK1 was dependent on the catalytic activity of the kinase as the inactive mutant ^{K127N}SGK1 did not enhance but slightly inhibited the transporter (55.9 ± 3.3 % of control, n = 18, Figure 9). This result indicates that phosphorylation processes are involved that culminate in stimulation of GLUT4 expression.

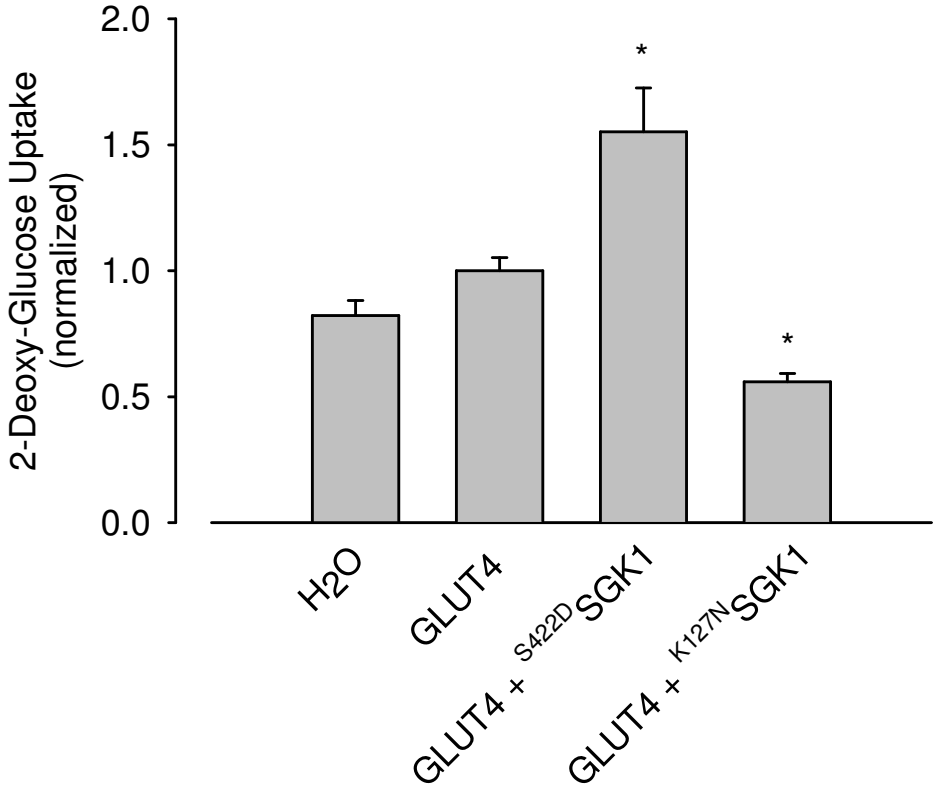

Figure 9. ^{S422D}SGK1 catalytical activity is required for the upregulation of GLUT4.
Xenopus oocytes were injected with GLUT4 alone or along with constitutively active ^{S422D}SGK1 or inactive ^{K127N}SGK1. H_2O injected oocytes served as control. 4 days after cRNA injection, labeled 2-DOG uptake was studied as a measure of GLUT4 activity. Arithmetic means ± SEM. * indicates statistically significant difference to uptake in *Xenopus* oocytes expressing GLUT4 alone. Uptake values were normalized in each batch of oocytes to the mean value obtained in oocytes expressing GLUT4 alone.

4.3 GLUT4 maximal transport rate is enhanced by ^{S422D}SGK1

To determine whether the kinase modulates GLUT4 kinetic properties, GLUT4 activity was measured at different 2-DOG concentrations in the absence and presence of ^{S422D}SGK1. Data obtained revealed that SGK1 enhances GLUT4 by modifying the maximal transport rate, Vmax, without affecting substrate affinity (Figure 10) (Table 3).

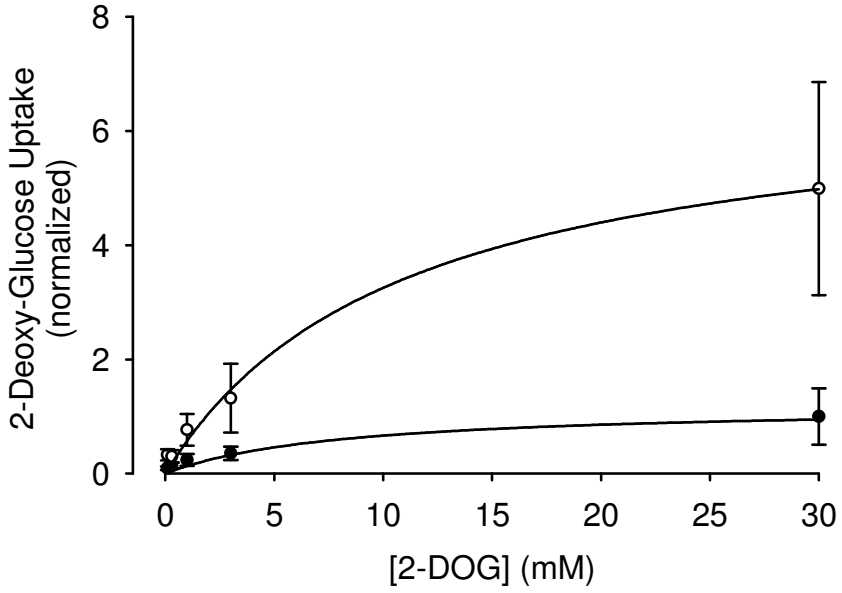

Figure 10. GLUT4 maximal transport rate is increased by ^{S422D}SGK1. *Xenopus* oocytes were injected with water or with GLUT4 alone or along with constitutively active ^{S422D}SGK1. 4 days after cRNA injection, 2-DOG uptake was measured at the indicated substrate concentrations. Uptake values of water-injected *Xenopus* oocytes were subtracted and results were normalized in each batch of oocytes to the V_{max} obtained in oocytes expressing GLUT4 alone. Arithmetic means ± SEM.

Injected cRNA	Km (mmol/l)	Vmax (normalised)
GLUT4	8.08 ± 0.95	1.20 ± 0.52
GLUT4+SGK1(SD)	10.85 ± 1.02	6.78 ± 2.32

Table 3. Kinetic parameters of GLUT4 coexpression of ^{S422D}SGK1 in X*enopus* oocytes.

