# Posttranslationale Regulation des Glucosesensorenzyms Glucokinase durch Interaktion mit dem bifunktionellen Enzym 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase

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## Kurzzusammenfassung

Das niedrigaffine glucosephosphorylierende Enzym Glucokinase vermittelt die Anpassung der zellulären glykolytischen Aktivität an die extrazelluläre Glucosekonzentration. Damit übernimmt die Glucokinase eine Schlüsselfunktion bei der glucose-induzierten Insulinsekretion in den β-Zellen des Pankreas wie auch im Glucosemetabolismus der Leber. Für die flexible Anpassung der Glucokinaseaktivität mit dem Ziel der Aufrechterhaltung der Blutglucosehomöostase sind posttranslationale Regulationsprozesse von besonderer Bedeutung. Das Glucokinase Regulatorprotein der Hepatocyten wurde bereits vor zwei Jahrzehnten als Bindungspartner der Glucokinase entdeckt. Durch die Interaktion wird die Glucokinase Enzymaktivität kompetitiv gehemmt und die Translokation des Enzyms in den Nukleus bei niedrigen Glucosekonzentrationen vermittelt. Vor einigen Jahren konnte mit dem bifunktionellen Enzym 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase, das in verschiedenen Isoformen in allen Geweben exprimiert wird und als Homodimer vorliegt, ein die Glucokinase aktivierender Interaktionspartner identifiziert werden. In der vorliegenden Arbeit konnte gezeigt werden, dass bei gleichzeitiger Überexpression der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase in Glucokinase überexprimierenden insulinproduzierenden Zellen die ATP/ADP Ratio erhöht und in der Folge die Insulinsekretion gesteigert wird. Weiterhin konnte die Steigerung der Glucokinase Enzymaktivität durch die Interaktion mit der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase mit aufgereinigten, rekombinanten Proteinen reproduziert werden. Die Beteiligung bislang unbekannter intrazellulärer Komponenten an der Komplexbildung kann dadurch nicht ausgeschlossen werden, ist jedoch nicht essentiell. Eine entscheidende Rolle bei der Modulation der Bindung der Glucokinase an die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase konnte für das Substrat Glucose nachgewiesen werden. Es konnte intrazellulär gezeigt werden, dass eine Erhöhung der Glucosekonzentration mit einer Erhöhung der Bindungsstärke der Interaktion einhergeht. Hierzu wurde ein fluoreszenzbasiertes *Mammalian Two-Hybrid* System etabliert, mit dem eine quantitative Analyse von Protein-Protein-Interaktionen in Säugetierzellen möglich ist. Mit diesem System konnte zudem für das Leberisoenzym der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase eine Abhängigkeit der Dimerisierung vom Phosphorylierungsstatus nachgewiesen werden. Die durchgeführten Studien belegen die physiologische Relevanz der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase als endogener Aktivator der Glucokinase und liefern somit neue Ansatzpunkte für die antihyperglykämische Therapie des Typ 2 Diabetes mellitus.

Schlagwörter: Diabetes mellitus, Glucokinase, 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase oder PFK-2/FBPase-2

## Abstract

The low-affinity glucose phosphorylating enzyme glucokinase mediates the adaptation of the glycolytic flux to the extracellular glucose concentration. Thus, glucokinase plays a key role during glucose-stimulated insulin secretion in pancreatic β-cells as well as in the carbohydrate metabolism of the liver. For maintaining glucose homeostasis posttranslational mechanisms are of particular importance for the precise regulation of glucokinase enzyme activity. The glucokinase regulatory protein of the hepatocytes has already been discovered as a glucokinase binding partner two decades ago. The protein competitively inhibits glucokinase enzyme activity and mediates the translocation of glucokinase into the nucleus at low glucose concentrations. Recently, the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase was identified as an activating glucokinase binding protein. This regulatory enzyme of glycolysis exists as a homodimer and is expressed in different isoforms in virtually every eukaryotic cell. The present work revealed that 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase overexpression in glucokinase expressing insulin-producing cells induces an increase in the cellular ATP/ADP ratio and subsequently in insulin secretion. Furthermore, the observed increase in glucokinase enzyme activity by co-overexpression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase could also be demonstrated using purified, recombinant proteins. Hitherto unidentified intracellular components might be involved in complex formation, however, they are not mandatory. An important role in the modulation of the interaction between glucokinase and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase could be attributed to the substrate glucose. An increase in the glucose concentration was accompanied by an increase in binding strength of the interaction. A fluorescence-based mammalian two-hybrid system was established for quantitative analysis of protein-protein interactions in cultured mammalian cells. The system proved to be capable to reveal glucose-dependent binding of glucokinase to 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Moreover, dimerization of the liver isoform of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase could be shown to be dependent on the serine-32 phosphorylation state of the enzyme. Thus, the present studies provide new knowledge upon the physiological relevance of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase as an endogenous glucokinase activator and may open the perspective of therapeutic targeting of this interaction in type 2 diabetes treatment.

Keywords: diabetes mellitus, glucokinase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase or PFK-2/FBPase-2

# Erklärung zur Dissertation

gemäß §6(1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität Hannover

für die Promotion zum Dr. rer. nat.

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel **„Posttranslationale Regulation des Glucosesensorenzyms Glucokinase durch Interaktion mit dem bifunktionellen Enzym 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase“** selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Hannover, den 16.12.2009

Sara Langer

Die vorliegende Dissertation wurde im September 2004 unter Anleitung von Herrn Prof. Dr. S. Lenzen am Institut für Klinische Biochemie der Medizinischen Hochschule Hannover begonnen und im Dezember 2009 abgeschlossen.

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Teilergebnisse der vorliegenden Dissertation sind bereits veröffentlicht.

Kurzbeiträge:

**Langer S., Baltrusch S., Smith W.E., Okar D.A. (2006).** Glucokinase and PFK-2/FBPase-2 interact to fulfil crucial roles in glucose induced insulin secretion in pancreatic beta cells and in glucose disposal in liver. *Diabetologia* **49**:[Suppl1]128, Europäischer Diabetes-Kongress, Kopenhagen, September 2006.

**Langer S., Okar D.A., Lenzen S., Baltrusch S. (2007).** Neue Aspekte der durch die PFK-2/FBPase-2 vermittelten Aktivierung der Glucokinase. *Diabetologie und Stoffwechsel* **2**: S 1, P342, Deutscher Diabetes-Kongress, Hamburg, Mai 2007.

**Langer S., Lenzen S., Baltrusch S. (2007).** Glucose-dependent interaction of glucokinase and PFK-2/FBPase-2 in insulin-producing cells. *Diabetologia* **50**:[Suppl1]S219, Europäischer Diabetes-Kongress, Amsterdam, September 2007.

**Langer S., Lenzen S., Baltrusch S. (2008).** Bedeutung der glucoseabhängigen Interaktion von Glucokinase und PFK-2/FBPase-2 in insulinproduzierenden Zellen. *Diabetologie und Stoffwechsel* **3**: S 1, A79, Deutscher Diabetes-Kongress, München, April/Mai 2008.

**Langer S., Kaminski M.T., Lenzen S., Baltrusch S. (2009).** Regulation of hepatic glucose homeostasis by glucokinase localization. *Diabetologia* **52**:[Suppl1]S239, Europäischer Diabetes-Kongress, Wien, September/Oktober 2009.

Veröffentlichungen:

**Baltrusch S., Langer S., Massa L., Tiedge M., Lenzen S. (2006).** Improved metabolic stimulus for glucose-induced insulin secretion through GK and PFK-2/FBPase-2 coexpression in insulin-producing RINm5F cells. *Endocrinology* **147**:5768-5776

**Smith W.E., Langer S., Wu C., Baltrusch S. und Okar D.A. (2007)**. Molecular coordination of hepatic glucose metabolism by the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase:glucokinase complex. *Molecular Endocrinology* **21(6)**:1478-1487

# Inhaltsverzeichnis

# 1. Einleitung

## 1.1. Glucosehomöostase

Die Blutglucosekonzentration bewegt sich beim gesunden Menschen in relativ engen Grenzen. Dabei wird die Erhaltung der Glucosehomöostase, also eines konstanten Blutglucosespiegels, vor allem durch hormonelle Regulationsmechanismen gewährleistet. Das wichtigste Kontrollelement des Energiestoffwechsels in der postresorptiven Phase ist das Peptidhormon Insulin, welches von den β-Zellen der Langerhans'schen Inseln des Pankreas sezerniert wird. Generell stimuliert Insulin anabole Stoffwechselwege. Es fördert in Muskulatur und Fettgewebe die Aufnahme von Glucose aus dem Blut über das Glucosetransportprotein GLUT4. In der Leber und der Muskulatur wird die Glykogensynthese stimuliert. Auch erhöht Insulin in der Leber die Glykolyserate, was wiederum die Fettsäuresynthese fördert. In der Fettzelle wird die Speicherung von Triacylglyceriden ausgelöst. Auf die Proteinsynthese hat Insulin einen allgemein stimulierenden Effekt, wobei es zusätzlich den intrazellulären Proteinabbau hemmt. Bei der Entfernung größerer Glucosemengen aus dem Blut kommt der Leber aufgrund ihrer hohen Glucosephosphorylierungskapazität eine besondere Rolle zu. Bei einem niedrigen Blutglucosespiegel sezernieren die α-Zellen des Pankreas das Peptidhormon Glucagon. Die Leber ist das Zielorgan des Glucagons. Dort stimuliert es den Glykogenabbau und die Gluconeogenese, so dass große Mengen Glucose von der Leber ans Blut abgegeben werden können. Gleichzeitig werden Glykogensynthese, Glykolyse und Fettsäuresynthese gehemmt. Zur Aufrechterhaltung der Blutglucosehomöostase tragen in der postabsorptiven Phase auch Muskulatur und Fettgewebe durch verminderte Glucoseverwertung bei.

## 1.2. Diabetes Mellitus

Der Diabetes mellitus ist eine Stoffwechselerkrankung, bei der eine Dysregulation des Glucosestoffwechsels zur Hyperglykämie führt. Die WHO definiert den Diabetes mellitus über eine Plasmaglucosekonzentration von nüchtern über 125 mg/dl (7 mmol/l) oder zwei Stunden nach oraler Glucosegabe über 200 mg/dl (11,1 mmol/l). Ab einer Blutglucose-

konzentration von über 180 mg/dl (10 mmol/l) kommt es nach Überschreiten der Nierenschwelle zur Glucosurie. Ursache für die Hyperglykämie ist ein absoluter (Typ 1 Diabetes mellitus) oder ein relativer (Typ 2 Diabetes mellitus) Insulinmangel. In den Industrienationen leiden inzwischen etwa 5 % der Bevölkerung an Diabetes mellitus, davon der weitaus überwiegende Anteil (90 %) an Diabetes mellitus Typ 2. Beim Diabetes mellitus Typ 1, der sich zumeist im Kindes- und Jugendalter manifestiert, resultiert der Insulinmangel aus einer autoimmunen Destruktion der insulinproduzierenden β-Zellen des Pankreas. Ein Diabetes mellitus Typ 2 entsteht aufgrund von Insulinsekretionsdefekten der β-Zellen und/oder aufgrund einer Insulinresistenz der Zielzellen. Der Diabetes mellitus Typ 2 tritt gewöhnlich erst im höheren Alter auf und ist primär nicht insulinpflichtig. Bei beiden Formen gibt es eine genetische Prädisposition, die gerade beim Diabetes mellitus Typ 2 für die Pathogenese eine wichtige Rolle spielt.

## *1.3. Glucoseinduzierte Insulinsekretion*

Insulin ist ein Peptidhormon, bestehend aus 51 Aminosäuren in zwei über Disulfidbrücken verknüpften Ketten. Es wird von den β-Zellen der Langerhans'schen Inseln des Pankreas synthetisiert, in Insulingranula gespeichert und bei Stimulation ausgeschüttet. Wie bei Sekretproteinen üblich, wird zunächst eine Vorstufe des Hormons gebildet (Präproinsulin), aus der im Endoplasmatischen Retikulum durch Abspaltung des Signalpeptids und Ausbildung der Disulfidbrücken das Proinsulin entsteht. Dieses wird im Golgi-Apparat in Granula verpackt, wobei es sich zu zink- und calciumhaltigen Hexameren zusammenlagert. In den Granula entsteht schließlich durch proteolytische Abspaltung des zwischen A- und B-Kette gelegenen C-Peptids das reife Insulin (Davidson 1988). Der wichtigste physiologische Stimulus für die Auslösung der Insulinsekretion ist der Anstieg der Blutglucosekonzentration nach Nahrungsaufnahme. Die ungestörte Stimulus-Sekretionskopplung in den β-Zellen ist Voraussetzung für die Aufrechterhaltung der Glucosehomöostase. Im Gegensatz zu anderen endokrinen Zelltypen entsteht das Signal für die glucoseinduzierte Insulinsekretion dabei im Metabolismus der β-Zelle (Lenzen 1990; Lenzen 1992; Lenzen & Panten 1988; Matschinsky 2002). Die β-Zellen nehmen, wie auch die Hepatocyten, die Glucose aus dem Blut über erleichterte Diffusion mit Hilfe des niedrigaffinen GLUT2 Glucosetransportproteins auf (Bell 1990; Kayano 1990; Lenzen & Tiedge 1994). Nach dem Eintritt in die Zelle erfolgt als erster Reaktionsschritt die Phosphorylierung der Glucose durch das Enzym Glucokinase. Die Reaktion stellt den geschwindigkeitsbestimmenden Schritt im Glucosemetabolismus der β-Zelle

dar und reguliert somit den Glucoseumsatz (Efrat 1994; Iynedjian 1993; Lenzen 1992; Lenzen & Panten 1988; Matschinsky 2002). Aus dem Glucoseabbau in Glykolyse, Citratzyklus und Atmungskette folgt die Erhöhung des intrazellulären ATP/ADP-Quotienten, was in der β-Zelle die Schließung eines ATP-sensitiven $K^+$-Kanals zur Folge hat. Der $K^+$-Kanal wird aus dem Kanalprotein KIR 6.2 und dem Sulfonylharnstoffrezeptor SUR 1, an dem sich die ATP-Bindungsstelle befindet, gebildet (Aguilar-Bryan 1998; Panten 1996). Die Schließung des $K^+$-Kanals führt zur Depolarisation der β-Zell-Plasmamembran, was wiederum die Öffnung von spannungsabhängigen $Ca^{2+}$-Kanälen zur Folge hat. Durch den $Ca^{2+}$-Einstrom wird schließlich die Exocytose der Insulingranula induziert (Abb. 1.1).

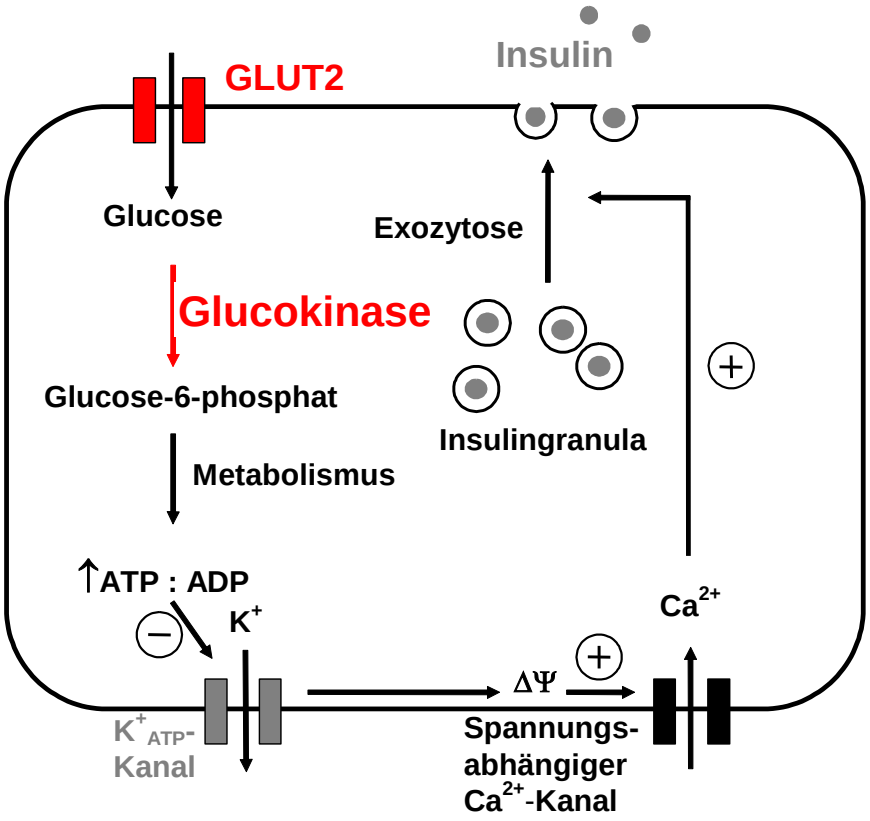


**Abb. 1.1 Modell des Mechanismus der glucoseinduzierten Insulinsekretion in den β-Zellen des Pankreas.**

## *1.4. Das Glucosesensorenzym Glucokinase*

Das niedrigaffine glucosephosphorylierende Enzym Glucokinase (Hexokinase Typ IV; EC 2.7.1.1) nimmt im komplexen Netzwerk zur Erhaltung der Blutglucosehomöostase eine zentrale Stellung ein. Der Leber ermöglicht die Expression der Glucokinase, bei steigender Blutglucosekonzentration große Mengen Glucose aufzunehmen, zu verstoffwechseln und

schließlich als Glykogen zu speichern. In den β-Zellen des Pankreas ist die Phosphorylierung der Glucose durch das Enzym Glucokinase der geschwindigkeitsbestimmende Schritt des Glucosemetabolismus und damit der glucoseinduzierten Insulinsekretion. Die Glucokinase gehört zur Familie der Hexokinasen, welche den Transfer der γ-Phosphatgruppe von ATP auf die 6-Hydroxylgruppe einer Hexose katalysieren. Die Hexokinasen liegen als Monomer vor. Die Gruppe der Hexokinasen Typ I-III hat ein Molekulargewicht von ca. 100 kDa. Sie zeigen eine hyperbole Enzymkinetik mit einer hohen Affinität zu dem Substrat Glucose mit $K_m$-Werten zwischen 20 und 130 µmol/l. Bei physiologischen Konzentrationen von Glucose-6-phosphat tritt eine Produkthemmung auf (Middleton 1990). Mit einem Molekulargewicht von etwa 55 kDa ist die Glucokinase ungefähr halb so groß wie die übrigen Hexokinasen und weist mit einem $S_{0,5}$-Wert um 8 mmol/l eine deutlich geringere Affinität für das Substrat Glucose auf (Iynedjian 1993; Lenzen 1990; Lenzen 1992; Matschinsky 1990; Printz 1993). Desweiteren zeigt die Glucokinase im Gegensatz zu den anderen Hexokinasen keine Produkthemmung durch physiologische Konzentrationen an Glucose-6-phosphat (Lenzen 1992) und eine kooperative sigmoidale Reaktionskinetik für das Substrat Glucose mit einem Hill-Koeffizienten von etwa 1,7 (Lenzen 1992; Printz et al. 1993; Tiedge 1997). Der Umkehrpunkt der sigmoidalen Aktivitätskurve liegt im Bereich von 5 mmol/l Glucose und damit nahe an der Schwelle für die Auslösung der glucoseinduzierten Insulinsekretion der β-Zelle. Jede Steigerung der Blutglucosekonzentration in diesem Bereich führt zu einer signifikanten Zunahme der Glucosephosphorylierung durch die Glucokinase und somit in den β-Zellen zur Insulinsekretion. Die beschriebenen Eigenschaften machen die Glucokinase zu einem idealen Kopplungsglied zwischen extrazellulären millimolaren Glucosekonzentrationen und den entsprechenden Fluxänderungen im Glucosestoffwechsel der β-Zelle.