4.4 ^{S422D}SGK1 stimulates GLUT4 plasma membrane abundance without affecting total GLUT4 proten levels

SGK1 might increase GLUT4 activity by promoting GLUT4 synthesis or by increasing GLUT4 abundance at the plasma membrane.

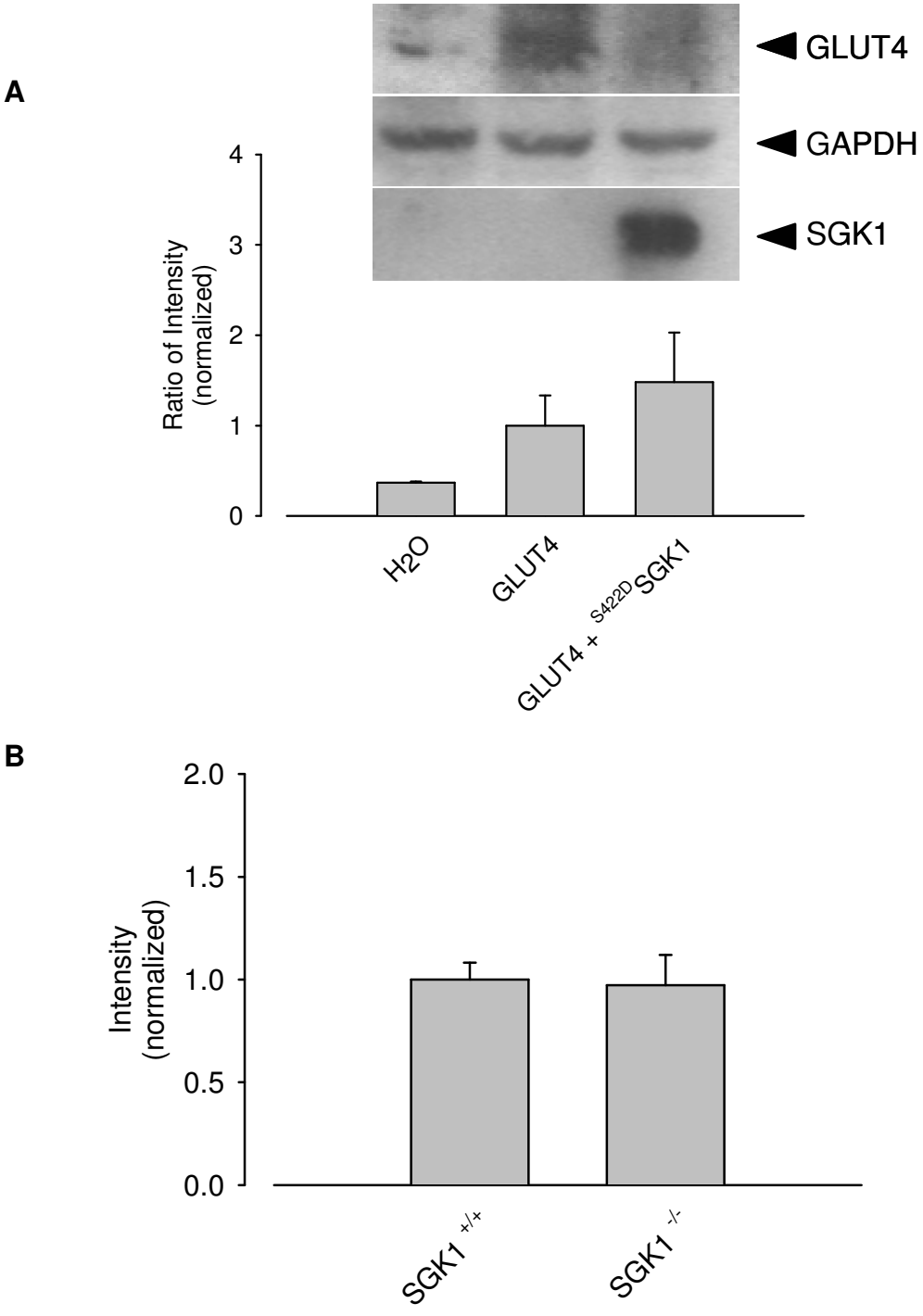

Figure 11. ^{S422D}SGK1 stimulates GLUT4 activity by promoting GLUT4 plasma membrane abundance without affecting total GLUT4 protein levels.

According to western blotting of whole oocyte lysates, enhanced GLUT4 activity is not the result of increased GLUT4 protein synthesis (Figure 11A). GLUT4 protein levels remained similarly unaffected in adipocytes isolated from *sgk1*$^{-/-}$ mice (Figure11B).

Total GLUT4 expression was assessed by western blotting of whole cell lysates from oocytes expressing GLUT4 alone or along with ^{S422D}SGK1 (A), and in adipocytes isolated from *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice (B). Arithmetic means ± SEM. GLUT4/GAPDH band intensities from three independent experiments were normalized in each batch to the mean value of GLUT4/GAPDH band intensity of cells expressing GLUT4 alone. For western blotting analysis of isolated adipocytes, GLUT4 band intensities from three independent experiments were normalized to the value of GLUT4 intensity in adipocytes isolated from *sgk1*$^{+/+}$ mice.

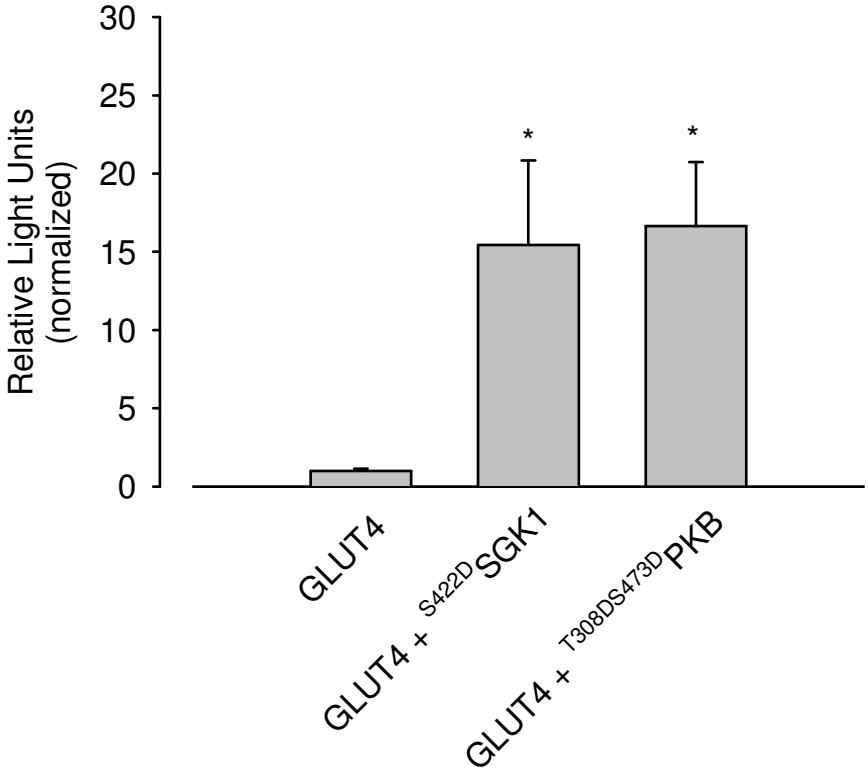

Figure 12. ^{S422D}SGK1 upregulates GLUT4 plasma membrane abundance in cell surface expression experiment. *Xenopus* oocytes were injected with GLUT4 alone or along with constitutively active ^{S422D}SGK1 or inactive ^{K127N}SGK1. H_2O injected oocytes served as control. 4 days after cRNA injection, labeled 2-DOG uptake was studied as a measure of GLUT4 activity. Arithmetic means ± SEM. * indicates statistically significant difference to uptake in *Xenopus* oocytes expressing GLUT4 alone. Uptake values were normalized in each batch of oocytes to the mean value obtained in oocytes expressing GLUT4 alone.

To test whether SGK1 enhances GLUT4 activity by altering its trafficking to the plasma membrane, GLUT4 cell surface abundance in *Xenopus* oocytes was examined upon coexpression of ^{S422D}SGK1 by quantitative immunoassays. Oocytes coexpressing ^{T308DS473D}PKB were used as a positive control. Figure 12 shows that the relative surface abundance of the transporter is more prominent in oocytes expressing ^{S422D}SGK1 or ^{T308DS473D}PKB along with GLUT4 than in oocytes expressing GLUT4 alone (n = 36).

4.5 GLUT4 transporter stimulation by ^{S422D}SGK1 is abrogated upon disruption of putative SGK phosphorylation site on GLUT4

GLUT4 bears a putative SGK1 phosphorylation site on its sequence at ^{274}Ser. To determine whether SGK1 modulates GLUT4 uptake by phosphorylating GLUT4, the serine at position 274 in GLUT4 was mutated into alanine (^{S274A}GLUT4) and its regulation evaluated upon coexpression of the kinase.

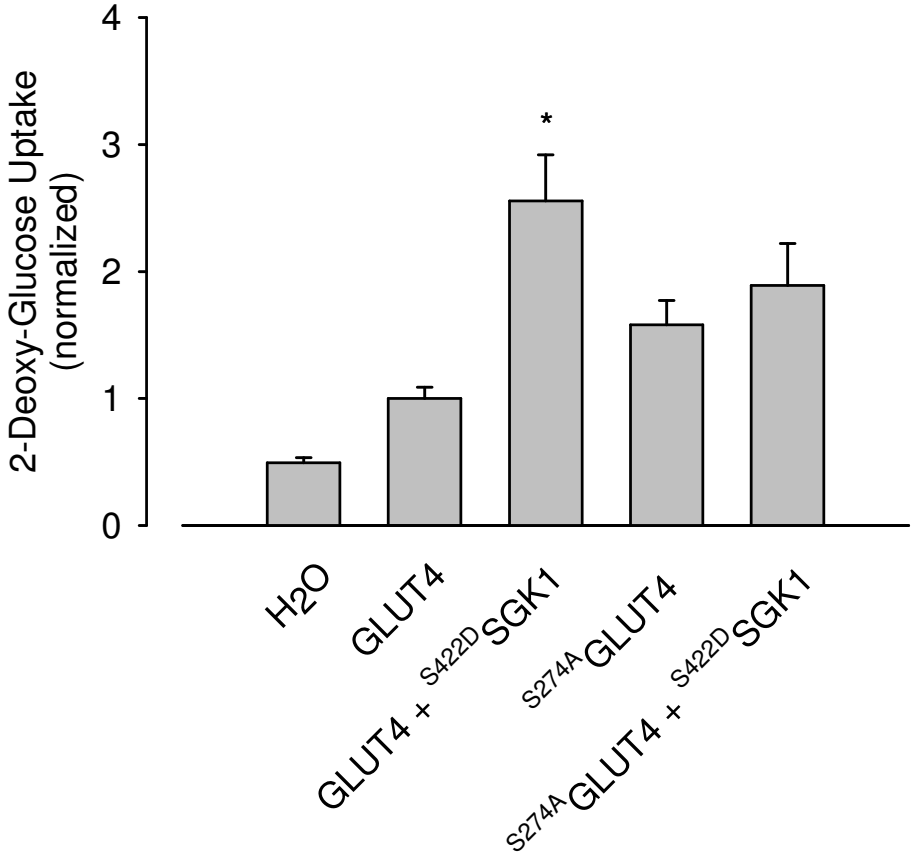

Figure 13. ^{S422D}SGK1 upregulates GLUT4 activity by the direct phosphorylation machanism. *Xenopus* oocytes were injected with wild-type GLUT4 or ^{S274A}GLUT4 alone or along with constitutively active ^{S422D}SGK1. 4 days after cRNA injection, labeled 2-DOG uptake was studied (A) and western blotting performed to demonstrate proper ^{S422D}SGK1 expression. Arithmetic means ± SEM. * indicates statistically significant difference to uptake in *Xenopus* oocytes expressing wild-type GLUT4 or ^{S274A}GLUT4 alone. Uptake values were normalized to the mean value obtained in oocytes expressing wild-type GLUT4 alone.

Ablation of the putative SGK phosphorylation site blunted significantly GLUT4 stimulation despite proper ^{S422D}SGK1 expression (120 ± 21 % of 2-DOG uptake measured in ^{S274A}GLUT4 expressing oocytes, n = 39, Figure 13).