## *1.5. Das Glucokinasegen*

Die Expression der Glucokinase erfolgt in den β-Zellen des Pankreas und in der Leber (Magnuson 1990), aber auch in α- und δ-Zellen des Pankreas, in enteroendokrinen Zellen des Magens und des Dünndarms, in speziellen Neuronen des Hypothalamus, in Epithelzellen der Atemwege sowie in der Hypophyse (Jetton 1994; Matschinsky 2009). Die Transkription der Glucokinase-mRNA erfolgt von einem Glucokinasegen, das aus zehn Exons und zwei gewebespezifischen Kontrollregionen besteht. Exon 2-10 werden in den verschiedenen Geweben identisch transkribiert. Die Kontrollregionen enthalten neben den Promotoren jeweils das erste Exon, wobei das neuroendokrine Promotorelement stromaufwärts und das

leberspezifische Promotorelement stromabwärts gelegen ist (Magnuson 1990; Magnuson 1989; Magnuson & Shelton 1989). Der primäre Regulator für den leberspezifischen Promotor ist Insulin (Iynedjian 1988). Der neuroendokrine Promotor unterliegt einer komplexen Regulation, wobei in pankreatischen β-Zellen Glucose der wesentliche Induktor der Glucokinase ist (Lenzen & Tiedge 1994; Matschinsky 2002).

## *1.6. Die Glucokinaseproteinstruktur*

Die Isoenzyme der humanen Glucokinase in der Leber und in den β-Zellen des Pankreas bestehen jeweils aus 465 Aminosäuren. Sie unterscheiden sich nur am N-Terminus in 11 der letzten 15 Aminosäuren (Andreone 1989; Magnuson & Shelton 1989). Beide Isoenzyme können in entsprechenden *Western Blot* Analysen des Cytosols als singuläre Bande von ca. 55 kDa detektiert werden (Iynedjian 1986). Das Expressionsniveau der Glucokinase ist sehr niedrig. In der Leber hat die Glucokinase einen Anteil von nur 0,1 % der Gesamtmasse aller löslichen Proteine und wird in der pankreatischen β-Zelle noch einmal um den Faktor 20 schwächer exprimiert (Iynedjian et al. 1986). In *E. coli* Bakterien konnte die Glucokinase rekombinant exprimiert und für funktionelle Studien eingesetzt werden (Gidh-Jain 1993; Pilkis 1994; Takeda 1993; Tiedge et al. 1997). Trotzdem gelang es erst vor wenigen Jahren, zwei N-terminal deletierte Formen des humanen Leberisoenzyms der Glucokinase zu kristallisieren und für Röntgenstrukturanalysen einzusetzen (Kamata 2004) (Abb. 1.2). Wie bereits zuvor in einem Strukturmodell, das aus den Röntgenstrukturdaten der zu 31 % mit der Glucokinase identischen Hexokinase B der Hefe abgeleitet worden war (Gidh-Jain et al. 1993; Pilkis et al. 1994; St Charles 1994; Takeda et al. 1993; Xu 1995; Xu 1995; Xu 1994), angenommen wurde, gliedert sich die humane Glucokinase in zwei Domänen. Zwischen der großen und der kleinen Domäne liegt ein tiefer Spalt, in dem das aktive Zentrum lokalisiert ist. Wie die Röntgenstrukturanalyse zeigte, wird die Bindungsstelle für das Substrat Glucose bei gleichzeitiger Bindung eines allosterischen chemischen Aktivators durch die Aminosäuren Thr-168 und Lys-169 der kleinen Domäne, Asn-204 und Asp-205 der Verbindungsregion II, sowie Glu-256 und Glu-290 der großen Domäne gebildet (Kamata 2004). Damit konnte eine Beteiligung der Aminosäuren Asn-204, Asp-205, Glu-256 und Glu-290 an der Glucosebindungsstelle bestätigt werden, die in früheren Studien aufgrund der Homologie zur Hexokinase B der Hefe postuliert worden war (St Charles et al. 1994). Treten die charakterisierten Aminosäuren mit den Hydroxylgruppen des Glucosemoleküls in Wechselwirkung, wird eine Konformationsänderung der Glucokinase induziert. Sie geht damit in die katalytisch

aktive geschlossene Form (*closed form*) über. Diese durch Substratbindung induzierte Konformationsänderung ist für die Phosphorylierungsaktivität der Glucokinase wie auch der übrigen Hexokinasen essentiell. Tatsächlich führen Mutationen der Aminosäuren Asn-204, Glu-256 und Glu-290 der Glucosebindungsstelle (Xu et al. 1994) zu einer signifikanten Abnahme der Glucoseaffinität und der Glucokinaseaktivität. Die Kristallisierung der katalytisch inaktiven Form der Glucokinase gab deutliche Hinweise darauf, dass die Konformationsänderung der Proteindomänen in Bezug auf die geschlossene Form deutlich größer ist als bei den bekannten Strukturen von Hexokinasen. Daher wurde die katalytisch inaktive Konformation als weit offene Form (*super-open form*) bezeichnet (Kamata et al. 2004) (Abb. 1.3). In kristallographischen Untersuchungen der 100 kDa Hexokinasen wurde eine weit offene Konformation des Enzyms nicht beobachtet. Bemerkenswerterweise ändert sich beim Übergang von der katalytisch aktiven zu der inaktiven Form nicht nur die Lage der großen und der kleinen Domäne zueinander, sondern auch innerhalb der kleinen Domäne kommt es zu deutlichen Umlagerungen. So ist die α13 Helix am C-terminalen Ende des Moleküls in der geschlossenen Form ein integraler Bestandteil der kleinen Domäne, wohingegen sie in der weit offenen Form aus der Domäne entlassen wird und zwischen großer und kleiner Domäne liegt. Weiterhin wurde eine hochflexible Struktur, welche für die Glucokinase spezifisch ist, in der Verbindungsregion I zwischen den Aminosäureresten Ser-64 und Gly-72 beschrieben. Die Verbindungsregion I befindet sich sowohl in der geschlossenen als auch in der weit offenen Form an der Oberfläche des Proteins. Zusammen mit Bereichen der großen (β1 Faltblatt und α5 Helix) und der kleinen Domäne (α13 Helix) bildet die Verbindungsregion I die Bindungsstelle eines synthetischen allosterischen Aktivators der Glucokinase, dessen Bindung bei hohen Konzentrationen (> 30 µmol/l) von der sigmoidalen zu einer hyperbolen Aktivitätskurve der Glucokinase führt. In der weit offenen Form der Glucokinase ist die Bindungsstelle des synthetischen Aktivators aufgrund der Konformationsänderungen nicht vorhanden (Kamata et al. 2004). Mithilfe der aus den Strukturdaten abgeleiteten Konformationen der Glucokinase entwickelten Kamata *et al*. ein kinetisches Modell, das eine Erklärung für die Kooperativität des monomeren Enzyms liefert (Abb. 1.4). Dabei wurde die Existenz einer dritten Konformation postuliert, entsprechend der offenen Konformation der Hexokinasen. Der Übergang von der offenen in die weit offene Konformation und umgekehrt erfolgt nach dem Modell langsam. In Abhängigkeit von der Glucosekonzentration überwiegt dabei der schnelle oder der langsame Katalysezyklus, was in der Summe zu einer sigmoidalen Reaktionskinetik führt.

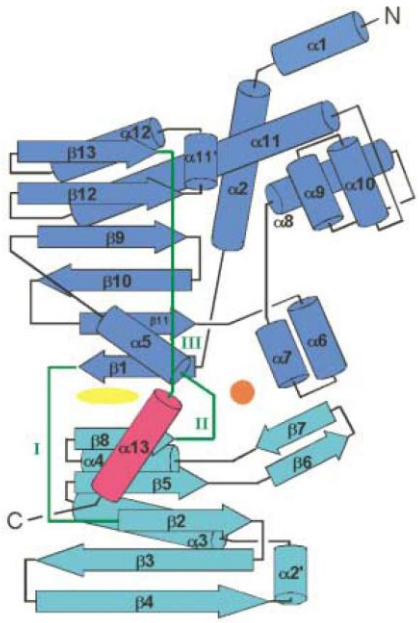


**Abb. 1.2 Schematische Darstellung von Domänenaufbau und Sekundärstruktur der Glucokinase im Komplex mit Glucose und einem synthetischen allosterischen Aktivator.** Die Glucokinase besteht aus der großen Domäne (blau) und der kleinen Domäne (cyan und magenta), die durch die Verbindungsregionen I-III (grün) verbunden sind. Die C-terminale Region der Glucokinase bildet die α13 Helix (magenta) und ist in der enzymatisch aktiven Form der Glucokinase integraler Bestandteil der kleinen Domäne. Die Bindungsstellen für Glucose (roter Kreis) und den allosterischen Aktivator (gelbes Oval) liegen im Bereich zwischen beiden Domänen (Kamata et al. 2004).

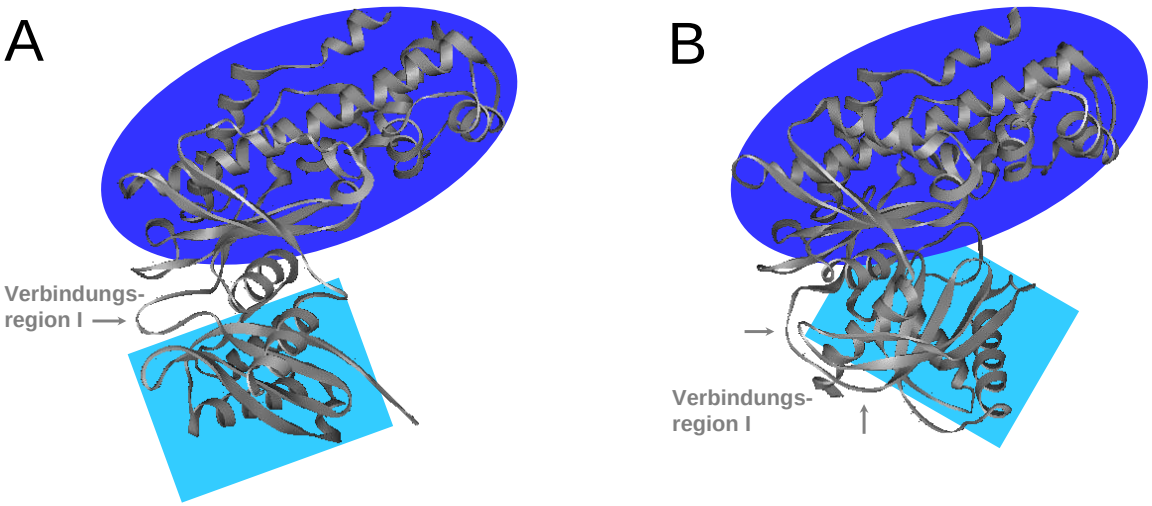


**Abb. 1.3 Strukturmodell der Glucokinase in der weit offenen und der geschlossenen Konformation.** Bändermodelle (A) der weit offenen (*Protein Databank Identification* 1V4T) und (B) der geschlossenen (*Protein Databank Identification* 1V4S) Konformation der Glucokinase mit der großen (blau) und der kleinen Domäne (cyan). Die Abbildung wurde erstellt mit dem *3D Molecule Viewer* (Invitrogen) (Baltrusch & Tiedge 2006).

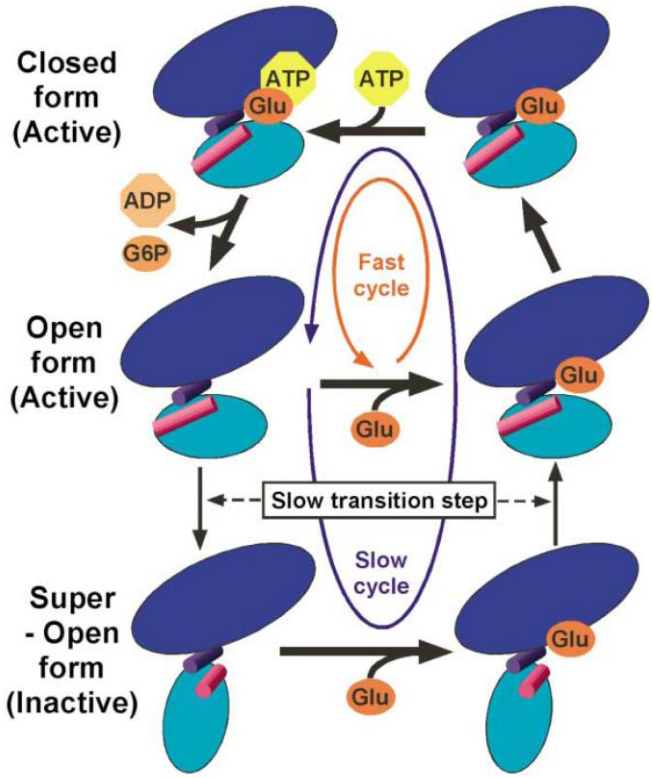


**Abb. 1.4 Kinetisches Modell der Glucokinase.** Neben der geschlossenen (*closed form*) und der weit offenen Konformation (*super-open form*) der Glucokinase, die aus Röntgenstrukturdaten abgeleitet wurden, wird die Existenz einer weiteren offenen Konformation (*open form*) des Enzyms angenommen. G6P, Glucose-6-phosphat; Glu, Glucose (Kamata et al. 2004).

## *1.7. Bindungspartner der Glucokinase*

Aufgrund der zentralen Rolle der Glucokinase bei der Aufrechterhaltung der Glucosehomöostase wird nicht nur die Genexpression des Enzyms gewebespezifisch reguliert. Sowohl in der Leber als auch in den β-Zellen des Pankreas haben außerdem posttranslationale Regulationsprozesse großen Einfluss auf die enzymatische Aktivität der Glucokinase. In Hepatocyten wird ein spezifisches Glucokinase Regulatorprotein exprimiert, welches durch Bindung an die Glucokinase deren Enzymaktivität kompetitiv inhibiert. Fructose-6-phosphat verstärkt die Bindung und damit die Inhibierung, wohingegen Fructose-1-phosphat und Glucose die Dissoziation des Komplexes bewirken (Agius & Stubbs 2000; Van Schaftingen 1989). Die Interaktion der Glucokinase mit dem Glucokinase Regulatorprotein bei niedrigen cytosolischen Glucosekonzentrationen bewirkt die Translokation des Proteinkomplexes vom Cytoplasma in den Zellkern (Bosco 2000; de la Iglesia 1999; Shiota 1999). Das Glucokinase Regulator-

protein der Leber wird in β-Zellen des Pankreas nicht exprimiert (Tiedge 1999). Zudem zeigt die Glucokinase in β-Zellen eine ausschließlich cytosolische Lokalisation. In Permeabilisierungsexperimenten konnte jedoch eine gebundene Fraktion der Glucokinase von einer frei beweglichen Fraktion mit jeweils unterschiedlicher intrinsischer Enzymaktivität unterschieden werden. Bei Glucosekonzentrationen unterhalb der zur glucoseinduzierten Insulinsekretion notwendigen Schwelle liegt die Glucokinase gebunden an Matrixproteine wie Tubulin (Baltrusch & Lenzen 2007; Tiedge et al. 1999), und/oder an sekretorische Granula vor (Rizzo 2002). Im Cytosol kommt es dann insbesondere bei hohen Glucosekonzentrationen zu einer Aktivitätssteigerung der Glucokinase. Das bifunktionelle Enzym 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase konnte als neuer Interaktionspartner der Glucokinase durch ein *Random Peptide Phage Display Library Screening* identifiziert werden (Baltrusch 2001). Die Interaktion mit der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase, die cytosolisch lokalisiert ist, führt zu einer Steigerung der intrinsischen Enzymaktivität der Glucokinase (Massa 2004). Diese Aktivierung der Glucokinase konnte in insulinproduzierenden Zellen sowohl für das Isoenzym der β-Zelle als auch für das Isoenzym der Leber in der Wildtypform und in einer kinaseaktiven mutierten Form der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase gezeigt werden (Massa et al. 2004). Die Steigerung der Enzymaktivität der Glucokinase erfolgte zudem unabhängig von der intrazellulären Konzentration des Fructose-2,6-bisphosphats, des Metaboliten, dessen Konzentration durch die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase reguliert wird (Massa et al. 2004). In den β-Zellen des Pankreas hat, anders als in Hepatocyten, der Ernährungszustand über die Regulation der Genexpression keinen signifikanten Einfluss auf den zellulären Gehalt an Glucokinase. Daher haben posttranslationale Mechanismen bei der Regulation der Enzymaktivität der Glucokinase in β-Zellen große Bedeutung. Die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase nimmt in dieser posttranslationalen Regulation als endogener Aktivator der Glucokinase eine wichtige Position ein (Baltrusch 2004) (Abb 1.5). In den letzten Jahren sind außerdem verschiedene chemisch synthetisierte Glucokinase-Aktivatoren beschrieben worden (Brocklehurst 2004; Efanov 2005; Grimsby 2003). Diese allosterischen Aktivatoren der Glucokinase können sowohl die glucoseinduzierte Insulinsekretion in den β-Zellen des Pankreas als auch die Glykogensynthese in der Leber steigern, und besitzen damit das Potential, eine krankheitsbedingt erhöhte Blutglucosekonzentration zu normalisieren (Guertin & Grimsby 2006; Leighton 2005; Printz & Granner 2005). Auch im Hinblick auf die noch notwendige Optimierung dieser synthetischen Glucokinase-Aktivatoren ist eine weitere Aufklärung der posttranslationalen Glucokinaseregulation von Interesse.