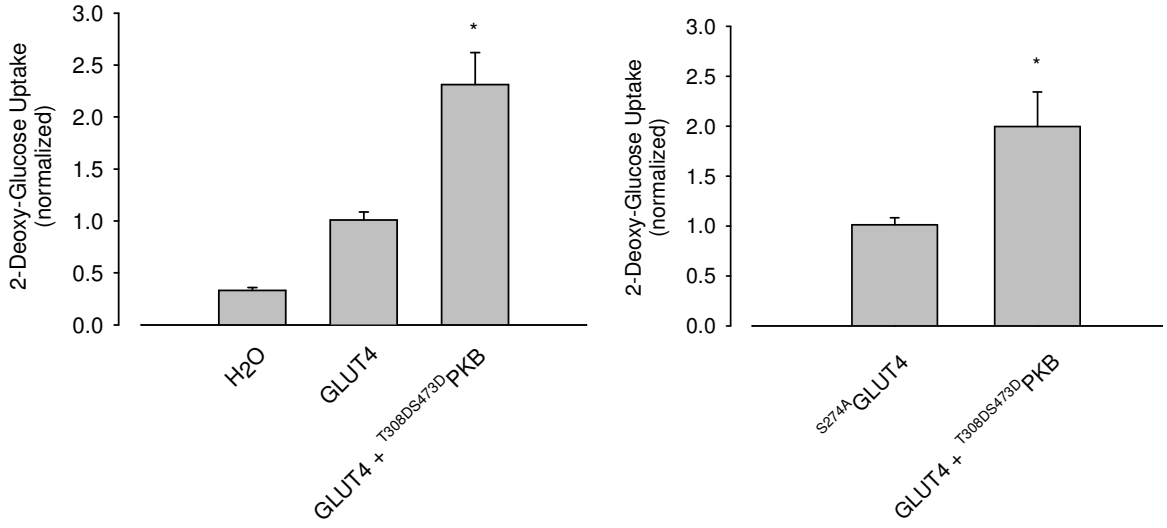

Figure 14. Direct phosphorylation machanism is not involved in the regulation of GLUT4 by PKB. *Xenopus* oocytes were injected with wild-type GLUT4 or ^{S274A}GLUT4 alone or along with constitutively active ^{T308DS473D}PKB. 4 days after cRNA injection, labeled 2-DOG uptake was studied in wild-type GLUT4 and in ^{S274A}GLUT4 expressing oocytes. Arithmetic means ± SEM. * indicates statistically significant difference to uptake in *Xenopus* oocytes expressing wild-type GLUT4 or ^{S274A}GLUT4 alone.

The effect of ^{T308DS473D}PKB on the GLUT4 phosphorylation-deficient mutant (^{S274A}GLUT4) was similarly analyzed. As shown in Figure 14, mutation of the putative phosphorylation site on GLUT4 did not alter the ability of ^{T308DS473D}PKB to activate GLUT4 (199.9 ± 34.4 % of 2-DOG uptake measured in ^{S274A}GLUT4 expressing oocytes, n = 33).

4.6 ^{S422D}SGK1 enhances 2-DOG transport in HEK-293 cells without affecting total protein expression levels of GLUT1

It was already observed the upregulation of GLUT1 by SGK1 in *Xenopus* oocytes. GLUT1 modulation by SGK1 was observed in mammalian cells (HEK-293). HEK-293 cells were transfected with pIRES2EGFP-^{S422D}SGK1, pIRES2EGFP^{K127N}SGK1 or with empty vector (as a control) and two days later 2-DOG uptake was measured in the presence and absence of GLUT1 inhibitor phloretin (0.1 mM).

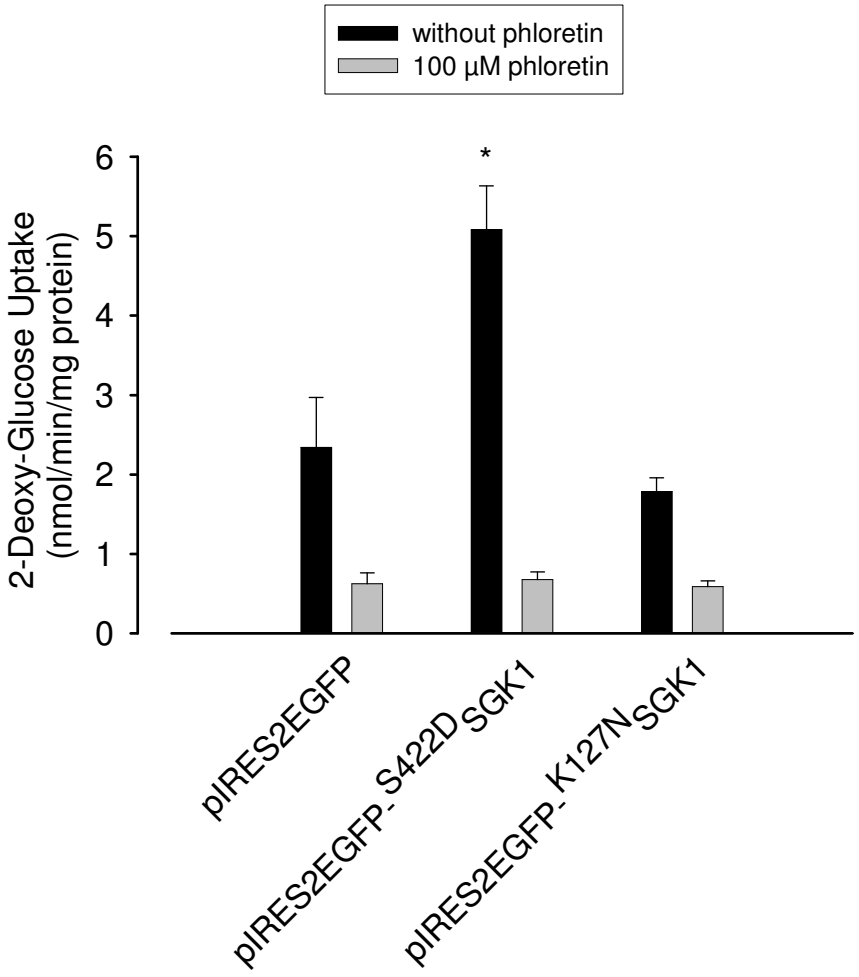

Figure 15. S422DSGK1 enhances 2-DOG transport in HEK-293 cells without affecting total GLUT1 expression levels. HEK-293 cells were transfected with pIRES2EGFP-^{S422D}SGK1, pIRES2EGFP^{K127N}SGK1 or empty vector and 2 days later, labeled 2-DOG uptake was studied in the presence and absence of GLUT1 inhibitor 0.1 mM phloretin

Tracer-flux studies revealed an increase in GLUT1 transport rate (from 2.3 ± 0.6nmol/min/mg protein, n = 3, to 5.1 ± 0.6 nmol/min/mg protein, n = 3, Figure 15) upon

coexpression of ^{S422D}SGK1. As observed in *Xenopus* oocytes, GLUT1 upregulation by SGK1 was dependent on the catalytic activity of the kinase as the effect was lacking upon expression of the catalytical inactive mutant ^{K127N}SGK1 (from 2.3 ± 0.6nmol/min/mg protein, n = 3, to 1.8 ± 0.2 nmol/min/mg protein, n = 3).

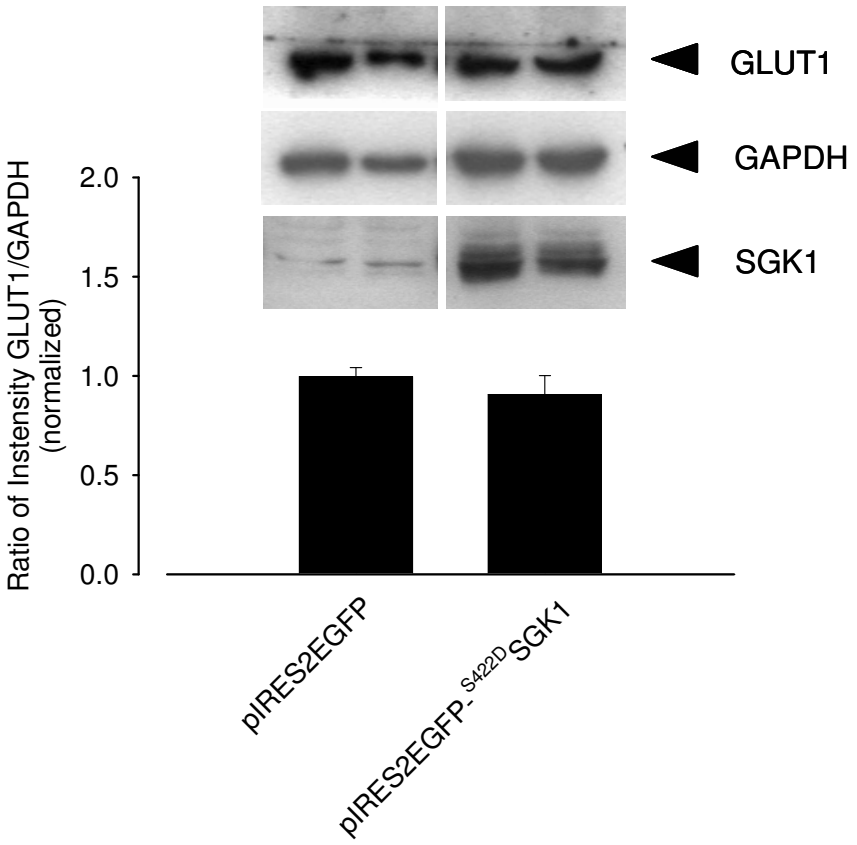

Figure 16. Western blots of whole cell lysates were performed . Arithmetic means ± SEM. * indicates statistically significant difference to uptake in HEK-293 cells transfected with the empty vector (*p < 0.05).

Western blotting of whole cell lysates indicated that the kinase fails to induce GLUT1 protein synthesis (Figure 16). For western blotting, GLUT1/GAPDH band intensities from three independent experiments were normalized in each transfection to the value of GLUT1/GAPDH band intensity of oocytes expressing GLUT1 alone.

4.7 Enhancement of GLUT1 maximal transport rate upon ^{S422D}SGK1 transfection into HEK-293 cells

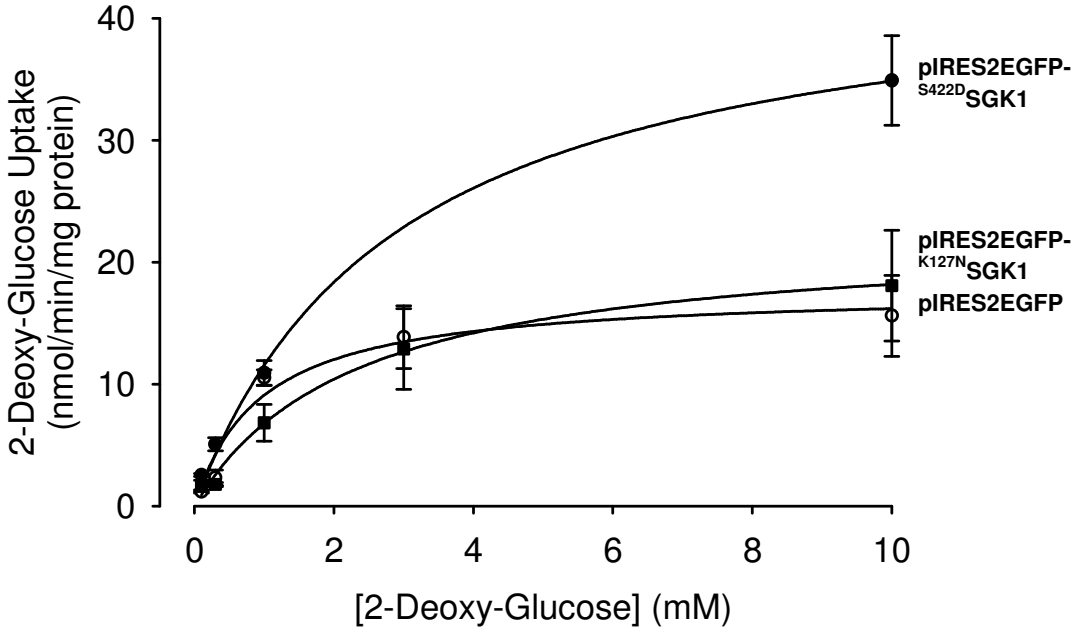

Figure 17. ^{S422D}SGK1 elevates GLUT1 maximal transport rate. HEK-293 cells were transfected with pIRES2EGFP-^{S422D}SGK1, pIRES2EGFP^{K127N}SGK1 or empty vector. 2 days after transfection, labeled 2-DOG uptake was studied at the indicated substrate concentrations. Arithmetic means ± SEM.