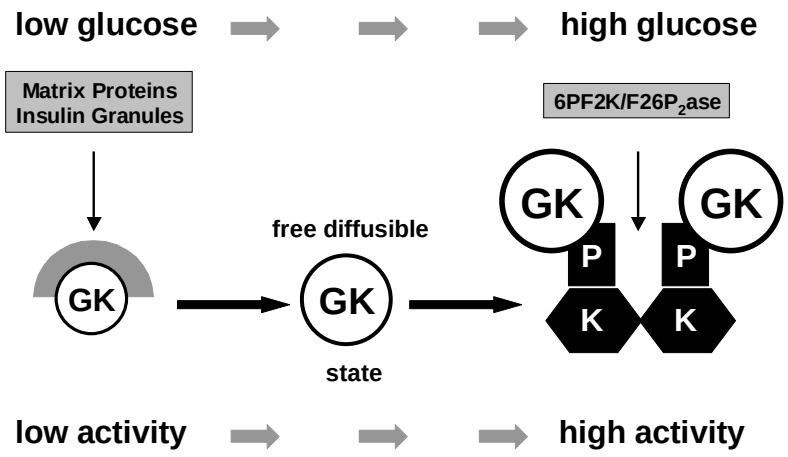


**Abb. 1.5 Aktivierung der Enzymaktivität der Glucokinase durch Interaktion mit dem 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase Protein in insulinproduzierenden Zellen.** Die Glucokinase wird reguliert durch die Interaktion mit spezifischen Proteinen, die die katalytische Funktion des Enzyms inhibieren (Matrixproteine, Insulingranula) oder aktivieren (6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase). Glucose bewirkt den Übergang von den inhibitorischen zu dem aktivierenden Bindungspartner. GK, Glucokinase; 6PF2K/F26$P_2$ase, 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase; K, Kinasedomäne; P, Bisphosphatasedomäne (Baltrusch 2004).

## *1.8. Das bifunktionelle Enzym 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase*

In der Regulation des Kohlenhydratstoffwechsels der Eukaryoten spielt Fructose-2,6-bisphosphat eine wichtige Rolle. Dieser Metabolit konnte in praktisch allen eukaryoten Geweben und Zellen nachgewiesen werden (Okar & Lange 1999). Das Fructose-2,6-bisphosphat ist der wirkungsvollste allosterische Aktivator des Enzyms 6-Phosphofructo-1-kinase, das die Schrittmacherreaktion der Glykolyse katalysiert. Gleichzeitig hemmt es in den Hepatocyten der Säugetiere die Fructose-1,6-bisphosphatase, welche ein Schlüsselenzym der Gluconeogenese ist. Bei hohen Konzentrationen an Fructose-2,6-bisphosphat findet in der Leber somit die Glykolyse und damit die Verstoffwechselung der Glucose statt. In Hepatocyten stimuliert eine Erhöhung der Fructose-2,6-bisphosphatkonzentration außerdem die Genexpression des Glucosesensorenzyms Glucokinase (Wu 2004). Der Fructose-2,6-bisphosphatspiegel wird bestimmt durch das Zusammenspiel von Synthese und Abbau, für das in Säugetieren eine einzige Enzymfamilie mit bifunktioneller Aktivität verantwortlich ist. Die N-terminal gelegene Kinasedomäne (EC 2.7.1.105) synthetisiert Fructose-2,6-bisphosphat aus Fructose-6-phosphat und ATP, die C-terminal gelegene Bisphosphatasedomäne (EC 3.1.3.46)

katalysiert die Hydrolyse des Fructose-2,6-bisphosphats zu Fructose-6-phosphat und Orthophosphat. Das bifunktionelle Enzym 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase nimmt somit eine zentrale Rolle in der Regulation des Kohlenhydratstoffwechsels ein (Okar 2001) (Abb. 1.6). Die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase unterliegt einer komplexen Regulation durch metabolische und hormonelle Signale, die insbesondere für das Isoenzym der Leber aufgeklärt werden konnten (Pilkis 1995). Da die Kinase- und die Bisphosphatasereaktion nebeneinander ablaufen, bestimmen die relativen enzymatischen Aktivitäten von Kinase und Bisphosphatase die Bilanz der Reaktion. Bei dem Isoenzym der Leber liegen sowohl der $K_m$-Wert der Kinase als auch der $K_i$-Wert der Bisphosphatase für das Fructose-6-phosphat im physiologischen Bereich. Somit ist die Konzentration des Fructose-2,6-bisphosphats in Hepatocyten eng an die des Fructose-6-phosphats und damit letztlich an den Blutglucosespiegel gekoppelt. Daneben wird das Isoenzym der Leber posttranslational durch Phosphorylierung reguliert (Pilkis et al. 1995). Die Phosphorylierung hat eine Aktivierung der Bisphosphatase und eine Inhibierung der Kinase zur Folge. Über diesen Weg wird die Mobilisierung der Glykogenreserven der Leber durch Glucagon vermittelt. Bindet Glucagon an seinen Rezeptor, so wird als *second messenger* cAMP gebildet. Die cAMP-abhängige Proteinkinase A phosphoryliert die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase am Aminosäurerest Serin-32. Aufgrund der resultierenden Verschiebung der Aktivität des bifunktionellen Enzyms in Richtung Bisphosphataseaktivität sinkt die Fructose-2,6-bisphosphatkonzentration. Umgekehrt bewirkt Glucose die Aktivierung der Xylulose-5-phosphat-abhängigen Proteinphosphatase 2A, welche die Dephosphorylierung der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase katalysiert, und ruft so einen dem Glucagon entgegengesetzten Effekt hervor (Okar et al. 2001). Das in den β-Zellen der Langerhans'schen Inseln des Pankreas exprimierte Isoenzym der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase wird im Gegensatz zum Isoenzym der Leber nicht über eine Proteinkinase A abhängige Phosphorylierung reguliert.

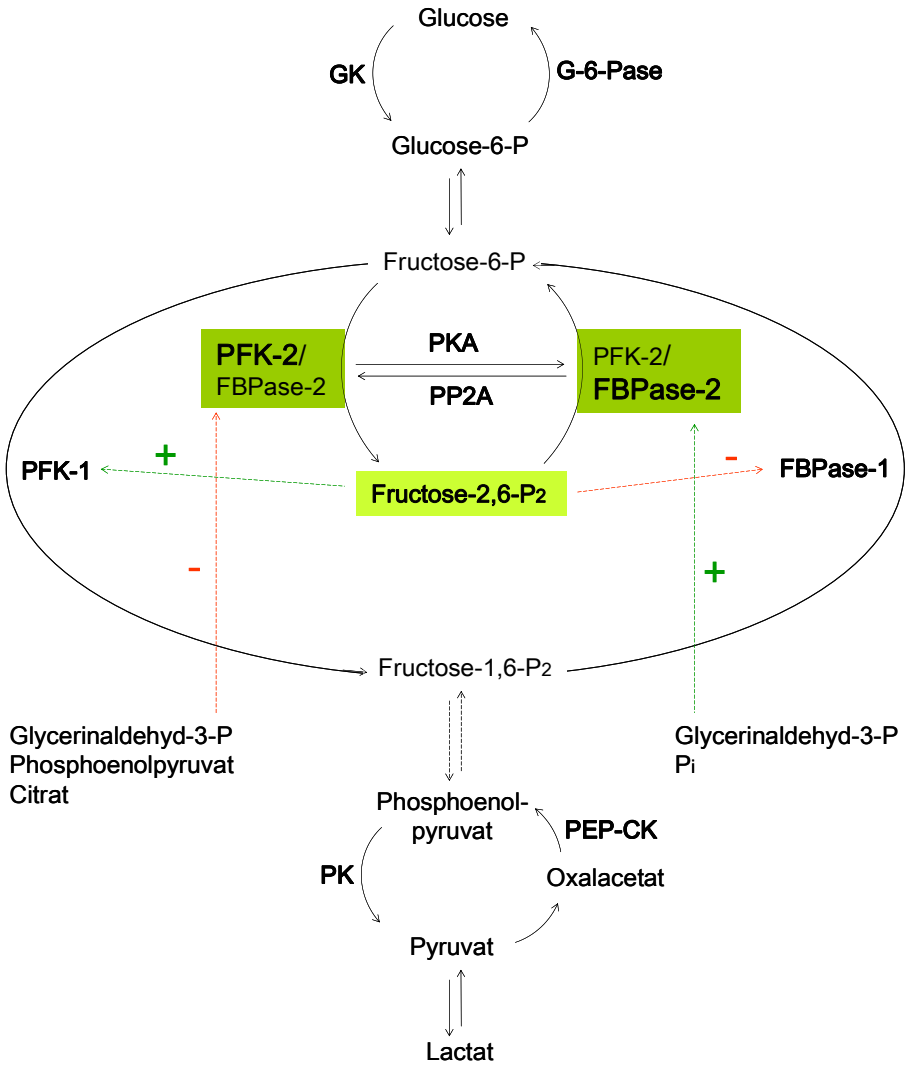


**Abb. 1.6 Die Rolle der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase im Intermediärstoffwechsel der Leberzelle.** FBPase-1, Fructose-1,6-bisphosphatase; FBPase-2, Fructose-2,6-bisphosphatase; Fructose-1,6-$P_2$, Fructose-1,6-bisphosphat; Fructose-2,6-$P_2$, Fructose-2,6-bisphosphat; Fructose-6-P, Fructose-6-phosphat; G-6-Pase, Glucose-6-phosphatase; GK, Glucokinase; Glucose-6-P, Glucose-6-phosphat; Glycerinaldehyd-3-P, Glycerinaldehyd-3-phosphat; PEP-CK, Phosphoenolpyruvat-Carboxykinase; PFK-1, 6-Phosphofructo-1-kinase; PFK-2, 6-Phosphofructo-2-kinase; $P_i$, Orthophosphat; PK, Pyruvatkinase; PKA, Proteinkinase A; PP2A, Proteinphosphatase 2A.

## *1.9. Genstruktur und Proteinstruktur der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase*

Die in den verschiedenen Geweben der Säugetiere vorliegenden Isoenzyme der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase werden von vier Genen, PFKFB1-PFKFB4, exprimiert (Okar et al. 2001). Die Kernregion mit N-terminal gelegener Kinasedomäne und C-terminal gelegener Bisphosphatasedomäne ist innerhalb der Isoenzyme hoch-

konserviert. Durch alternatives Spleißen entstehen Isoformen des Enzyms mit N- und C-terminalen Regionen variabler Länge. Über die terminalen Regionen erfolgt auch die gewebespezifische Regulation der Enzymaktivitäten durch Interkonversion. Die mRNA des Isoenzyms der Rattenleber wird von dem PFKFB1 Genlocus transkribiert. Das Isoenzym besteht als Monomer aus 470 Aminosäureresten und hat ein Molekulargewicht von 55 kDa (El-Maghrabi 1982). Seine Genexpression wird hormonell, in erster Linie durch Glucocorticoide (Lange 1992) und Insulin (Pierreux 1998), reguliert. Die mRNA des Isoenzyms der β-Zelle der Ratte wird dagegen vom PFKFB2 Genlocus transkribiert. Es sind zwei Spleißvarianten des Isoenzyms der β-Zelle bekannt, die als Monomer aus 474 bzw. aus 481 Aminosäureresten bestehen (Baltrusch et al. 2001). Die Expression der erstgenannten Variante wurde ebenfalls für das Rattengehirn beschrieben (Watanabe 1994). Die letztgenannte Variante unterscheidet sich zur erstgenannten nur durch eine Folge von acht zusätzlichen Aminosäureresten in der Verbindungsregion zwischen Kinase- und Bisphosphatasedomäne, die durch alternatives Spleißen von Intron 8 entsteht (Baltrusch et al. 2001). Das 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase Protein liegt als Homodimer vor, welches durch Wechselwirkungen zwischen den N-terminalen Kinasedomänen stabilisiert wird (Okar et al. 2001). Bisher gelang nur die Röntgenstrukturanalyse der Isoform des Rattenhodens der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase (Hasemann 1996). Dabei zeigte sich keine deutliche Annäherung der Bisphosphatasedomänen im Dimer. Für das in seiner Primärstruktur unveränderte Isoenzym der Leber liegt nur eine Kristallstruktur der separat exprimierten Bisphosphatasedomäne vor. Allerdings konnte in Hefe *Two-Hybrid* Experimenten eindeutig die Interaktion zwischen zwei Kinasedomänen, nicht aber zwischen zwei Bisphosphatasedomänen gezeigt werden (Baltrusch et al. 2001). Ferner liegt die rekombinant exprimierte Fructose-2,6-bisphosphatasedomäne als Monomer vor, was gegen eine Beteiligung an der Dimerisierung des bifunktionellen Enzyms spricht (Okar et al. 2001). Für die Bindung des Enzyms an die Glucokinase ist eine innerhalb der Isoenzyme hoch konservierte Region in der Fructose-2,6-bisphosphatasedomäne mit dem Consensusmotiv (SL)KVWT verantwortlich. Aufgrund der entgegengesetzten Lokalisation der Bindungsstelle im 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase Homodimer erscheint daher eine gleichzeitige Bindung von zwei Molekülen der Glucokinase möglich (Baltrusch et al. 2001) (Abb. 1.7). In Hefe *Two-Hybrid* Experimenten zeigte sowohl das Isoenzym der β-Zellen des Pankreas als auch das Isoenzym der Leber eine Bindung an die Glucokinase, wobei die Interaktion eindeutig der Fructose-2,6-bisphosphatasedomäne zugeordnet werden konnte (Baltrusch et al. 2001).

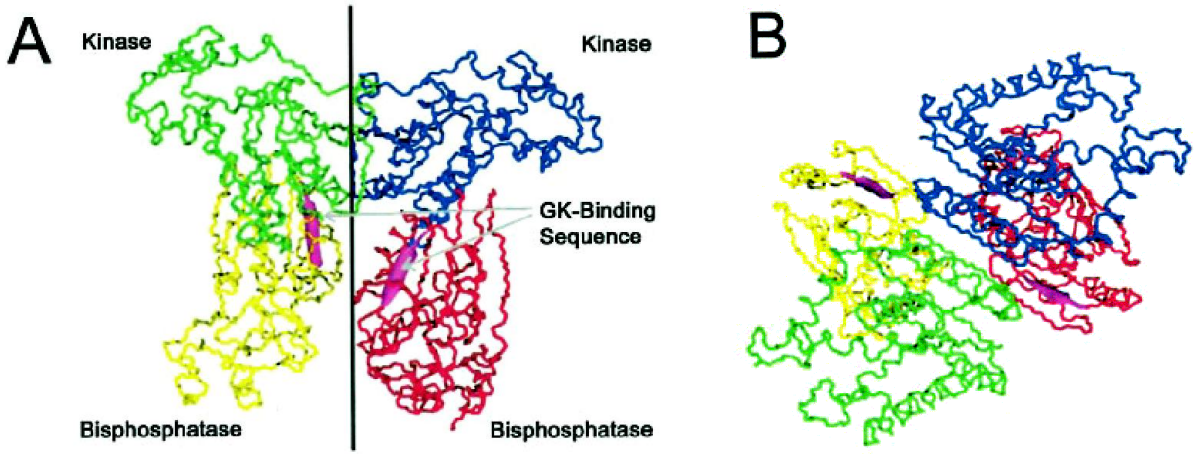


**Abb. 1.7 Räumliche Struktur des 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase Dimers mit den Glucokinase bindenden Konsensussequenzen.** Die Koordinaten stammen aus Röntgenstrukturdaten des Kristalls des Isoenzyms der Hoden der Ratte (Research Collaboratory for Structural Bioinformatics Data Bank). Die Darstellung wurde mit der Swiss PDB Viewer and POV Ray Software erstellt. Die Kinasedomänen sind in grün und blau dargestellt, die Bisphosphatasedomänen in gelb und rot. Die Glucokinase bindende Sequenz ist jeweils in magenta dargestellt. (A) Die Mitte zwischen den beiden Monomeren des Enzyms ist durch die vertikale Linie gekennzeichnet. (B) Das Enzym ist in Aufsicht auf die Kinasedomänen dargestellt. GK, Glucokinase (Baltrusch et al. 2001).

## 1.10. Fragestellung

Das bifunktionelle Enzym 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase wurde als ein neuer intrazellulärer Bindungspartner der Glucokinase, des Schlüsselenzyms der glucose-induzierten Insulinsekretion in den β-Zellen des Pankreas und des Glucosemetabolismus in der Leber, identifiziert. Die Überexpression der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase Isoform sowohl der β-Zelle als auch der Leber führte in insulinproduzierenden Zellen zu einem Anstieg der intrinsischen Glucokinase Enzymaktivität. Damit stellt die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase einen endogenen Aktivator der Glucokinase dar.

**(1)** Da das Signal für die Insulinsekretion im Stoffwechsel der β-Zelle entsteht, sollte der Einfluss der Interaktion zwischen Glucokinase und 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase auf den Intermediärstoffwechsel von insulinproduzierenden Zellen untersucht werden. Dabei sollten in insulinproduzierenden RINm5F Zellklonen, die neben der Glucokinase der humanen β-Zelle die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase Isoform der β-Zelle der Ratte stabil überexprimieren, der zelluläre Gehalt an ATP und ADP sowie die glucoseinduzierte Insulinsekretion gemessen werden. Die Messungen sollten nach Kultivierung der Zellen mit unterschiedlichen Glucosekonzentrationen im Kulturmedium durchgeführt werden.

**(2)** Es sollte weiterhin untersucht werden, ob die in insulinproduzierenden Zellen beobachtete Aktivierung der Glucokinase Enzymaktivität gleichermaßen auch mit rekombinant exprimierten, aufgereinigten Proteinen auftritt. Hierdurch sollte geklärt werden, ob es möglicherweise bislang unbekannte intrazelluläre molekulare Komponenten gibt, die die Interaktion zwischen der Glucokinase und der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase stabilisieren.

**(3)** Da eine direkte Bindung der Glucokinase an die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase im Hefe *Two-Hybrid* System gezeigt wurde, sollte der molekulare Mechanismus der Wechselwirkung in Säugetierzellen analysiert werden. Zu diesem Zweck sollte ein *Mammalian Two-Hybrid* System in verschiedenen Säugetierzelllinien etabliert werden. In insulinproduzierenden Zellen sollte insbesondere mithilfe des Systems geklärt werden, welchen Einfluss die Glucosekonzentration auf die Interaktion der Proteine hat. Dieser Frage

sollte außerdem mithilfe von fluoreszierenden Fusionsproteinen in Fluoreszenz Resonanz Energietransfer Messungen (FRET) und Kolokalisationsstudien nachgegangen werden.

**(4)** Die Dimerisierung der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase auf der Basis einer homophilen Interaktion der 6-Phosphofructo-2-kinase konnte ebenfalls im Hefe *Two-Hybrid* System nachgewiesen werden. Damit eröffnet sich ein Modell des über die 6-Phosphofructo-2-kinase stabilisierten dimeren 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase Proteins. Für das bifunktionelle Enzym der Rattenleber wird postuliert, dass die Dimerisierung durch Phosphorylierung des Enzyms moduliert wird. Um diese Hypothese zu überprüfen, sollten das *Mammalian Two-Hybrid* System sowie photoaktivierbare fluoreszierende Fusionsproteine verwendet werden. Insbesondere sollte der Einfluss der cAMP-abhängigen Phosphorylierung von Ser-32 auf die Wechselwirkungen innerhalb eines Dimers der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase Isoform der Leber der Ratte untersucht werden.

# 2. Publikationen

Simone Baltrusch, Sara Langer, Laura Massa, Markus Tiedge und Sigurd Lenzen (2006) **Improved metabolic stimulus for glucose-induced insulin secretion through GK and PFK-2/FBPase-2 coexpression in insulin-producing RINm5F cells.** *Endocrinology* 147(12): 5768-5776 (*Copyright 2006, The Endocrine Society)*

W. Ed Smith, Sara Langer, Chaodong Wu, Simone Baltrusch und David A. Okar (2007) **Molecular coordination of hepatic glucose metabolism by the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase:glucokinase complex.** *Molecular Endocrinology* 21(6):1478-1487 (*Copyright 2007, The Endocrine Society)*

Sara Langer, Martin Kaminski, Sigurd Lenzen und Simone Baltrusch (2009) **Endogenous activation of glucokinase by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase is glucose dependent.** *Zur Publikation vorbereitet. In revidierter Form zur Publikation in* Molecular Endocrinology *angenommen am 09.07.2010. (Copyright 2010, The Endocrine Society)*

Sara Langer, David A. Okar, Sigurd Lenzen und Simone Baltrusch (2009) **Dimer interface rearrangement of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase rat liver isoenzyme by cAMP-dependent Ser-32 phosphorylation.** *Zur Publikation vorbereitet.*

Simone Baltrusch, Heike Schmitt, Sara Langer und Sigurd Lenzen (2009) **Activation of glucokinase by the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and the chemical compound LY2121260.** *Zur Publikation vorbereitet.*

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doi: 10.1210/en.2006-0694

# Improved Metabolic Stimulus for Glucose-Induced Insulin Secretion through GK and PFK-2/FBPase-2 Coexpression in Insulin-Producing RINm5F Cells

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**The glucose sensor enzyme glucokinase plays a pivotal role in the regulation of glucose-induced insulin secretion in pancreatic β-cells. Activation of glucokinase represents a promising concept for the treatment of type 2 diabetes. Therefore, we analyzed the glucokinase activation through its physiological interaction partner, the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) and the resulting effect on glucose metabolism in insulin-producing cells. In RINm5F-GK-PFK-2/FBPase-2 cells stably overexpressing glucokinase plus islet PFK-2/FBPase-2, colocalization between both enzymes as well as elevation of glucokinase activity were significantly increased at a stimulatory glucose concentration of 10 mmol/liter. RINm5F-GK-PFK-2/FBPase-2 cells showed under this culture condition a significant increase in glucose utilization and in the ATP/ADP ratio compared with RINm5F-GK cells, which only overexpress glucokinase. Also glucose-induced insulin secretion was elevated in RINm5F-GK-PFK-2/FBPase-2 cells in comparison to RINm5F-GK cells. Furthermore, pyruvate accumulation and lactate production in RINm5F-GK-PFK-2/FBPase-2 cells were significantly lower at both 10 and 30 mmol/liter glucose than in RINm5F-GK and RINm5F cells. The significant improvement of glucose metabolism after PFK-2/FBPase-2 overexpression is apparently not exclusively the result of high glucokinase enzyme activity. Stabilization of the closed glucokinase conformation by PFK-2/FBPase-2 may not only activate the enzyme but also improve metabolic channeling in β-cells. (*Endocrinology* 147: 5768–5776, 2006)**

THE GLUCOSE PHOSPHORYLATING enzyme glucokinase, in pancreatic β-cells and liver, but also in enteroendocrine cells, pituitary cells, and neurons, has the glucose sensor function due to its cooperative kinetics and high control strength in the regulation of glycolysis (1–8). In β-cells, glucokinase catalyzes the rate-limiting step of glucose-induced insulin secretion (6). Therefore, the elucidation of the mechanisms underlying glucokinase regulation in β-cells on the gene transcription level and especially by post-translational mechanisms has gained particular interest (8). The glucose-dependent glucokinase conformational change between the closed and super-open form plays a fundamental role in the glucokinase activity modulation (9). Furthermore, glucokinase binding partners like β-cell matrix proteins and insulin secretory granules may affect cytoplasmic compartmentation of glucokinase (10–14). Interestingly, recently discovered small chemical compounds activate glucokinase by binding to an allosteric site of the enzyme protein. These so-called glucokinase activators have the ability to increase glucose-stimulated insulin release and thereby provide the rationale for a promising new concept of type 2 diabetes pharmacotherapy (9, 15–17). Glucokinase activation is also physiologically mediated by interaction with the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) (18, 19). β-Cells express the brain isoform of PFK-2/FBPase-2, which is not regulated by phosphorylation and dephosphorylation (20). In recent studies, we could show that overexpression of PFK-2/FBPase-2 in insulin-producing cells results in a significant increase of glucokinase enzyme activity and glucose oxidation, indicating a beneficial effect on glucose-induced insulin secretion (18).

Therefore, the aim of our present study was to characterize key features of glucose metabolism in insulin-producing RINm5F cells overexpressing glucokinase plus islet PFK-2/FBPase-2 (RINm5F-GK-PFK-2/FBPase-2 cells) or glucokinase alone (RINm5F-GK cells). The results show that activated glucokinase plays a central role in the flux control of glycolysis and improves oxidative glucose metabolism and glucose-induced insulin secretion with a concomitant decrease in pyruvate and lactate accumulation.

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Abbreviations: F-2,6-$P_2$, Fructose-2,6-bisphosphate; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; PFK-2/FBPase-2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; siRNA, small interfering RNA; TxRed, Texas Red.

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## Materials and Methods

### *Materials*

The enhanced chemiluminescence detection system, autoradiography films, and radiochemicals were from Amersham Pharmacia Biotech (Freiburg, Germany). Forskolin was from ICN Biomedicals (Irvine, CA). All reagents of analytical grade were from Merck (Darmstadt, Germany). All tissue culture equipment was from Invitrogen (Karlsruhe, Germany).

### *RINm5F cell culture*

Insulin-producing RINm5F cells overexpressing glucokinase (RINm5F-GK cells) were generated by stable transfection of the human β-cell glucokinase cDNA as described previously (11, 21). RINm5F-GK

cells overexpressing PFK-2/FBPase-2 were generated by a second stable transfection of the cDNA for rat liver (RINm5F-GK-PFK-2/FBPase-2 L 11), for the rat liver S32A/H258A double mutant (RINm5F-GK-PFK-2/FBPase-2 LM 12), or for rat islets (RINm5F-GK-PFK-2/FBPase-2 I 4) as described previously (18). Cells were grown in RPMI 1640 medium supplemented with 10 mmol/liter glucose, 10% (vol/vol) fetal calf serum (FCS), penicillin, and streptomycin in a humidified atmosphere at 37 C and 5% $CO_2$. The medium for RINm5F-GK cells was additionally supplemented with 250 $\mu$g/ml G418, and the medium for RINm5F-GK-PFK-2/FBPase-2 cells with 250 $\mu$g/ml G418 and 250 $\mu$g/ml Zeocin. For gene silencing, RINm5F-GK-PFK-FBPase-2 islet (I 4) cells were seeded in six-well microplates at a density of $1.5 \times 10^5$. On the next day, cells were transfected with either 1.5 $\mu$g islet PFK-2/FBPase-2 cDNA-specific small interfering RNA (siRNA) or control siRNA and 4 $\mu$l jetSI-ENDO (Polyplus, Illkirch, France) for 4 h in RPMI 1640 medium in the absence of FCS according to the manufacturer's instructions. Thereafter, cells were incubated for 48 h in RPMI 1640 medium supplemented with 10 mmol/liter glucose and 5% FCS. The islet PFK-2/FBPase-2 cDNA specific siRNA duplex was designed against a target sequence of the islet PFK-2/FBPase-2 cDNA (CCA GAG TAA GAT TGT CTA CTA, GenBank accession no. S67900) by the HiPerformance Design Algorithm licensed from Novartis AG (QIAGEN, Hilden, Germany).

### *Glucose phosphorylation and glucokinase enzyme activity*

Glucose phosphorylating activity was measured at various glucose concentrations (1, 1.56, 3.12, 6.25, 12.5, and 25 mmol/liter) in soluble cellular fractions of RINm5F, RINm5F-GK, and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells by an enzyme-coupled photometric assay as described previously (22). One unit of enzyme activity was defined as 1 $\mu$mol glucose-6-phosphate formed from glucose and ATP per minute at 37 C. Enzyme activity was expressed as units per mg cellular protein. Glucokinase activity was determined by subtracting the hexokinase activity measured at 1 mmol/liter glucose from the activity measured at 100 mmol/liter glucose.

### *Western blot analyses*

Glucokinase and PFK-2/FBPase-2 Western blot analyses and glucokinase activity measurements were performed from identical samples to allow a direct comparison between protein expression and enzyme activity. The cells were homogenized in PBS (pH 7.4), and insoluble material was pelleted by centrifugation. The protein concentration was quantified by a Bio-Rad protein assay. Forty micrograms of cellular protein were fractionated by reducing 10% SDS-PAGE and electroblotted to polyvinylidine difluoride membranes. The membranes were stained by Ponceau to verify the transfer of comparable amounts of cellular protein. Nonspecific binding sites of the membranes were blocked by nonfat dry milk overnight at 4 C. Glucokinase and PFK-2/FBPase-2 immunodetection was performed as described (11, 18).

### *Immunostaining*

Cells were seeded on glass cover slips and grown for 24 h in medium with 10 mmol/liter glucose. Thereafter, cells were incubated for the next 24 h in medium with either 3 or 10 mmol/liter glucose. Finally, cells were washed twice with PBS (pH 7.4), fixed with ice-cold acetone for 5 min, and treated for 20 min with 0.2% Triton X-100 and 1% BSA in PBS. Cover slips were washed three times with PBS and incubated for 1 h with the glucokinase antibody (11) and the FBPase-2 antibody (18), both diluted 1:1000 in PBS supplemented with 0.1% Triton X-100 and 1% BSA. Thereafter, cover slips were washed three times with PBS and incubated for 1 h with fluorescein isothiocyanate (FITC) donkey antirabbit antibody for glucokinase and Texas Red (TxRed) donkey antichicken antibody for FBPase-2, both diluted 1:200 in PBS supplemented with 0.1% Triton X-100 and 1% BSA. Finally, cover slips were washed three times with PBS and mounted with ProLong antifade reagent in mounting medium (Molecular Probes Invitrogen Detection Technologies, Eugene, OR) onto slides. Images were taken with an Olympus IX81/cell system as described previously (23). S 492/18 and S 572/23 excitation filters were used for FITC and TxRed, respectively, and a DAPI/FITC/TxRed triple band beam splitter and emitter (AHF Analysentechnik, Tübingen, Germany). Colocalization was calculated with Image J 1.32 (W. Rasband, National Institutes of Health) using the plug-in module Colocalization Finder (C. Laummonerie, Strasbourg, France).

### *Measurements of fructose-2,6-bisphosphate (F-2,6-$P_2$)*

RINm5F, RINm5F-GK, RINm5F-GK-PFK-2/FBPase-2 (L 11), RINm5F-GK-PFK-2/FBPase-2 (LM 12), and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells were grown for 24 h in medium with 10 mmol/liter glucose. Thereafter, cells were incubated for 2 h in the presence of 10 $\mu$M forskolin. Finally, cells were permeabilized by 10 $\mu$g/ml $\alpha$-toxin from *Staphylococcus aureus* (Sigma, Taufkirchen, Germany) as described (18). F-2,6-$P_2$ was determined in the supernatant by an enzyme-coupled method using pyrophosphate-dependent fructose-6-phosphate kinase (Sigma).

### *Glucose metabolism*

The glucose utilization rate was assessed as the production of $^3H_2O$ from D-[5-$^3$H]glucose. Glucose metabolism was measured as described (24, 25) in batches of $5 \times 10^5$ cells over a 1-h incubation at 37 C in 40 $\mu$l of Krebs-Ringer buffer containing various glucose concentrations (0.5, 1, 2, 5, and 10 mmol/liter). Total radioactivity added to the cells was 0.4 $\mu$Ci/ml. Cellular metabolism was arrested by the addition of 50 $\mu$l of 0.2 M HCl, and the produced $^3H_2O$ was captured by 500 $\mu$l $H_2O$. After overnight incubation at 37 C, wells containing cells were removed and the scintillation liquid was added. The radioactivity was counted in a liquid-scintillation spectrometer.

### *Determination of ATP and ADP*

Cells were seeded in six-well microplates at a density of $5 \times 10^5$ cells and grown for 24 h in medium with 10 mmol/liter glucose. Thereafter, cells were incubated for the next 48 h in medium with either 3 or 10 mmol/liter glucose. Analysis of ATP was performed with the ATPlite Detection Assay System (PerkinElmer Life Sciences, Zaventem, Belgium) according to the manufacturer's instructions. This system is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin (26). The attached mammalian cell lysis solution releases the adenine nucleotides and inactivates endogenous ATP degrading enzymes. For determining the ADP content, the sum of the ATP content and the ADP content was measured by conversion of ADP to ATP with pyruvate kinase and phosphoenolpyruvate for 15 min. Light emission was recorded as a 1-sec integral in a microplate using a Victor$^2$ luminometer (Wallac, Freiburg, Germany) and the protein concentration was quantified by a Bio-Rad (Hercules, CA) protein assay. ATP/ADP values were calculated in relation to ATP standard values and protein content.

### *Measurements of pyruvate and lactate*

Cells were grown for 72 h in medium with 10 mmol/liter glucose or for 24 h in medium with 10 mmol/liter glucose and for the next 48 h in medium with 30 mmol/liter glucose. Thereafter, batches of $2 \times 10^6$ cells were incubated for 1 h at 37 C and 300 rpm in 1 ml Krebs-Ringer buffer in the presence of various glucose concentrations (0.5, 1, 2, 5, and 10 mmol/liter). The cellular solution was gently centrifuged to remove the cells, and the supernatant was heated to 95 C for a period of 5 min to denature proteins. Thereafter, pyruvate production was determined by measuring the oxidation of reduced nicotinamide adenine dinucleotide in the presence of lactate dehydrogenase, and lactate production was determined by measuring the reduction of nicotinamide adenine dinucleotide in the presence of lactate dehydrogenase, glutamate, and glutamate-pyruvate transaminase as described (27, 28).

### *Insulin secretion and insulin content*

Cells were seeded in six-well microplates at a density of $5 \times 10^5$ cells and grown for 48 h in medium with 10 mmol/liter glucose. Cells were then washed twice with medium without glucose and preincubated for 1 h in medium without glucose. Insulin secretion during a 1-h incubation period was measured in Krebs-Ringer buffer without glucose or in the presence of various glucose concentrations (1, 3, 5, 10, and 25 mmol/liter) or in the presence of 25 mmol/liter KCl. Thereafter, for measure-

ments of insulin secretion, the incubation buffer was carefully removed and gently centrifuged to delete detached cells. Insulin content was measured in the soluble fraction of the homogenized cells. Insulin was determined by RIA using a rat insulin standard.

### *MTT cell viability assay*

RINm5F, RINm5F-GK, and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells were seeded in 96-well microplates at a density of 5,000 cells per well in 100-$\mu$l medium with 10 mmol/liter glucose and cultured for 24 h. Thereafter, cells were incubated for the next 48 h in medium with 3, 10, or 30 mmol/liter glucose. The cell viability was determined using a microtiter plate-based MTT assay as described (29). The decrease of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) activity is a reliable metabolically based test for quantification of cell viability (30).

### *Statistical analyses*

The data are expressed as means ± SEM. Statistical analyses were performed by ANOVA followed by Bonferroni's test for multiple comparison or Student's *t* test using the Prism analysis program (GraphPad Software, Inc., San Diego, CA).

## Results

### *Relationship between glucokinase activation and PFK-2/FBPase-2 enzyme level*

Stable overexpression of the islet PFK-2/FBPase-2 isoform in RINm5F-GK cells resulted in a significant increase in the PFK-2/FBPase-2 protein level to 364% and in a significant increase in glucokinase enzyme activity to 178%, whereas glucokinase protein expression levels were not affected (18). The $S_{0.5}$ values for glucose phosphorylation were 3.2 mmol/liter in RINm5F-GK cells and 3.1 mmol/liter in RINm5F-GK-PFK-2/FBPase-2 (I 4) cells, and were thus lower than in pancreatic islets probably due to the high activity levels of low-$K_m$ hexokinases (18). Although RINm5F cells showed no demonstrable increase in the phosphorylating activity in response to glucose, glucokinase overexpressing RINm5F-GK cells exhibited an increase in the phosphorylating activity at 3.12 mmol/liter and higher glucose concentrations (Fig. 1).

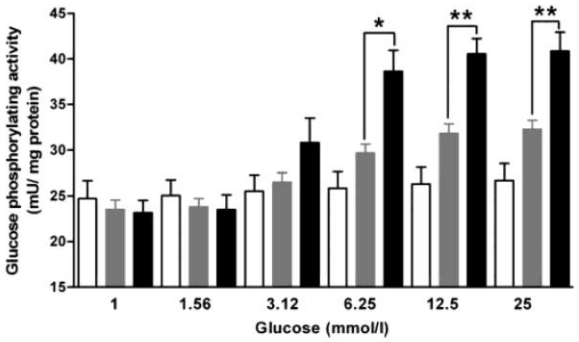


FIG. 1. Effects of glucokinase overexpression alone and glucokinase plus islet PFK-2/FBPase-2 co-overexpression in RINm5F-cells on the glucose phosphorylating activity. Cells were grown overnight at 10 mmol/liter glucose. Thereafter, RINm5F cells (*white bars*), RINm5F-GK cells (*gray bars*), and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells (*black bars*) were homogenized by sonication, and phosphorylating activities were determined spectrophotometrically in the presence of various glucose concentrations (1, 1.56, 3.12, 6.25, 12.5, and 25 mmol/liter). Shown are means ± SEM from four individual experiments. *, $P < 0.05$; **, $P < 0.01$ compared with RINm5F-GK cells (ANOVA/Bonferroni's test).

Interestingly, only at these glucose concentrations did RINm5F-GK-PFK-2/FBPase-2 (I 4) show a higher phosphorylating activity than RINm5F-GK cells (Fig. 1). Thus, the increase of phosphorylating activity in RINm5F-GK-PFK-2/FBPase-2 (I 4) cells compared with RINm5F-GK cells was apparently conferred by activation of the glucokinase enzyme and seems to be independent of the hexokinase enzyme. Furthermore, insulin-producing RINm5F-GK-PFK-2/FBPase-2 (I 4) cells were transfected with islet PFK-2/FBPase-2 cDNA-specific siRNA, which resulted in a reduction of the PFK-2/FBPase-2 protein level by 48% compared with cells transfected with control siRNA (Fig. 2A). The increase in glucokinase enzyme activity in RINm5F-GK-PFK-2/FBPase-2 (I 4) cells to 178% could be significantly reduced to 133% by transfection with the islet PFK-2/FBPase-2 cDNA specific siRNA, but not with control siRNA (Fig. 2B). Glucokinase protein expression levels were not affected by down-regulation of PFK-2/FBPase-2 (Fig. 2C). Thus, glucokinase was activated by PFK-2/FBPase-2 on the posttranslational level.