Tranfected DNA	K_m (mmol/l)	V_{max} (nmol/min/mg protein)
pIRES2EGFP	0.95±0.44	17.74±2.31
pIRES2EGFP-^{S422D}SGK1	2.89±0.85	44.93±4.40(P<0.05)
pIRES2EGFP-^{K127N}SGK1	2.30±1.38	22.37±4.81

Table 4. Kinetic parameters of GLUT1 upon transfection of pIRES2EGFP-^{S422D}SGK1 pIRES2EGFP-^{K127N}SGK1, or empty vector into HEK -293 cells.

Kinetic analysis of 2-DOG uptake in transfected HEK-293 cells revealed that ^{S422D}SGK1 enhances GLUT1 by modifying the maximal transport rate, *Vmax* (from 17.7 ± 2.3 nmol/min/mg protein, n = 3, to 44.9 ± 4.4 nmol/min/mg protein, n = 3) thereby corroborating the results obtained in the *Xenopus* oocyte expression system (Figure 17 and Table 4).

4.8 GLUT1 mediated glucose transport is reduced in adipocytes isolated from SGK1 knockout mice

Tracer flux studies in adipocytes isolated from *sgk1+/+* and *sgk1-/-* mice demonstrated the role of SGK1 in glucose transport regulation. As depicted in Figure 18A, 2-DOG uptake was lower (55.67 ± 16.46 %) in adipocytes isolated from *sgk1-/-* mice compared to *sgk1+/+* mice despite similar total GLUT1 expression levels (Figure 18B).

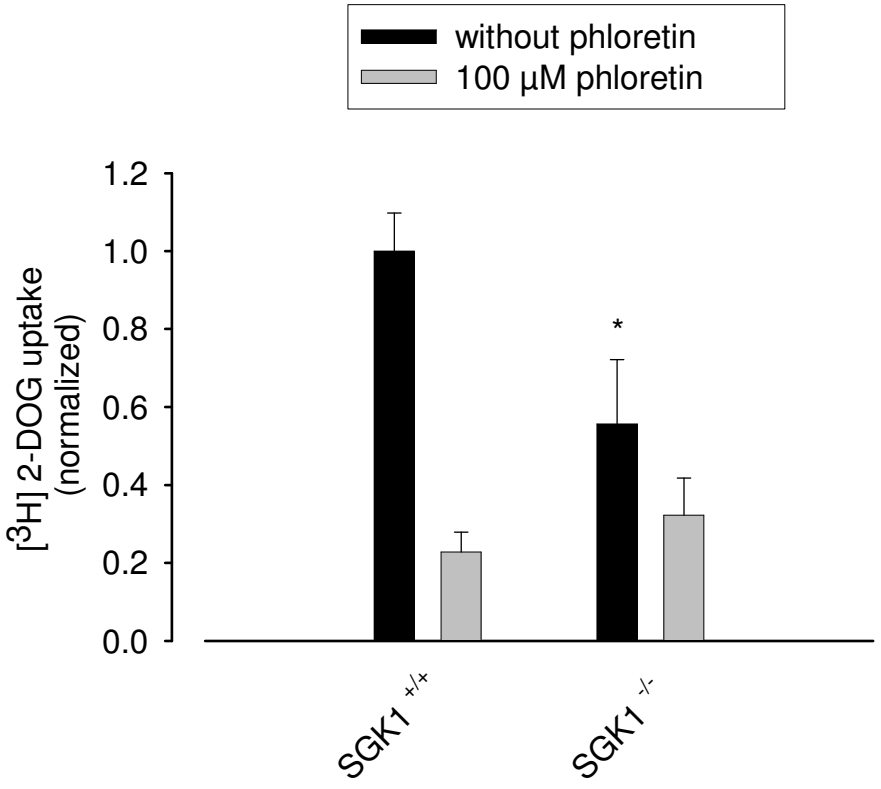

Figure 18A. Glucose transport is reduced in adipocytes isolated from *sgk1-/-* mice
Adipocytes were isolated from *sgk1+/+* and *sgk1-/-* mice and 2-DOG uptake studied in the presence and absence of 0.1 mM phloretin (in A) or western blots of whole cell lysates were performed (in B). Arithmetic means ± SEM. * indicates statistically 25 significant difference to uptake in adipocytes isolated from *sgk1+/+* mice (*$p < 0.05$).