### *F-2,6-$P_2$ metabolism in RINm5F cells*

Overexpression of the islet PFK-2/FBPase-2 isoform in RINm5F-GK-PFK-2/FBPase-2 (I 4) cells did not affect the F-2,6-$P_2$ concentration in comparison to RINm5F-GK cells. The protein kinase A activator forskolin provoked a nonsignificant decrease of the F-2,6-$P_2$ concentration by 26% in RINm5F-GK-PFK-2/FBPase-2 (I 4) cells, which was also observed in RINm5F-GK (23%) cells and untransfected RINm5F (24%) cells (Fig. 3). Thus, neither glucokinase nor the overexpression of the tissue-specific islet PFK-2/

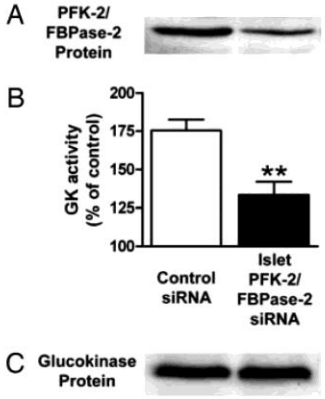


FIG. 2. Down-regulation of PFK-2/FBPase-2 in RINm5F-GK-PFK-2/FBPase-2 (I 4) cells by siRNA. Cells were transfected with islet PFK-2/FBPase-2 cDNA-specific siRNA (*black bar*) or with control siRNA (*white bar*) for 4 h in RPMI 1640 medium in the absence of FCS. Thereafter, cells were incubated for 48 h in RPMI 1640 medium supplemented with 10 mmol/liter glucose and 5% FCS. For Western blot analyses, 40 $\mu$g cellular protein was analyzed per lane by immunoblotting using a specific antibody against FBPase-2 (A) or glucokinase (C). Shown are representative blots of four independent experiments. Glucokinase enzyme activities (B) were measured spectrophotometrically in cell extracts after sonication. Data are expressed as percentage of enzyme activity measured in RINm5F-GK control cells. Shown are means ± SEM from four individual experiments. **, $P < 0.01$ compared with cells transfected with control siRNA (Student's *t* test).

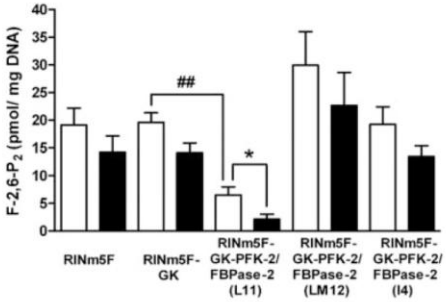


FIG. 3. Effects of forskolin on the F-2,6-$P_2$ content in RINm5F, RINm5F-GK, RINm5F-GK-PFK-2/FBPase-2 (L 11), RINm5F-GK-PFK-2/FBPase-2 (LM 12), and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells. Cells were grown for 24 h in medium with 10 mmol/liter glucose. Thereafter, cells were incubated for 2 h in the absence (*white bars*) or presence (*black bars*) of 10 $\mu$M forskolin. Finally, cells were permeabilized by 10 $\mu$g/ml $\alpha$-toxin from *S. aureus*, and the F-2,6-$P_2$ was determined spectrophotometrically. Shown are means ± SEM from four to seven individual experiments. *, $P < 0.05$ compared with the respective untreated cells; ##, $P < 0.01$ compared with RINm5F-GK cells (Student's *t* test).

FBPase-2 isoform affected the regulation of the F-2,6-$P_2$ concentration in insulin-producing cells.

Although overexpression of the liver PFK-2/FBPase-2 isoform in RINm5F-GK-PFK-2/FBPase-2 (L11) cells significantly reduced the cellular F-2,6-$P_2$ concentration by 66%, RINm5F-GK-PFK-2/FBPase-2 (LM 12) cells overexpressing a phosphorylation insensitive mutant showed a 53% higher concentration of F-2,6-$P_2$ than RINm5F-GK-PFK-2/FBPase-2 (I 4) cells. This indicates alterations in the F-2,6-$P_2$ concentration due to expression of another PFK-2/FBPase-2 isoform in insulin-producing cells. Furthermore, in comparison with RINm5F-GK cells there was a comparable increase in the PFK-2/FBPase-2 protein level to 364% in RINm5F-GK-PFK-2/FBPase-2 (I 4) cells and to 362% in RINm5F-GK-PFK-2/FBPase-2 (L11) cells, whereas in RINm5F-GK-PFK-2/FBPase-2 (LM 12) cells the liver mutant PFK-2/FBPase-2 protein level was elevated to 415%. Considering this somewhat higher expression level of the latter clone, the RINm5F-GK-PFK-2/FBPase-2 (LM 12) cells showed only a 38% higher concentration of F-2,6-$P_2$ compared with RINm5F-GK-PFK-2/FBPase-2 (I 4) cells. Forskolin significantly reduced the F-2,6-$P_2$ concentration by 68% in cells overexpressing the liver PFK-2/FBPase-2 isoform, whereas RINm5F-GK-PFK-2/FBPase-2 (LM 12) cells showed only a decrease of the F-2,6-$P_2$ concentration by 24% comparable to RINm5F-GK-PFK-2/FBPase-2 (I 4), RINm5F-GK, and RINm5F cells (Fig. 3). Thus, the low F-2,6-$P_2$ concentration and the inhibitory effect of forskolin in RINm5F-GK-PFK-2/FBPase-2 (L 11) cells could be mediated through the regulatory phosphorylation site for protein kinase A in the liver isoform, which is not present in the PFK-2/FBPase-2 islet isoform.

### *Glucose-dependent interaction of glucokinase with islet PFK-2/FBPase-2*

PFK-2/FBPase-2 and glucokinase immunofluorescence was detectable in the cytoplasm of RINm5F, RINm5F-GK, and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells. Colocalization between glucokinase and PFK-2/FBPase-2, indicating interaction between both proteins, was observed in RINm5F-GK and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells cultured at 10 mmol/liter glucose (data not shown). Interestingly, RINm5F-GK-PFK-2/FBPase-2 (I 4) cells showed less colocalization between glucokinase and PFK-2/FBPase-2 at 3 mmol/liter glucose (Fig. 4A) than at 10 mmol/liter glucose (Fig. 4B). Quantitative analyses revealed a significant 3-fold increase of colocalization in cells cultured at 10 mmol/liter glucose in comparison to cells cultured at 3 mmol/liter glucose (Fig. 4C).

### *Effects of islet PFK-2/FBPase-2 overexpression on glucose utilization in RINm5F-GK cells*

Production of $^3H_2O$ from D-[5-$^3$H]glucose was measured to estimate the overall rate of glycolysis. Rates of glucose utilization increased in a concentration-dependent manner in RINm5F-GK and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells. Glucose utilization rates were significantly higher in RINm5F-GK-PFK-2/FBPase-2 (I 4) cells than in RINm5F-GK cells at 5 and 10 mmol/liter glucose (Fig. 5). Thus, islet PFK-2/FBPase-2 overexpression resulted in an enhanced glycolytic flux in insulin-producing cells.

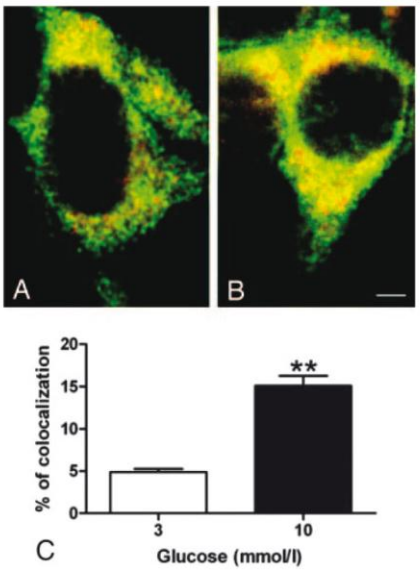


FIG. 4. Effects of glucose on the colocalization between glucokinase and PFK-2/FBPase-2 in RINm5F-GK-PFK-2/FBPase-2 (I 4) cells. Cells were seeded on glass cover slips and grown for 24 h in medium with 10 mmol/liter glucose. Thereafter, cells were incubated for the next 24 h in medium with either 3 (A) or 10 (B) mmol/liter glucose. Finally, cells were fixed and double immunostained for glucokinase (*green*) and PFK-2/FBPase-2 (*red*). *Yellow color* indicates colocalization. C, Colocalization was calculated by Image J/ Colocalization Finder (C. Laummonerie, Strasbourg, France). Shown are means ± SEM from three individual experiments. **, $P < 0.01$ compared with cells cultured at 3 mmol/liter glucose (Student's *t* test).

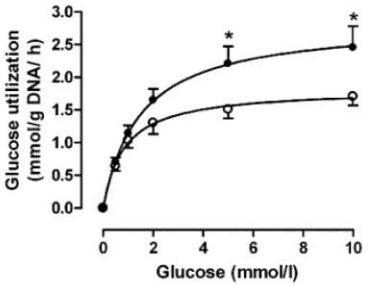


FIG. 5. Effects of islet PFK-2/FBPase-2 overexpression on glucose utilization in RINm5F-GK cells. RINm5F-GK cells (*white circles*) and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells (*black circles*) were grown in medium with 10 mmol/liter glucose. After 48 h, cells were incubated for 1 h at 37 C in Krebs-Ringer buffer without glucose. Thereafter, glucose metabolism was measured in the presence of various glucose concentrations (0.5, 1, 2, 5, and 10 mmol/liter). Glucose utilization was calculated from the production of $^3H_2O$. Shown are means ± SEM from seven individual experiments. *, $P < 0.05$ compared with RINm5F-GK cells (Student's $t$ test).

### *ATP/ADP ratio, ATP content, and ADP content of RINm5F, RINm5F-GK, and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells*

The intracellular ATP/ADP ratio (Fig. 6A) was not different in RINm5F cells cultured at 3 mmol/liter or at 10 mmol/liter glucose due to a comparable ATP and ADP content (Fig. 6, B and C). In contrast, glucokinase overexpressing RINm5F-GK cells showed a 14% higher and glucokinase plus islet PFK-2/FBPase-2 co-overexpressing RINm5F-GK-PFK-2/FBPase-2 (I 4) cells a 32% higher ATP/ADP ratio at 10 mmol/liter glucose than at 3 mmol/liter glucose (Fig. 6A), mediated by an increase in the ATP content (Fig. 6B) and a decrease in the ADP (Fig. 6C) content. Although at 3 mmol/liter glucose the ATP/ADP ratio was comparable in control and transfected RINm5F cells, at 10 mmol/liter glucose RINm5F-GK cells and in particular RINm5F-GK-PFK-2/FBPase-2 (I 4) cells showed a significantly higher ATP/ADP ratio than RINm5F cells (Fig. 6A). Incubation of control and transfected RINm5F cells at 30 mmol/liter glucose resulted in comparable ATP/ADP ratios as observed for cells cultured at 10 mmol/liter glucose (data not shown).

### *Cell viability of RINm5F, RINm5F-GK, and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells*

The viability of RINm5F cells was not affected by incubation for 48 h at 3 mmol/liter glucose (105 ± 12%) or at 30 mmol/liter glucose (94 ± 9%) in comparison to 10 mmol/liter glucose (100 ± 12%) in the culture medium. RINm5F-GK cells and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells also showed no difference in the cell viability after incubation at 3 mmol/liter glucose (99 ± 9 and 97 ± 9%) or 30 mmol/liter glucose (98 ± 6 and 97 ± 10%) compared with 10 mmol/liter (data not shown). Thus, neither glucokinase nor glucokinase plus islet PFK-2/FBPase-2 overexpression provoked glucose toxicity in RINm5F cells. Glucokinase enzyme activity was differentially regulated by high glucose concentrations in the culture medium in RINm5F-GK and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells.

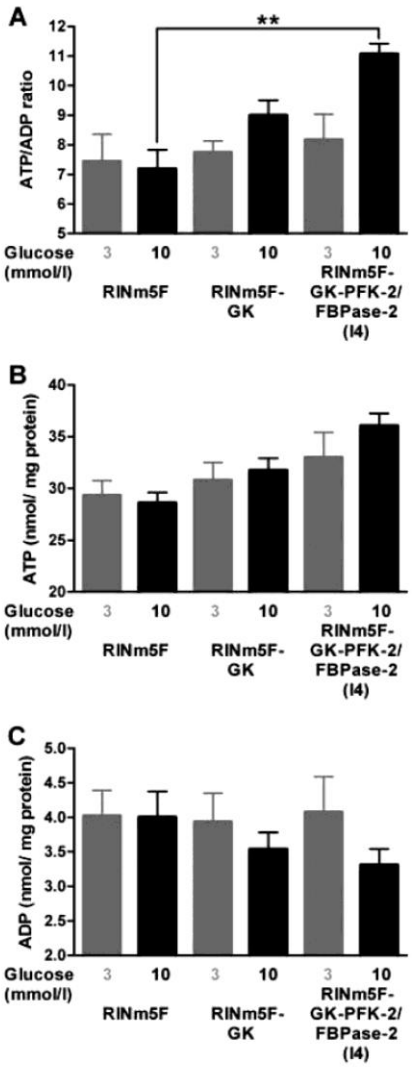


FIG. 6. ATP/ADP ratio (A), ATP content (B), and ADP content (C) in RINm5F, RINm5F-GK, and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells cultured at 3 and 10 mmol/liter glucose. Cells were grown for 24 h in medium with 10 mmol/liter glucose. Thereafter, cells were incubated for the next 48 h in medium with either 3 (*gray bars*) or 10 mmol/liter (*black bars*) glucose. After permeabilization of the cells, ATP and ADP contents were measured by a luminometric assay, and the ATP/ADP ratio was calculated. Shown are means ± SEM from three to five individual experiments. **, $P < 0.01$ compared with RINm5F cells (ANOVA/Bonferroni's test).

RINm5F-GK-PFK-2/FBPase-2 (I 4) cells cultured at 30 mmol/liter glucose showed 15% higher glucokinase enzyme activities in comparison to cells incubated at 10 mmol/liter glucose. In contrast, glucokinase enzyme activity in RINm5F-GK cells was reduced by 10% in cells cultured at 30 mmol/liter glucose in comparison to cells incubated at 10 mmol/liter glucose (data not shown).

*Effects of glucokinase overexpression and glucokinase plus islet PFK-2/FBPase-2 co-overexpression on pyruvate and lactate production in RINm5F cells*

For further characterization of glucose metabolism, RINm5F, RINm5F-GK, and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells were cultured for 48 h at 10 or 30 mmol/liter glucose (Fig. 7). Both lactate and pyruvate production increased, dependent on the glucose concentration in all cell types. Although pyruvate production was comparable in RINm5F and RINm5F-GK cells cultured at 10 mmol/liter glucose (Fig. 7A), the lactate production of RINm5F cells was slightly higher, with significantly higher lactate values at glucose concentrations of 0.5 and 2 mmol/liter in comparison to RINm5F-GK cells (Fig. 7B). Pyruvate and lactate production was significantly lower in RINm5F-GK-PFK-2/FBPase-2 (I 4) cells than in RINm5F-GK cells cultured at 10 mmol/liter glucose (Fig. 7, A and B). Pyruvate production was decreased in RINm5F and RINm5F-GK cells but not in RINm5F-GK-PFK-2/FBPase-2 (I 4) cells at 30 mmol/liter glucose in comparison to 10 mmol/liter glucose. Thus, at high glucose concentration, the pyruvate production in RINm5F-GK-PFK-2/FBPase-2 (I 4) cells was lower, but not significantly different from RINm5F-GK cells (Fig. 7C). Lactate production increased in all cells after incubation at 30 mmol/liter glucose. RINm5F cells showed in comparison with RINm5F-GK cells significantly lower lactate values at glucose concentrations of 5 and 10 mmol/liter (Fig. 7D). Notably, lactate production in RINm5F-GK-PFK-2/FBPase-2 (I 4) cells was significantly lower at all glucose concentrations when compared with RINm5F-GK cells (Fig. 7D).

*Insulin secretion in RINm5F-GK cells overexpressing islet PFK-2/FBPase-2*

Although RINm5F cells showed a basal insulin secretion rate of 18.5% in relation to insulin content and no demonstrable insulin secretory response to glucose (data not shown), glucokinase overexpressing RINm5F-GK cells as well as glucokinase plus islet PFK-2/FBPase-2 co-overexpressing RINm5F-GK-PFK-2/FBPase-2 (I 4) cells were responsive to glucose (Fig. 8). RINm5F-GK and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells exhibited basal insulin secretion rates of 10.9 and 11.1%, respectively, and a comparable insulin secretion at 0 and 1 mmol/liter glucose (Fig. 8). Notably, however, RINm5F-GK-PFK-2/FBPase-2 (I 4) cells showed a higher rate of insulin secretion than RINm5F-GK cells in response to 3, 5, 10, and 25 mmol/liter glucose (Fig. 8). Insulin secretion in response to KCl (25 mmol/liter), a nonnutritional stimulus, was not different in both cell types, and insulin content was not affected by overexpression of islet PFK-2/FBPase-2 in RINm5F-GK cells (data not shown).

## Discussion

Regulation of the glucose sensor enzyme glucokinase in pancreatic β-cells is complex and only partially understood. Posttranslational mechanisms are important for the adaptation of the glucokinase enzyme activity to the physiological requirements of the β-cell (6, 7, 11). Although glucose is the main stimulus in the process of glucokinase regulation (2, 5) other modulators are crucial to confer a precise adaptation of glucokinase enzyme activity to the needs of metabolic stimulus-secretion coupling. Intracellular structures such as insulin secretory granules and soluble proteins have been considered as potential regulators of glucokinase activity (10–14, 20, 31, 32). The bifunctional enzyme PFK-2/FBPase-2 is a cytoplasmic binding partner of glucokinase, which posttranslationally activates the enzyme in β-cells (18, 20). Overexpression of PFK-2/FBPase-2 resulted in a significant increase in glucokinase enzyme activity in insulin-producing RINm5F-GK and INS1 cells (18). Our present results indicate that, besides the physiological up-regulation of glucokinase

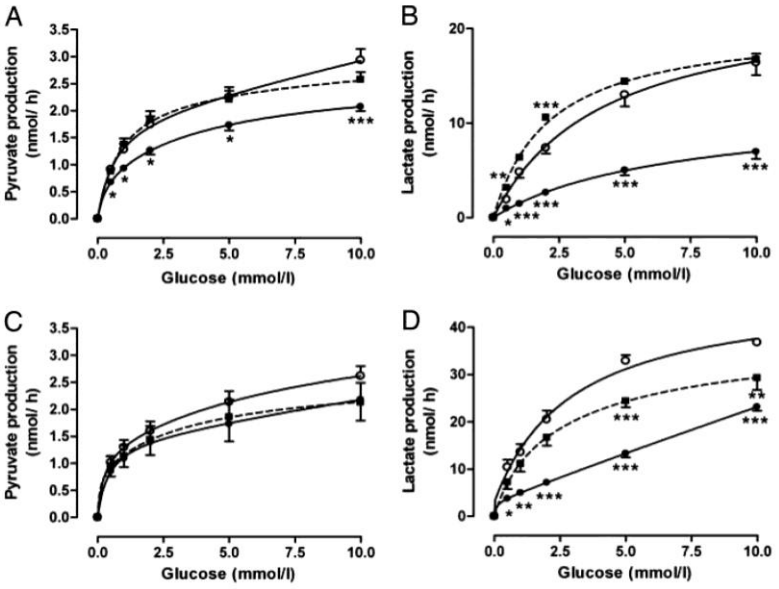


FIG. 7. Pyruvate and lactate production in RINm5F, RINm5F-GK, and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells. Measurements were performed in RINm5F cells (*black squares/dashed line*), RINm5F-GK cells (*white circles/closed line*), and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells (*black circles/closed line*). Cells were either grown for 72 h in medium with 10 mmol/liter glucose (A, B) or grown for 24 h in medium with 10 mmol/liter glucose and for the next 48 h in medium with 30 mmol/liter glucose (C, D). Thereafter, cells were incubated for 1 h at 37 C and 300 rpm in Krebs-Ringer buffer in the presence of various glucose concentrations (0.5, 1, 2, 5, and 10 mmol/liter). After centrifugation lactate and pyruvate contents were measured spectrophotometrically in the supernatant. Shown are means ± SEM from four to seven individual experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with RINm5F-GK cells (ANOVA/Bonferroni's test).