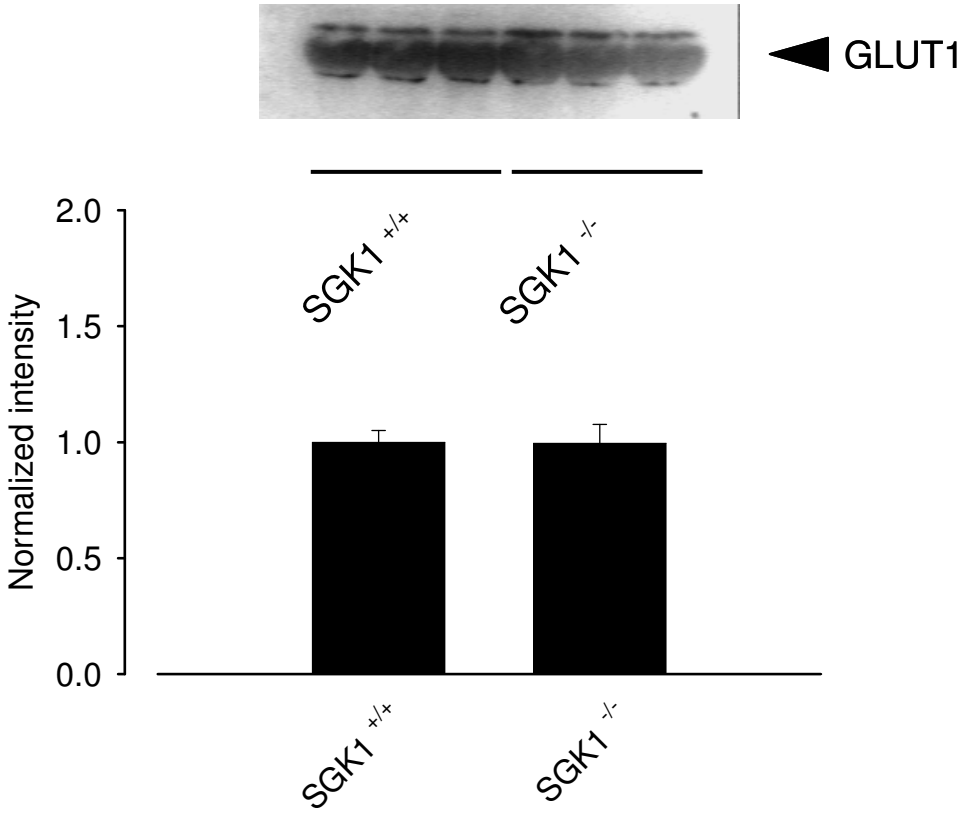

Figure 18B. Glucose transport is reduced in adipocytes isolated from *sgk1-/-* mice
Adipocytes were isolated from *sgk1+/+* and *sgk1-/-* mice and 2-DOG uptake studied in the presence and absence of 0.1 mM phloretin (in A) or western blots of whole cell lysates were performed (in B). Arithmetic means ± SEM. * indicates statistically 25 significant difference to uptake in adipocytes isolated from *sgk1+/+* mice (*$p < 0.05$). For western blotting, GLUT1 band intensities from three independent experiments were normalized to the value of GLUT1 intensity in adipocytes isolated from *sgk1+/+* mice.

5. Discussion

Insulin-induced translocation of GLUT4 and GLUT1 to the plasma membrane requires the activity of phosphatidylinositol 3-kinase. The phosphoinositide signal is transmitted through PDK1 and its downstream targets PKB and atypical PKCs (118-122). The role of these kinases in regulating the gain in GLUT4 surface abundance has been supported by the use of dominant-negative mutants {Bandyopadhyay, 2000 171 /id;Cong, 1997 4502 /id}. Although PKB and atypical PKCs are involved in the insulin-stimulated GLUT4 translocation they do not fully account for the recruitment of GLUT4 to the plasma membrane (123). Thus, additional signalling pathways may participate through PI3K activation. The role of PI-3 kinase in insulin-dependent and insulin-independent stimulation of GLUT1 and GLUT4 translocation has been confirmed by several studies using pharmacological (wortmannin) inhibitin and genetic knockout of the kinase. The effect of PI-3 kinase on GLUT4 trafficking is mediated through PKB. PKB is, however, atleast in some cells, not required for the PI-3 dependent signaling of GLUT1. Thus some other PI-3 dependent protein kinases are presumably involved in the regulation of GLUT1 and insulin mediated GLUT4 signaling.

SGK1 is a further downstream molecule in the PI3K signalling cascade that regulates cell surface expression of several metabolite transporters and ion channels.

In the present study we demonstrate that SGK1 induces the activity of GLUT4 heterologously expressed in *Xenopus* oocytes. Like its close relative PKB, SGK1 stimulates GLUT4-mediated transport by augmenting GLUT4 abundance in the plasma membrane. Adipocytes express GLUT1 and GLUT4 (124). Whereas GLUT4 is present in intracellular compartments in unstimulated adipocytes, GLUT1 is mainly located in the plasma membrane. The differential targeting of mammalian GLUT1 and GLUT4 is maintained when these proteins are expressed in *Xenopus* oocytes (125) which suggests that the cellular apparatus controlling the intracellular targeting of GLUT4 is conserved in *Xenopus* oocytes and that oocytes are a good expression system to elucidate the mechanism of GLUT4 targeting. Other investigators have examined GLUT4-mediated transport in oocytes to evaluate the effect of insulin and IGF-I on glucose uptake and glucose transporter distribution (126;127). Additional advantages of oocytes are that specific GLUT transporters can be tested individually and that endogenous glucose transport is negligibly low. Furthermore exogenous SGK1 expression did not significantly increase intrinsic glucose

uptake in control oocytes demonstrating that it is not effective through activation of endogenous transporters.

In theory, SGK1 might increase GLUT4 activity by promoting GLUT4 synthesis. However, the stimulation of glucose transport in *Xenopus* oocytes in which GLUT4 cRNA has been injected points to posttranscriptional regulation of GLUT4. Enhanced GLUT4 activity is also not due to the result of increased GLUT4 translation (Figure 2A, B).

The kinase exerts its effects at least in part by phosphorylating GLUT4 directly as indicated by our coexpression studies. A direct GLUT4 phosphorylation by PKCζ has been demonstrated in GLUT4-containing vesicles purified from low density microsomes of rat adipocytes. GLUT4 phosphorylation was increased in insulin treated cells suggesting that transporter phosphorylation might be required in GLUT4 translocation mechanism in adipocytes. Although SGK1 and PKB recognize the same phosphorylation site on their target proteins, there is no evidence that PKB phosphorylates GLUT4 directly. This could be explained by the fact that residues neighbouring the putative phosphorylation site can influence the phosphorylation efficiency by the kinases (128). ^{274}Ser in the GLUT4 sequence is followed by a leucine. Kobayashi et al. (128) demonstrated by *in vitro* kinase assays that SGK1 phosphorylates targets with a leucine residue next to the phosphorylation site more efficiently than PKB. Our observations on the ability of PKB to stimulate the phosphorylation-deficient GLUT4 mutant (^{S274A}GLUT4) support the notion that PKB effects do not require the putative phosphorylation site on GLUT4. SGK1-dependent stimulation of GLUT4 transporters may be of relevance in the cellular uptake of glucose into muscle and adipose cells. In fact SGK1 dependent regulation of glucose transport is reflected by delayed glucose uptake into skeletal muscle and fat tissue in the *sgk1* null mouse following an intraperitoneal glucose tolerance test. It has been already shown that SGK1 upregulates the facilitative glucose transporter GLUT1 in its constitutive form by using *Xenopus* oocytes model system. On the other hand, the kinase dead SGK1 does not upregulate the activity of GLUT1 which suggests that the catalytical activity of the kinase is required for the upregulatory mechanism.

Phosphorylation dependent events have previously been shown to modulate GLUT1 activity. Protein kinase C isozymes stimulate the activity of GLUT1 by recruiting the transporter to the cell surface. The kinase stimulates GLUT1 by enhancing the transporter plasma membrane abundance through phosphorylation of an unknown intermediate protein.