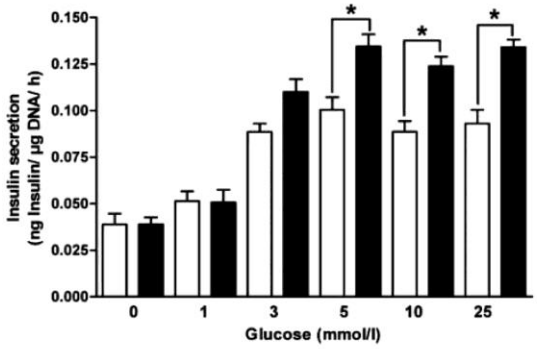


FIG. 8. Effects of islet PFK-2/FBPase-2 overexpression on insulin secretion from RINm5F-GK cells. RINm5F-GK cells (*white bars*) and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells (*black bars*) were incubated for 1 h at 37 C without or in the presence of various glucose concentrations (1, 3, 5, 10, and 25 mmol/liter). Thereafter, insulin was measured in the supernatant. Shown are means ± SEM from four to six individual experiments. *, $P < 0.05$ compared with RINm5F-GK cells (ANOVA/Bonferroni's test).

through endogenous PFK-2/FBPase-2 in β-cells, a further activation can be achieved by an additional expression of PFK-2/FBPase-2. Thus, the glucokinase enzyme activity level correlates with the PFK-2/FBPase-2 protein expression level. In islet PFK-2/FBPase-2 overexpressing insulin-producing RINm5F-GK cells, down-regulation of PFK-2/FBPase-2 by siRNA resulted in a concomitant decrease of glucokinase enzyme activity.

Because of the metabolic key role of glucokinase, changes in the enzyme activity status will directly affect the glycolytic flux and the initiation of insulin-secretion in pancreatic β-cells. This pivotal function is in particular illustrated in patients carrying activating or inactivating glucokinase gene mutations (7, 8, 33). As activation of glucokinase is a promising concept for treatment of type 2 diabetes, this enzyme proved to be an interesting target for antidiabetic drugs (15–17). The recently developed small chemical compounds activate glucokinase by binding to an allosteric site of the enzyme protein. The glucokinase activators RO281675, RO0274375, and LY2121260 are especially interesting molecules, because they are able to increase the $V_{max}$ value of glucokinase along with a decrease in the glucose $S_{0.5}$ (15, 17, 34). A sole decrease of the $S_{0.5}$ for glucose would result in an undesirable left-shift in the characteristic sigmoidal response curve of the enzyme. Thus, an increase of the $V_{max}$ value of glucokinase, which improves the sensor function within the millimolar glucose concentration range, is a major goal of glucokinase activation (34). Interestingly, glucokinase activation through PFK-2/FBPase-2 mirrors the physiological regulation with an increase in the $V_{max}$ value, whereas the $S_{0.5}$ for glucose remains unchanged (18). The activation of glucokinase enzyme activity by PFK-2/FBPase-2 improved metabolic stimulus-secretion coupling in insulin-producing cells (18). Importantly, the glucokinase activators RO281675, RO0274375, and LY2121260 induced a stimulation of glucose-induced insulin secretion in rat pancreatic islets (15, 17, 34).

It is well known that a reasonable overexpression of glucokinase protein in β-cells increases glucokinase enzyme activity and concomitantly glucose metabolism (21, 35). However, it is not clear whether high amounts of glucokinase protein will have the same effect upon glucose metabolism in β-cells as activation of constitutive physiological glucokinase protein levels. Activated glucokinase is present in the closed conformation and prevents the conformational transition to the super-open form as shown by crystallographic analyses (9). This is an important aspect based on the fact that impaired insulin secretion and a loss of cell viability have been described in insulin-producing cells after an excessive overexpression of glucokinase (36). Moreover, recently an increase in oxidative stress has been reported to aggravate glucose toxicity in insulin-secreting cells even after moderate overexpression of glucokinase (37).

In the present study, glucose metabolism has been analyzed in RINm5F-GK cells overexpressing the endogenous glucokinase activator PFK-2/FBPase-2 and compared with RINm5F-GK and RINm5F cells. These experiments have been performed in insulin-producing cells with the islet PFK-2/FBPase-2 isoform, which, in contrast to the liver isoform, is not regulated by phosphorylation and dephosphorylation (20, 38). Changes in the F-2,6-$P_2$ level obtained by overexpression of another PFK-2/FBPase-2 in RINm5F-GK cells, namely the liver or liver mutant isoform, revealed differences to the islet PFK-2/FBPase-2 isoform. Nevertheless, however, this overexpression in insulin-producing cells cannot mirror the metabolic regulation in hepatocytes. Clearly, control of the cellular F-2,6-$P_2$ concentration and glucokinase regulation by PFK-2/FBPase-2 in β-cells differ from the regulatory principles in liver (39–42). In β-cells, F-2,6-$P_2$ is of a subordinate physiological relevance, and the enzyme activity is dominated by the PFK-2 domain (43, 44). Our present results show that overexpression of the islet PFK-2/FBPase-2 isoform did not affect the regulation of the cellular F-2,6-$P_2$ concentrations and thus had no direct effect on glucose metabolism in insulin-producing cells.

The activation of glucokinase by PFK-2/FBPase-2 proved to be glucose dependent. Overexpression of islet PFK-2/FBPase-2 in RINm5F-GK cells resulted in a greater stimulatory effect of glucose on glucokinase enzyme activity when the glucose concentration was increased from 2 to 10 mmol/liter (18). Importantly, our present results demonstrate also a glucose-dependent increase in the colocalization between glucokinase and PFK-2/FBPase-2 in these cells. Thus, there is evidence that the binding site of glucokinase is mainly accessible for an interaction with PFK-2/FBPase-2 in the closed conformation of the enzyme when the substrate glucose is bound (9). This may explain why activation of glucokinase by PFK-2/FBPase-2 affects only the $V_{max}$ of the enzyme, but not the $S_{0.5}$ for glucose, and has to be elucidated in further experiments.

Overall glucose metabolism is up-regulated in RINm5F-GK cells overexpressing the glucokinase activator PFK-2/FBPase-2. Thus, the increase in glucokinase enzyme activity not only enhances glucose phosphorylation but also improves significantly the coupling between glycolysis and oxidative flux. Moreover, this improved metabolic coupling could also be demonstrated by a lesser accumulation of pyruvate and conversion to lactate in RINm5F-GK cells overex-

pressing PFK-2/FBPase-2 both in normal and high glucose culture. It should be noted that RINm5F cells exhibit high lactate dehydrogenase activities, thereby provoking anaerobic conversion of pyruvate into lactate (25). The improved metabolism in RINm5F-GK-PFK-2/FBPase-2 (I 4) cells is also demonstrated by an increase in the ATP/ADP ratio at 10 mmol/liter glucose compared with RINm5F-GK and RINm5F cells. In control RINm5F cells, glucose metabolism is dominated by considerable levels of high-affinity hexokinases (21). With glucokinase overexpression in RINm5F-GK cells, the β-cell characteristic responsiveness to millimolar glucose concentration could be established. RINm5F-GK cells showed both significantly lower basal insulin release than RINm5F cells and a threshold for glucose-induced metabolism, which is somewhat lower than in β-cells probably due to the high levels of low-$K_m$ hexokinases. Nevertheless, RINm5F-GK cells showed an increase in the ATP/ADP ratio as well as in glucose-stimulated insulin secretion in dependence on the millimolar glucose concentration. Importantly, additional overexpression of PFK-2/FBPase-2 in RINm5F-GK-PFK-2/FBPase-2 (I 4) cells caused a doubling of glucokinase activities with concomitant increases of the metabolic flux rates without any effect upon cell viability. This resulted in a higher ATP/ADP ratio and in a significant increase in glucose-stimulated insulin secretion compared with RINm5F-GK cells. This is in accordance with a report that the glucokinase activator RO0281675 augmented cellular respiration in parallel with the enhancement of glucose-stimulated insulin secretion (34).

In contrast to the liver, the glucose sensor function in β-cells is dependent upon relatively low glucokinase activities whose controlled activation through PFK-2/FBPase-2 or small chemical compounds seems to have a more integrative effect on glucose metabolism than excessive overexpression of the enzyme. Probably, the closed conformation of glucokinase may play a pivotal role in the macromolecular channeling of glycolytic intermediates. Metabolic compartmentation is a generally accepted biological phenomenon (45–47), but due to its great complexity its details are not well elucidated. Based on conformational data (9), it seems to be plausible that stabilized glucokinase is favorable for dynamic complex formation in the channeling process.

The present study supports the assumption that activation of glucokinase through interaction with the bifunctional enzyme PFK-2/FBPase-2 is an important element of posttranslational glucokinase regulation in pancreatic β-cells (8, 48–50). Furthermore, glucokinase activation is an attractive therapeutic strategy, and promising activating small chemical compounds have been described recently (15, 17, 34). The beneficial effect on key metabolic parameters in pancreatic β-cells by PFK-2/FBPase-2-mediated glucokinase activation will contribute to a better understanding of β-cell glucokinase regulation and promote further studies on the metabolic mechanisms underlying coupling between glucokinase activation and glucose-induced insulin secretion.

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## References

1. **Meglasson MD, Matschinsky FM** 1984 New perspectives on pancreatic islet glucokinase. Am J Physiol 246:E1–E13
2. **Lenzen S, Panten U** 1988 Signal recognition by pancreatic B-cells. Biochem Pharmacol 37:371–378
3. **Magnuson MA** 1990 Glucokinase gene structure. Functional implications of molecular genetic studies. Diabetes 39:523–527
4. **Iynedjian PB** 1993 Mammalian glucokinase and its gene. Biochem J 293:1–13
5. **Matschinsky F, Liang Y, Kesavan P, Wang L, Froguel P, Velho G, Cohen D, Permutt MA, Tanizawa Y, Jetton TL, Niswender K, Magnuson MA** 1993 Glucokinase as pancreatic β cell glucose sensor and diabetes gene. J Clin Invest 92:2092–2098
6. **Matschinsky FM** 1996 Banting lecture 1995. A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. Diabetes 45:223–241
7. **Matschinsky FM** 2002 Regulation of pancreatic β-cell glucokinase: from basics to therapeutics. Diabetes 51(Suppl 3):S394–S404
8. **Matschinsky FM, Magnuson MA, Zelent D, Jetton TL, Doliba N, Han Y, Taub R, Grimsby J** 2006 The network of glucokinase-expressing cells in glucose homeostasis and the potential of glucokinase activators for diabetes therapy. Diabetes 55:1–12
9. **Kamata K, Mitsuya M, Nishimura T, Eiki J, Nagata Y** 2004 Structural basis for allosteric regulation of the monomeric allosteric enzyme human glucokinase. Structure 12:429–438
10. **Toyoda Y, Yoshie S, Shironoguchi H, Miwa I** 1999 Glucokinase is concentrated in insulin-secretory granules of pancreatic B-cells. Histochem Cell Biol 112:35–40
11. **Tiedge M, Steffeck H, Elsner M, Lenzen S** 1999 Metabolic regulation, activity state, and intracellular binding of glucokinase in insulin-secreting cells. Diabetes 48:514–523
12. **Rizzo MA, Magnuson MA, Drain PF, Piston DW** 2002 A functional link between glucokinase binding to insulin granules and conformational alterations in response to glucose and insulin. J Biol Chem 277:34168–34175
13. **Rizzo MA, Piston DW** 2003 Regulation of β cell glucokinase by S-nitrosylation and association with nitric oxide synthase. J Cell Biol 161:243–248
14. **Arden C, Harbottle A, Baltrusch S, Tiedge M, Agius L** 2004 Glucokinase is an integral component of the insulin granules in glucose-responsive insulin secretory cells and does not translocate during glucose stimulation. Diabetes 53:2346–2352
15. **Grimsby J, Sarabu R, Corbett WL, Haynes NE, Bizzarro FT, Coffey JW, Guertin KR, Hilliard DW, Kester RF, Mahaney PE, Marcus L, Qi L, Spence CL, Tengi J, Magnuson MA, Chu CA, Dvorozniak MT, Matschinsky FM, Grippo JF** 2003 Allosteric activators of glucokinase: potential role in diabetes therapy. Science 301:370–373
16. **Brocklehurst KJ, Payne VA, Davies RA, Carroll D, Vertigan HL, Wightman HJ, Aiston S, Waddell ID, Leighton B, Coghlan MP, Agius L** 2004 Stimulation of hepatocyte glucose metabolism by novel small molecule glucokinase activators. Diabetes 53:535–541
17. **Efanov AM, Barrett DG, Brenner MB, Briggs SL, Delaunois A, Durbin JD, Giese U, Guo H, Radloff M, Gil GS, Sewing S, Wang Y, Weichert A, Zaliani A, Gromada J** 2005 A novel glucokinase activator modulates pancreatic islet and hepatocyte function. Endocrinology 146:3696–3701
18. **Massa L, Baltrusch S, Okar DA, Lange AJ, Lenzen S, Tiedge M** 2004 Interaction of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) with glucokinase activates glucose phosphorylation and glucose metabolism in insulin-producing cells. Diabetes 53:1020–1029
19. **Baltrusch S, Wu C, Okar DA, Tiedge M, Lange A** 2004 Interaction of GK with the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF2K/F26P2ase). In: Matschinsky FM, Magnuson MA, eds. Glucokinase and glycemic disease. Basel, Switzerland: Karger; 262–274
20. **Baltrusch S, Lenzen S, Okar DA, Lange AJ, Tiedge M** 2001 Characterization of glucokinase-binding protein epitopes by a phage-displayed peptide library. Identification of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase as a novel interaction partner. J Biol Chem 276:43915–43923
21. **Tiedge M, Elsner M, McClenaghan NH, Hedrich HJ, Grube D, Klempnauer J, Lenzen S** 2000 Engineering a glucose responsive surrogate cell for insulin replacement therapy of experimental insulin-dependent diabetes. Hum Gen Ther 11:403–414
22. **Lenzen S, Tiedge M, Panten U** 1987 Glucokinase in pancreatic B-cells and its inhibition by alloxan. Acta Endocrinol Copenh 115:21–29

23. **Baltrusch S, Francini F, Lenzen S, Tiedge M** 2005 Interaction of glucokinase with the liver regulatory protein is conferred by leucine-asparagine motifs of the enzyme. Diabetes 54:2829–2837
24. **Lenzen S, Panten U** 1980 2-Oxocarboxylic acids and function of pancreatic islets in obese-hyperglycaemic mice. Biochem J 186:135–144
25. **Alcazar O, Tiedge M, Lenzen S** 2000 Importance of lactate dehydrogenase for the regulation of glycolytic flux and insulin secretion in insulin-producing cells. Biochem J 352:373–380
26. **Hampp R** 1986 Methods of enzymatic analysis. In: Bergmeyer HU, ed. 3rd ed. Weinheim, Germany: VCH Verlagsgesellschaft Weinheim; 370–379
27. **Noll F** 1986 Methods of enzymatic analysis. In: Bergmeyer HU, ed. 3rd ed. Weinheim, Germany: VCH Verlagsgesellschaft Weinheim; 582–588
28. **Lamprecht W, Heinz F** 1986 Methods of enzymatic analysis. In: Bergmeyer HU, ed. 3rd ed. Weinheim, Germany: VCH Verlagsgesellschaft Weinheim; 570–577
29. **Tiedge M, Lortz S, Drinkgern J, Lenzen S** 1997 Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. Diabetes 46:1733–1742
30. **Mosmann T** 1983 Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55–63
31. **Murata T, Katagiri H, Ishihara H, Shibasaki Y, Asano T, Toyoda Y, Pekiner B, Pekiner C, Miwa I, Oka Y** 1997 Co-localization of glucokinase with actin filaments. FEBS Lett 406:109–113
32. **Shiraishi A, Yamada Y, Tsuura Y, Fijimoto S, Tsukiyama K, Mukai E, Toyoda Y, Miwa I, Seino Y** 2001 A novel glucokinase regulator in pancreatic $\beta$ cells: precursor of propionyl-CoA carboxylase $\beta$ subunit interacts with glucokinase and augments its activity. J Biol Chem 276:2325–2328
33. **Gloyn AL** 2003 Glucokinase (GCK) mutations in hyper- and hypoglycemia: maturity-onset diabetes of the young, permanent neonatal diabetes, and hyperinsulinemia of infancy. Hum Mutat 22:353–362
34. **Grimsby J, Matschinsky FM, Grippo JF** 2004 Discovery and action of glucokinase activators. In: Matschinsky FM, Magnuson MA, eds. Glucokinase and glycemic disease. Basel, Switzerland: Karger; 360–378
35. **Wang H, Iynedjian P** 1997 Modulation of glucose responsiveness of insulinoma $\beta$-cells by graded overexpression of glucokinase. Proc Natl Acad Sci USA 94:4372–4377
36. **Wang H, Iynedjian PB** 1997 Acute glucose intolerance in insulinoma cells with unbalanced overexpression of glucokinase. J Biol Chem 272:25731–25736
37. **Wu L, Nicholson W, Knobel SM, Steffner RJ, May JM, Piston DW, Powers AC** 2004 Oxidative stress is a mediator of glucose toxicity in insulin-secreting pancreatic islet cell lines. J Biol Chem 279:12126–12134
38. **Okar DA, Manzano A, Navarro-Sabate A, Riera L, Bartrons R, Lange AJ** 2001 PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose-2,6-bisphosphate. Trends Biochem Sci 26:30–35
39. **Wu C, Okar DA, Newgard CB, Lange AJ** 2001 Overexpression of 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase in mouse liver lowers blood glucose by suppressing hepatic glucose production. J Clin Invest 107:91–98
40. **Wu C, Okar DA, Newgard CB, Lange AJ** 2002 Increasing fructose 2,6-bisphosphate overcomes hepatic insulin resistance of type 2 diabetes. Am J Physiol Endocrinol Metab 282:E38–E45
41. **Wu C, Okar DA, Stoeckman AK, Peng LJ, Herrera AH, Herrera JE, Towle HC, Lange AJ** 2004 A potential role for fructose-2,6-bisphosphate in the stimulation of hepatic glucokinase gene expression. Endocrinology 145:650–658
42. **Payne VA, Arden C, Wu C, Lange AJ, Agius L** 2005 Dual role of phosphofructokinase-2/fructose bisphosphatase-2 in regulating the compartmentation and expression of glucokinase in hepatocytes. Diabetes 54:1949–1957
43. **Sener A, Van Schaftingen E, Van de Winkel M, Pipeleers DG, Malaisse-Lagae F, Malaisse WJ, Hers HG** 1984 Effects of glucose and glucagon on the fructose 2,6-bisphosphate content of pancreatic islets and purified pancreatic B-cells. A comparison with isolated hepatocytes. Biochem J 221:759–764
44. **Burch PT, Berner DK, Najafi H, Meglasson MD, Matschinsky FM** 1985 Regulatory role of fructose-2,6-bisphosphate in pancreatic islet glucose metabolism remains unsettled. Diabetes 34:1014–1018
45. **Srere PA, Ovadi J** 1990 Enzyme-enzyme interactions and their metabolic role. FEBS Lett 268:360–364
46. **Ovadi J, Srere PA** 2000 Macromolecular compartmentation and channeling. Int Rev Cytol 192:255–280
47. **Ovadi J, Saks V** 2004 On the origin of intracellular compartmentation and organized metabolic systems. Mol Cell Biochem 256–257:5–12
48. **Sarabu R, Grimsby J** 2005 Targeting glucokinase activation for the treatment of type 2 diabetes—a status review. Curr Opin Drug Discov Devel 8:631–637
49. **Printz RL, Granner DK** 2005 Tweaking the glucose sensor: adjusting glucokinase activity with activator compounds. Endocrinology 146:3693–3695
50. **Leighton B, Atkinson A, Coghlan MP** 2005 Small molecule glucokinase activators as novel anti-diabetic agents. Biochem Soc Trans 33:371–374