According to our results, SGK1 could play a similar role in the regulation of GLUT1 as PKB plays in the regulation of GLUT4. In some studies on adipocytes, constitutively active PKB has been able to upregulate GLUT4 but not GLUT1 (129). Insulin, however, upregulated both GLUT1 and GLUT4, and both effects were abrogated by inhibition of PI3 kinase (129) . On the other hand, dominant inhibitory PKB has been shown to inhibit the insulin-mediated increase in GLUT1 protein abundance (130) , and PKB has been reported to stimulate GLUT1 translocation to the cell membrane (131), suggesting a role of PKB in the regulation of GLUT1. Moreover, insulin may (129;129) or may not (132) stimulate GLUT1. Thus, the regulation of GLUT1 may depend on the experimental condition and may be supported by PKB and/or SGK1. It should be kept in mind that the transcription of SGK1 is highly variable and subject to a number of regulators (81;132) , and thus SGK1-dependent effects may be similarly variable. GLUT1 modulation by SGK1 in cultured mammalian cells and adipocytes isolated from *sgk1*$^{-/-}$ mice points to a physiological role of the kinase in glucose transport regulation.

Facilitated GLUT1 transporter deficiency syndrome defines a prototype of a novel group of disorders resulting from impaired glucose transport across blood barriers. It is caused by the defect of glucose transport into the brain, mediated by the glucose transporter GLUT1.The hallmark of this disease is a low glucose concentration in the CSF. Clinical features are variable and include seizures, developmental delay, acquired microcephaly, hypotonia, dystonia and spasticity. Increased expression of this gene has been shown in the rat cortex at 24 hours after focal cerebral ischemia. Notably, SGK1 expression is upregulated by cerebral ischemia and may help to enhance cellular glucose uptake into the ischemic tissue. Moreover, the enhanced cerebral SGK1 expression in enriched environment my serve to adjust glucose uptake into several tissues in the SGK1 knockout mouse following an intraperitoneal glucose tolerance test.

The SGK1-dependent regulation of GLUT1 may participate in the adjustment of cellular glucose uptake to the demand. Similarly to GLUT4, SGK1 activates GLUT1 by promoting the transporter´s cell surface expression without impacting the total GLUT1 protein abundance. In contrast to GLUT4, the putative SGK1 phosphorylation site on GLUT1 is not essential for transport modulation by the kinase.

SGK1-dependent stimulation of GLUT4 transporters may be of relevance in the cellular uptake of glucose into muscle and adipose cells. In fact SGK1 dependent regulation of

glucose transport is reflected by delayed glucose uptake into skeletal muscle and fat tissue in the *sgk1* null mouse following an intraperitoneal glucose tolerance test .

In conclusion, SGK1 stimulates glucose transport by enhancing the abundance of the facilitative glucose transporter GLUT4 in the cell membrane at least in part via direct GLUT4 phosphorylation. Whereas in the case of GLUT1, it enhances the transporter in the plasma membrane through phosphorylation of a hitherto unknown intermediate protein. The SGk1 dependent regulation of GLUT1 may participate in the adjustment of cellular glucose uptake to the demand. The observations disclose a novel PI3K target participating in the regulation of glucose transport.

6. References

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Curriculum Vitae

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Academic training

February 2004-October 2007	**PhD student** "Regulation of metabolite transport by the serum and glucocorticoid induced kinases SGK " Prof. Florian Lang. Eberhard-Karls University of Tuebingen, Germany,
2003	**Project Assistant** Molecular Diversity and Drug Design: Drug targeting of Fab Z involved in the Fatty Acid Biosyntheis of Malarial Parasite *Plasmodium falciparum* Prof. K.Suguna, IISC, India.
2002	**Project Assistant** "A new insecticidal transgenic *Cicer arietinum* colonizer" Prof. Vaithilingam Sekar's lab, Molecular Microbiology Department, School of Biotechnology, Madurai Kamaraj University, India.
2002	**M. Sc.** Microbiology, Bharathidasan University, Trichy, Tamilnadu, India. "16S rDNA sequencing for the identification of unculturable microorganisms"

April 1999 **B. Sc.**
Microbiology. Madurai Kamaraj University
Madurai, Tamilnadu, India.

Methodological expertise

- **Molecular Biology / Biochemistry**

Heterologous protein expression in *Xenopus laevis* oocytes

Site-directed mutagenesis

Isolation of DNA/RNA from bacteria

DNA sequencing

cDNA synthesis

RT-PCR

Isolation of pancreatic beta cells from mice

Transfection of Insulin secreting cell lines

Cell surface expression analysis of membrane proteins by chemiluminescence

Detection of protein phosphorylation by immunoprecipitation / immunoblotting

Co-immunoprecipitation for protein interactions

Protein purification by chromatography

- **Functional Methods**

Animal care and surgery of *Xenopus laevis* oocytes

Heterologous expression and functional characterization of recombinant proteins in *Xenopus laevis* oocytes

Radiolabeled tracer flux measurements

Two electrode voltage-clamp

Tranining in Patch clamping in beta cells and INS-1 cell lines

- **Basic microbiological techniques**

Publications (peer review)

1 **Jeyaraj S**, Bohmer C, Lang F, Palmada M; Role of SGK1 kinase in regulating glucose transport via glucose transporter GLUT4. Biochem. Biophys. Res. Commun. 356(3):629-35, 2007

2 Palmada M, Boehmer C, Akel A, Rajamanickam J, **Jeyaraj S**, Keller K, Lang F; SGK1 kinase upregulates GLUT1 activity and plasma membrane expression. Diabetes 55 4(2):421-7, 2006

3 Palmada M, Speil A, **Jeyaraj S**, Bohmer C, Lang F; The serine/threonine kinases SGK1, 3 and PKB stimulate the amino acid transporter ASCT2. Biochem. Biophys. Res. Commun. 331(1):272-7, 2005

4 Paul J, **Sankarganesh Jeyaraj**, Huber SM, Seebohm G, Böhmer C, Lang F, Kremsner P, and Kun JFJ Alterations in the cytoplasmic domain of CLCN2 result in altered gating kinetics. Accepted in Cell. Physiol. Biochem

5 Modulation of the volgate-gated potassium channel Kv 1.5 by SGK1 Kinase involves inhibition of channel ubiquitination (Manuscript in preparation).

Awards and Scholarships

1999 **"Best student award"** for Under Graduate studies in Madurai Kamaraj University, Madurai, India.

2000-2002 "**National Merit Scholarship"** was awarded for the Post Graduate programme by the Government of India during 2000-2002.

Seminars and conferences

April 2007: **Oral talk** on "Mechanisms involved in SGK1 dependent regulation of glucose transporter GLUT4" German Physiological Society Congress 2007 in Hannover, Germany.

March 2006: **Poster presentation** in the The Federation of European Physiological Societies and German Society of Physiology 2006 in Munich, Germany.

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