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# Molecular Coordination of Hepatic Glucose Metabolism by the 6-Phosphofructo-2-Kinase/Fructose-2,6-Bisphosphatase:Glucokinase Complex

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**Glucokinase (GK) and 6-phosphofructo-2-kinase (PFK-2)/fructose-2,6-bisphosphatase (FBP-2) are each powerful regulators of hepatic carbohydrate metabolism that have been reported to influence each other's expression, activities, and cellular location. Here we present the first physical evidence for saturable and reversible binding of GK to the FBP-2 domain of PFK-2/FBP-2 in a 1:1 stoichiometric complex. We confirmed complex formation and stoichiometry by independent methods including affinity resin pull-down assays and fluorescent resonance energy transfer. All suggest that the binding of GK to PFK-2/FBP-2 is weak. Enzymatic assays of the GK:PFK-2/FBP-2 complex suggest a concomitant increase of the kinase-to-bisphosphatase ratio of bifunctional enzyme and activation of GK upon binding. The kinase-to-bisphosphatase ratio is increased by activation of the PFK-2 activity whereas FBP-2 activity is unchanged. This means that the GK-bound PFK-2/FBP-2 produces more of the biofactor fructose-2,6-bisphosphate, a potent activator of 6-phosphofructo-1-kinase, the committing step to glycolysis. Therefore, we conclude that the binding of GK to PFK-2/FBP-2 promotes a coordinated up-regulation of glucose phosphorylation and glycolysis in the liver, *i.e.* hepatic glucose disposal. The GK:PFK-2/FBP-2 interaction may also serve as a metabolic signal transduction pathway for the glucose sensor, GK, in the liver. Demonstration of molecular coordination of hepatic carbohydrate metabolism has fundamental relevance to understanding the function of the liver in maintaining fuel homeostasis, particularly in managing excursions in glycemia produced by meal consumption. (*Molecular Endocrinology* 21: 1478–1487, 2007)**

THE BIFUNCTIONAL ENZYME 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBP-2; Bif) is an important regulator of cellular fuel metabolism, especially in the liver (1, 2). Its primary function is to control the intracellular content of the biofactor fructose-2,6-bisphosphate (F-2,6-$P_2$), a potent activator of 6-phosphofructo-1-kinase (PFK-1) (3). Activation of PFK-1 *in vivo* by increased hepatic F-2,6-$P_2$ has been conclusively demonstrated (4, 5). In addition to confirming that increased hepatic F-2,6-$P_2$ activates glycolysis and reduces glycemia, some reports also suggested that this metabolic regulator coordinates hepatic glycolysis with lipid metabolism (5). Predicated on yeast 2 hybrid and phage display results suggesting that liver PFK-2/FBP-2 bound to glucokinase (GK; hexokinase 4) (6) and using a graded overexpression of Ser-32-dephosphorylated rat liver PFK-2/FBP-2, Payne *et al.* (7) have demonstrated the retention of GK in the cytosol of mouse liver is promoted by the bifunctional enzyme. Serine-32-dephosphorylated hepatic PFK-2/FBP-2 is also a net producer of F-2,6-$P_2$ and is generated from phosphoserine-32 bifunctional enzyme by the action of protein phosphatases that are stimulated by glucose via xylulose-5-phosphate (8) and/or insulin signaling (9). Conversely, PFK-2/FBP-2 is phosphorylated by protein kinase A, activated in response to glucagon via increased cellular cAMP (10).

GK, or hexokinase 4, also has a major role in regulating fuel metabolism. It has been described as a glucose sensor, primarily on the basis of its function in pancreatic $\beta$-cells where it couples the abundance of postprandial serum glucose to the release of insulin (11). How GK functions as a glucose sensor in the liver has been less obvious because it has not been clear to which cellular response the GK signal is coupled. Regardless, because the glucose transporter 2 in hepatocytes is not strongly regulated, hepatic GK essentially controls glucose uptake, which suggests a more

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Abbreviations: AF, Alexa Fluor; FBP-2, fructose-2.6-bisphosphatase; FI, fluorescence intensity; F-2,6-$P_2$, fructose-2,6-bisphosphate; FRET, fluorescent resonance energy transfer; GK, glucokinase; HK1, yeast hexokinase 1; NADP, nicotinamide adenine dinucleotide phosphate; Ni:NTA, nickel-nitrilotriacetic acid; PEG, polyethylene glycol; PFK-1, phosphofructo-1-kinase; PFK-2, 6-phosphofructo-2-kinase; PKI, protein kinase A inhibitor; TPI, triose phosphate isomerase; XSFI, excess fluorescence intensity.

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fundamental role than simply sensing glucose (12). Due to the role of the liver in maintaining systemic glucose homeostasis, perhaps it is not surprising that the orchestration of the complex metabolic responses to nutritional and hormonal signals requires the close association of two very powerful metabolic regulators such as GK and PFK-2/FBP-2. Yet, despite all of the *in vivo* evidence that these proteins work together, no direct evidence of their binding was detected (7, 13). This is an essential point as there are many ways in which proteins can interact without binding, especially on the time scale (days) required for studies, such as those of Payne *et al.* (7), which use adenovirus-mediated overexpression of cellular proteins. A more recent report by Garcia-Herrero *et al.* (14) has demonstrated pull down of $^{35}$S-labeled PFK-2/FBP-2 by affinity resin-bound GK from extracts of Hep2G cells, but even these crucial observations do not conclusively settle the issue of whether GK and PFK-2/FBP-2 directly interact.

For these reasons we set out to investigate the *in vitro* binding of GK to the FBP-2 domain and PFK-2/FBP-2. For this purpose we chose the Alexa Fluor (AF) series of dyes (Molecular Probes, Inc., Eugene, OR), which have very well-defined absorbance and fluorescence spectra and are available with a variety of reactive groups directed toward different moieties of the surface of the proteins. We used two amine-reactive AF dyes that also support fluorescent resonance energy transfer (FRET) with AF-488 as donor and AF-594 as acceptor. The nonradiative FRET is distinct from radiative transfer of fluorescent energy, which is due to the excitation of the acceptor by the light fluoresced by the donor, because it is a direct transfer of energy between the excited states (15). The FRET signal is detected as fluorescence intensity (FI) at the emission wavelength of the acceptor with excitation of the donor. FRET is inversely proportional to the sixth power of the distance between the donor and acceptor (15). The only way the dyes on different proteins can produce a FRET signal is if they bind to each other because these dyes must come within approximately 50 A to support FRET. Because we have not undertaken the protein engineering required to assure single-site labeling of these enzymes, the labeling reactions produce multiply labeled proteins, which severely impedes quantitative FRET studies aimed at determining the relative orientation of the proteins in the complex. Even so, by exploiting a qualitative use of FRET, we devised a series of experiments in which we titrated the FBP-2 domain (ND249) tagged with an acceptor fluorophore (AF-594) with GK tagged with a donor (AF-488). Because we measured the FI of the acceptor while exciting the donor, if FRET occurs during the titration, the FI will be limited by complex formation at high concentrations of titrant. In addition to reconfirming the weak binding, this method also determines the stoichiometry of the complex.

We also determined whether and how the binding of GK and PFK-2/FBP-2 affects their activities. These critical data highlight the switch-like properties of GK and PFK-2/FBP-2, which provide for at least a portion of the acute reversal of hepatic carbohydrate metabolism during the immediate response to refeeding after a fast. According to theory, each molecular contribution to biological switching adds with the others, until at the macroscopic level, a near step-function switch can be attained by a subset of cellular proteins (16–18). Whether such constitutes a molecular machine is a question for other publications, but we are not the first to suggest this possibility for the PFK-2/FBP-2 and F-2,6-$P_2$ (19). By this analysis, the physical junction of these two biological switches, especially within the context of the *in vivo* studies, has a fundamental role in providing the kind of synergistic molecular interactions that, in sum, produce efficient biological switching within the liver.

## RESULTS

### GK Binds Specifically to the FBP-2 Domain of PFK-2/FBP-2

The fluorescent labeling of GK and ND249 (an N-terminal deletion of 249 amino acids from the rat liver bifunctional enzyme), the FBP-2 domain, allowed us to trace these proteins in nickel-nitrilotriacetic acid (Ni:NTA) resin pull-down assays using $H_6$PFK-2/FBP-2 and $H_6$GK, respectively. Figure 1A shows that including $H_6$GK promotes the retention of ND249*594 (the * indicates fluorescent tag and the number identifies which AF dye was used) by the resin, indicating that GK binds to the FBP-2 domain. Furthermore, the ability of a 5-fold molar excess of unlabeled ND249 to elute the ND249*594 from the pellet (Fig. 1B) confirms that the binding is reversible. When the binding of ND249*488 to $H_6$GK was challenged by proteins other than unlabeled ND249 (Fig. 1C), neither the glycolytic enzymes triose phosphate isomerase (TPI) and yeast hexokinase 1 (HK1), nor BSA could effectively wash ND249*488 from the complex, thus confirming that the binding of the FBP-2 domain to GK is specific. The binding of GK to the intact bifunctional enzyme was assessed by the pull-down assay summarized in Fig. 2, where $H_6$PFK-2/FBP-2 was shown to bind to GK*594 or GK*488. With this set of experiments we have exhaustively assessed the GK:PFK-2/FBP-2 complex with each binding partner as the bait and the prey, while demonstrating that the interaction is specific.

### The Molar Stoichiometry of the Complex Is 1:1

Because the pull-down assays suggested that the binding of PFK-2/FBP-2 and GK is a relatively weak interaction, we sought to both reconfirm its formation and determine the stoichiometry by titrating ND249*594 with GK*488 while observing the emission spectrum of the acceptor (AF-594) produced by excit-

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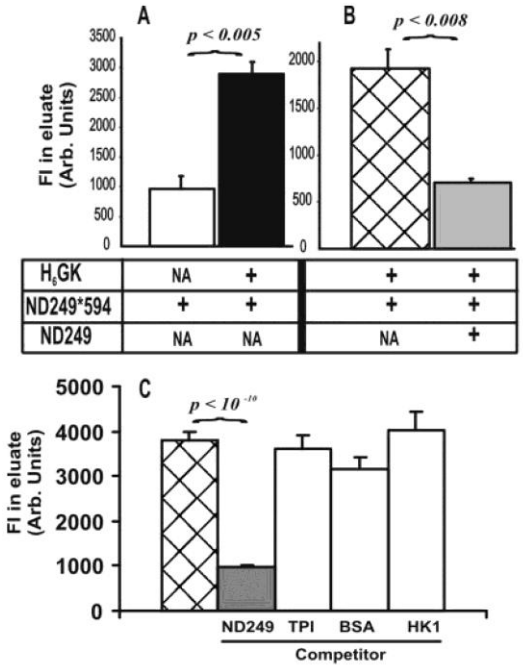


**Fig. 1.** Affinity Resin Pull-Down Assays

Panel A demonstrates that $H_6$GK can pull down the liver FBP-2 domain, ND249*594, because more fluorescent protein was eluted from the resin when the bait was included (*solid bar*) relative to when it was not (*open bar*). Panel B shows that a 5-fold molar excess of unlabeled ND249 displaced the ND249*594 from the resin (*gray bar*). The data are background corrected, *i.e.* the *hatched bar* is the difference between the bars in panel A and accounts for the nonspecific binding of ND249 to the resin. All data are averages over three experiments conducted using the same stock proteins. Panel C reiterates that ND249 (*gray bar*) washes ND249*488 (*hatched bar*) from the pellet and the control proteins (*open bars*) do not. Competitor proteins were: TPI, yeast hexokinase 1 (HK1), and BSA. n = 9 for *hatched* and *gray bars*; n = 3 for the *open bars*. The SEM is indicated for each bar and the *P* values were calculated by Student's *t* test. Result: ND249 binding to GK is reversible and saturable. NA, Not added; Arb., arbitrary.

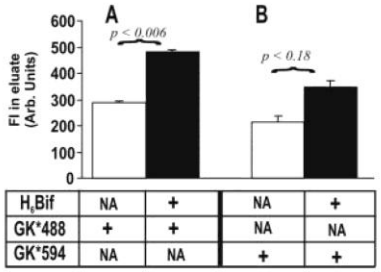


**Fig. 2.** Affinity Resin Pull-Down Results

Panels A and B show results using two differently labeled GK preparations. *Open bars* are nonspecific binding and *solid bars* are from samples including the bait. The average FI determined for each fluorophore over four determinations are reported with SEM; indicated *P* values were calculated by Student's *t* test.. Result: GK binds to PFK-2/FBP-2. NA, Not added; Arb., arbitrary.

ing the donor (AF-488), as shown in Fig. 3, A and B. Initially, we adjusted the conditions of the amine-reactive labeling reactions to minimize the dye-protein ratio (data not shown), but these did not provide sufficient sensitivity to reproducibly detect protein binding, so we completed the studies with fluorescently labeled proteins (GK*488 and ND249*594) with dye-to-protein ratios from 3–4. The representative fluorescence spectra in Fig. 3, A and B, show enhanced FI in the mixed samples. This excess fluorescence intensity (XSFI) may be seen more clearly by subtracting the spectra as detailed in *Materials and Methods*. Representative difference spectra for a complete titration are given in Fig. 3C. Note the similarity of the two highest GK*488 points, suggesting saturation. By plotting XSFI at 621 nm as a function of GK*488 concentration, as in Fig. 3D, the nonlinear result suggests that some portion of the energy transferred between the fluorophores is due to FRET. Because the donor and acceptor must be rather close in space to accomplish FRET, these data confirm the formation of a GK:FBP-2 complex. The representative data in Fig. 3D were acquired by titrating a 5 $\mu$M sample of ND249*594 with GK*488. The titration curve plateaus near the 1:1 molar stoichiometry, thus determining the stoichiometry of the complex.

### Molecular Coordination of Hepatic Glucose Metabolism

We hypothesized that formation of the GK:PFK-2/FBP-2 complex, in and of itself, serves to increase both the activity of GK and the kinase-to-bisphosphatase ratio of PFK-2/FBP-2. To test this hypothesis we investigated whether the activities of the enzymes were influenced by formation of the protein-protein complex. When GK activity was monitored as a function of PFK/2/FBP-2, as shown in Fig. 4, a significant activation of GK was observed at a 10-fold molar excess of PFK-2/FBP-2. When purified GK was added to PFK-2/FBP-2 *in vitro*, no significant change in PFK-2 or FBP-2 activities was detected. Considering that the interaction we were investigating had previously been predicted based on the results of *in vivo* studies, we reasoned that a putative missing component exists in the cell that serves to facilitate the binding of the proteins, thus increasing the relative population of the bound state. Therefore, we obtained extracts of mouse livers from 16-h fasted animals and 30-min post bolus glucose infusion as mimic of the fed state, and we used these in hopes of adding back the missing component(s). The extracts were prepared to preserve total protein. Each extract was diluted to 1 mg/ml total protein with extract buffer. These diluted fed liver extracts contained more F-2,6-$P_2$ than did those fasted livers (1.77 $\pm$ 0.03 pM in fed liver extracts *vs.* undetectable in fasted liver extracts), which reflects the hepatic response to refeeding. These values are

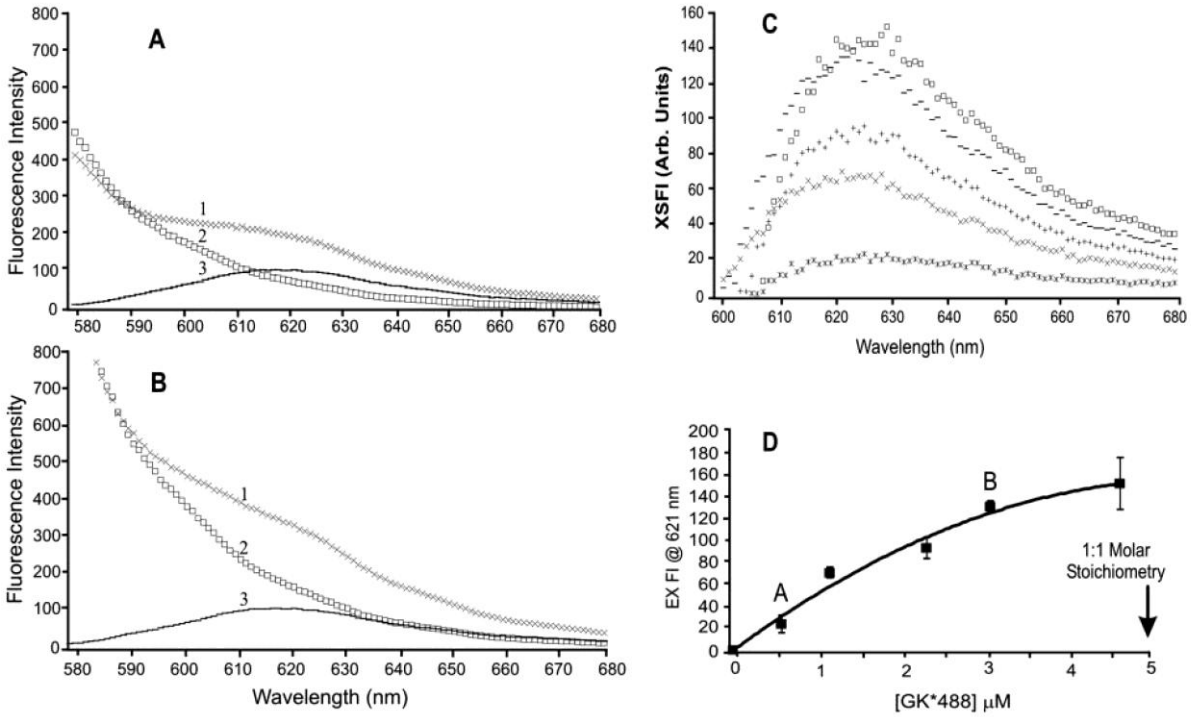


**Fig. 3.** FRET Titration of ND249*594 with GK-488

Panels A and B are representative raw data showing the fluorescence emission spectra from the microplate: 1) mixed sample; 2) GK*488 alone; and 3) ND249*594 alone. The labels in panel D indicate where the data in panels A and B fall in the titration curve. At each point, the amount of GK*488 (traces 1 and 2) is the same, whereas that of ND249*594 is constant over all points (trace 3). The difference spectra (see Equation 1) for an entire titration series is given in panel C. The FRET titration curve is plotted in panel D, which clearly shows a plateau near a 1:1 molar stoichiometry. The average over four determinations ± SEM are reported with a spline fit. Arb., Arbitrary.

lower than typically reported because the method we used to prepare the liver extracts was not optimal for preservation of F-2,6-$P_2$, which requires extraction with alkali (20).

The absolute PFK-2 activity of endogenous PFK-2/FBP-2 in mouse liver extracts was low, and the specific activity decreased as more extract was included in the assays, as shown in Fig. 5A. These demonstrated no significant correlation with nutritional state. This result is, perhaps, not surprising because the concentration of PFK-2/FBP-2 in the extracts is low, and previous studies have shown that it must be partially purified by polyethylene glycol (PEG) precipitation to clearly reveal the effects of changing nutritional state. We interpret the data in Fig. 5A as the dilution of some PFK-2 inhibitory component of the liver extracts that relieves inhibition more effectively when it is more highly diluted, *i.e.* when smaller aliquots of the extracts are used. The nature of this putative inhibitor remains unknown. Experimentally we could not use less than 10 $\mu$l of the extracts, because although the specific activity was high, the absolute activities were quite low. Interestingly, the addition of purified GK to liver extracts produces higher PFK-2 activity, as shown in Fig. 5, B and C, for fed and fasted extracts, respectively. Addition of the GK dialysis buffer did not activate the endogenous PFK-2, nor did the GK stock exhibit any PFK-2 activity (data not shown). The paired *t* test is justified because we included the extract controls in each assay. Of the eight total conditions, five are significantly different to at least 90% probability, three to greater than 95%. The increased PFK-2 activity in the presence of GK was more prominent for fasted liver extracts (Fig. 5C) relative to fed (Fig. 5B),

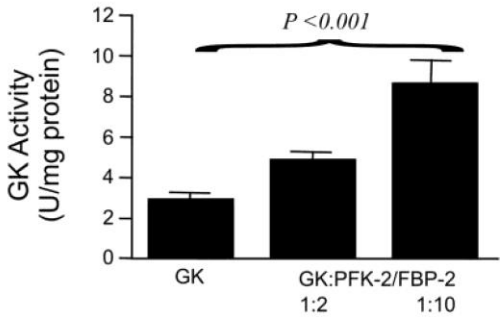


**Fig. 4.** GK Is Activated by PFK-2/FBP-2

The specific activity of GK is increased by addition of PFK-2/FBP-2 at equimolar and 10-fold molar excess. The increase attains significance at the 1:10 point. The averages over four experiments are reported ± SEM.

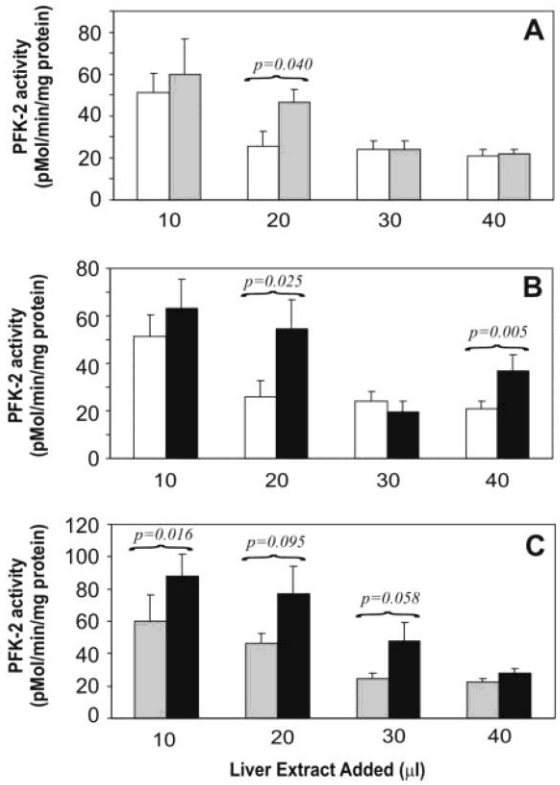


**Fig. 5.** PFK-2 in Liver Extracts Is Activated by Added GK

The specific PFK-2 activity in liver extracts from 16-h fasted (*gray bars*) and fed (infused with a glucose bolus of 2 g/kg body weight over 30 min) (*open bars*) mouse livers are shown in panel A. The *P* value reported in panel A is unpaired, whereas all others are from paired *t* tests. Panels B (fed extracts) and C (fasted extracts) show that when 5 μM GK was added (*solid bars*) to either liver extract, the PFK-2 was significantly activated in five of eight conditions. When buffer was substituted for the added GK, no activation was detected. Data reported are the averages over all assays at each amount of extract ± SEM. n = 12 for fasted extracts and 14 for fed.

but essentially the effect of GK on the PFK-2 activity was independent of the nutritional state. Paired Student's *t* tests using all data for each nutritional state, regardless of the amount of extract assayed, gave $P = 0.006$ and 0.001 for fed and fasted extracts, respectively. Thus, the activation of endogenous PFK-2 in liver extracts by added GK is significant, yet correlates only slightly with nutritional state. The trend is toward more significant activation of extract PFK-2 by GK in the fasted state, perhaps reflecting the loss of GK upon fasting, but this suggestion has many caveats. When proteins other than GK were added to fed and fasted liver extracts (Fig. 6, A and B), no activation of endogenous PFK-2 was observed; in fact, addition of TPI, HK1, or BSA appeared to facilitate loss of PFK-2 activity in the extracts, again independent of nutritional state. The addition of protein kinase A inhibitor (PKI) to the liver extracts did not activate PFK-2 either, suggesting that changes in the posttranslational modification state of PFK-2/FBP-2 are not occurring during our assays. Little influence of GK on FBP-2 activity in either PFK-2/FBP-2 or ND249 was observed *in vitro*. Because the liver extracts quenched the fluorescence of oxidized nicotinamide adenine dinucleotide phosphate (NADP)+ that is the read-out of the FBP-2 assay, we were precluded from investigating the effect of these on FBP-2 activity.

## DISCUSSION

Based on the results of these studies we conclude that GK binds reversibly to bifunctional enzyme at a site in the FBP-2 domain to form a 1:1 stoichiometric complex. Moreover, the pull-down assays demonstrate that when the proteins were mixed in equimolar proportions, the extent of complex formation is essentially the same whether we used PFK-2/FBP-2 or the ND249 bisphosphatase domain as binding partner for GK. This localizes the GK binding site to the FBP-2 domain, consistent with the prediction of Baltrusch *et al.* (6). This is the first evidence of direct binding between GK and PFK-2/FBP-2 proteins. Because the bifunctional enzyme functions as a dimer, this implies the heterotetrameric structure, $GK_2$:$Bif_2$, for the full complex. In contrast, because the ND249 is monomeric, a dimeric structure, GK:ND249, is implied. Within the context of the *in vivo* studies (7), our results confirm direct binding of GK and PFK-2/FBP-2 as a mechanism by which these two enzymes interact within the hepatocyte. Very recently, this interaction has been observed in the human hepatoma cell line Hep2G by affinity resin pull down of $^{35}$S-labeled PFK-2/FBP-2 by GK (14), yet those crucial studies did not preclude the participation of another protein in the complex. This is an important point because the interaction of PFK-2/FBP-2 with the scaffolding protein 14-3-3 has been reported (21).

Our most interesting observations are the enzyme kinetic data that suggest formation of the PFK-2/FBP-2:GK complex activates GK and PFK-2, whereas the FBP-2 is, apparently, unchanged. The data in Figs. 5 must be interpreted with care, however, due to the inclusion of live liver extracts in the PFK-2 assays. The specific PFK-2 activities in various amounts of the liver extracts (Fig. 5A) demonstrate that the more dilute the extract, the higher the specific activity. These data formalize long-standing anecdotal observations that PFK-2 activity is inhibited by some component in cell extracts. This observation does not undermine any previous reports about the activities of PFK-2/FBP-2 from tissue or cell lysates because the bifunctional enzyme was routinely concentrated and partially purified by PEG precipitation in those studies. Here we sought to maintain all components of the extracts because we sought to complement the interaction between GK and PFK-2/FBP-2. The observed effect of

dilution upon PFK-2 specific activity compelled us to assure that when we added purified GK to the extracts (*closed bars* in Fig. 5) the extent of dilution was exactly the same as in the blanks (*open* and *gray bars* in Fig. 5). This same condition also applied to the addition of control proteins to the extracts (Fig. 6). None of these control proteins showed any activation of PFK-2, reiterating that the effect of GK is specific and significant. Moreover, our inclusion of PKI in the control experiments suggests that changes in the phosphorylation state of PFK-2/FBP-2 are not at the root of the activation of PFK-2 by GK. It is surprising, perhaps, that we see no significant correlation of these effects with the nutritional state. This may be related to the nature of the live liver extracts, which are not optimal for assay of the bifunctional enzyme due to the lack of precipitation by PEG, which would be incompatible with our purposes in these studies. These caveats leave open the question of which particular component(s) in the liver extracts promote the activation of PFK-2 by GK. Taken together with the observed weak binding between GK and PFK-2/FBP-2, the PFK-2 activation data suggest at least two possibilities: 1) activation of PFK-2 by binding of GK requires some other component in the liver extracts; or 2) some component in the liver extracts promotes binding of GK to PFK-2/FBP-2. Although the former cannot be conclusively eliminated, the latter is consistent with weak binding *in vitro*, and published observations that suggest the *in vivo* binding is more extensive (6, 7, 13, 14).

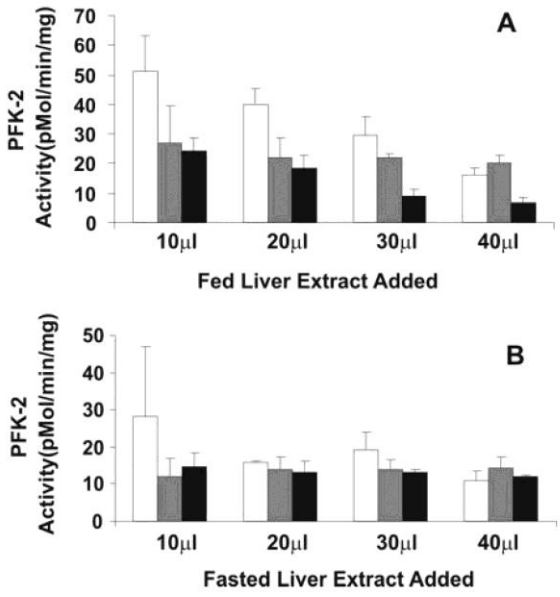


**Fig. 6.** PFK-2 in Liver Extracts Is Not Activated by Adding Proteins Other Than GK

The *open bars* in either panel are the extracts diluted with buffer. A, Fed liver extracts with either BSA (*gray bars*) or HK1 (*solid bars*) added to the extracts. B, Fasted liver extracts with either TPI (*gray bars*) or PKI (*solid bars*) added to the extracts. n = 6 for *open bars*; n = 3 for all others. No statistically significant differences were observed at any point.

Conversely, the activation of GK by binding of PFK-2/FBP-2 (Fig. 4) does not require the presence of liver extracts. The requirement of a 10-fold molar excess of PFK-2/FBP-2 is consistent with weak binding, because such implies a low population of the bound state.

The binding of GK to PFK-2/FBP-2 both increases the phosphorylation of glucose and the net synthesis of F-2,6-$P_2$, implying a molecular coordination of two sequential steps in hepatic glucose metabolism that directly couple serum glucose to glycolysis, as depicted in Fig. 7. We do not mean to suggest that the liver is primarily responsible for the clearance of excess blood glucose, because the role of skeletal muscle in this regard has been well established. At the same time, we cannot discount the possibility that the liver is responsible for some glucose disposal, as the early work of Wu *et al.* (22) suggested that the reduction in serum glucose was closely associated with increasing hepatic F-2,6-$P_2$ and lactate content, even before the effect of adenoviral-mediated overexpression of PFK-2/FBP-2 was maximal. Moreover, the 1[$^{13}$C]glucose magnetic resonance spectroscopy study of Choi *et al.* (23) clearly detected a more active PFK-1 in the livers of living mice that were overexpressing a mutant PFK-2/FBP-2 that produced chronically elevated hepatic F-2,6-$P_2$. At the same time, if our observation of weak binding between GK and PFK-2/FBP-2 is reflected *in vivo*, it may argue against the GK/PFK-2/FBP-2 complex contributing quantitatively to the increase in metabolic flux from serum glucose to glycolysis because it implies a relatively low population of the bound state. However, it may be that another factor in the liver promotes binding of GK to PFK-2/FBP-2, increasing the population of the bound state. At the same time, the reported effects of elevated hepatic F-2,6-$P_2$ on gene expression (24), GK

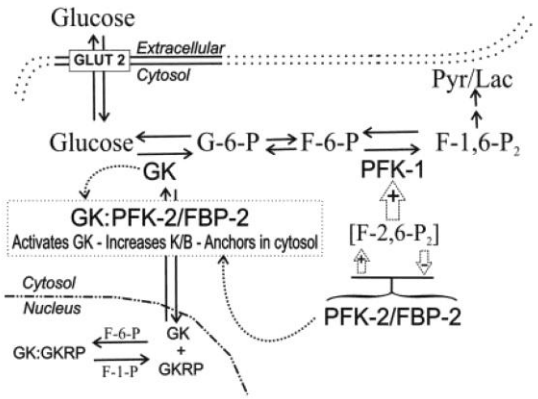


**Fig. 7.** Schematic Model of the Role of GK:PFK-2/FBP-2 in Hepatic Metabolism

Formation of the complex directly couples increased glucose phosphorylation uptake in the liver with the commitment to glycolysis via F-2,6-$P_2$. G-6-P, Glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1-P, fructose-1-phosphate; GLUT2, glucose transporter 2; GKRP, GK regulatory protein.

translocation (7), and Akt phosphorylation state in mouse liver (25) suggest that the binding of bifunctional enzyme to GK may elicit profound regulatory effects even if it cannot quantitatively increase hepatic glucose disposal. From this perspective, the glucose-sensing capabilities of GK are adapted to the regulation of liver metabolism by interaction with PFK-2/FBP-2.

The molecular coordination revealed by our experiments is very interesting, because it serves to amplify the switch-like behavior implied by the translocation of GK in response to the regulatory dephosphorylation of Ser-32 in PFK-2/FBP-2 (3, 26). As others have pointed out, the ability of a biological system to exhibit switch-like properties, such as those that underlie the acute hepatic response to refeeding, is no small feat (18, 27). The theoretical discussion of Hervagault and Canu (28) suggests that bistability underpins fast biological switching, and it has been suggested that these can be built up by the synergistic interaction of many different mechanisms. It is clear that GK, PFK-2/FBP-2, and F-2,6-$P_2$ work together to provide one component of the biological switch that allows the liver to accomplish the acute response to refeeding after a fast. The GK regulatory protein is clearly a major player in this switching mechanism, and it is only brevity that precludes its inclusion in our discussion (29). Therefore, our results underscore the potential of targeting hepatic F-2,6-$P_2$ content as a desirable point of intervention to ameliorating the perturbations of carbohydrate and lipid metabolism that are associated with obesity and diabetes. We have also identified a new target for the design of novel pharmaceuticals because stabilizing the GK:PFK-2/FBP-2 complex will increase hepatic glucose disposal.

## MATERIALS AND METHODS

### Experimental Animals

No animals were killed for these studies. All liver extracts obtained were from excess sample generated by Dr. Wu in the course of his work at the University of Minnesota. Accordingly, all protocols were reviewed and approved by the Institutional Animal Care and Use Committees at the University of Minnesota.

### Expression and Purification of Proteins

All proteins were expressed from plasmid vectors in *Escherichia coli* (BL21DE3, M15, and XK1Blue) and subsequently purified by either the histidine-binding resin Ni:NTA affinity chromatography (for His-tagged;$H_6$ proteins), strepavidin (for Strep-Tag PFK-2/FBP-2), or slight modification of established chromatographic methods (for non-$H_6$ proteins) (30–32). We modified the purification methods for non-$H_6$ proteins by implementing them on the BioCAD Perfusion Chromatography System and POROS resins, which increased the yield and minimized the time required for protein production. SDS-PAGE and standard kinetic assays were used to assess the quality of the purified proteins and all data reported were derived from fresh samples of at least 90% purity (see supplemental Figs. 1 and 2 published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). We have produced six different proteins for use in these studies, *i.e*. human liver GK and $H_6$GK, rat liver PFK-2/FBP-2, $H_6$PFK-2/FBP-2, Strep-Tag PFK-2/FBP-2, and ND249. The ND249 bisphosphatase domain is an N-terminal deletion of 249 amino acids from the rat liver bifunctional enzyme sequence that has been extensively studied by x-ray crystallography and nuclear magnetic resonance spectroscopy. Proteins were expressed from either pET 3a, pET 16b, pASK-IBA7, or pQE30, plasmids for ND249, PFK-2/FBP-2, $H_6$PFK-2/FBP-2, Strep-Tag PFK-2/FBP-2, and $H_6$GK, respectively [Novagen (Madison, WI) or QIAGEN (Chatsworth, CA)]. The pLysS system was used for expression of PFK-2/FBP-2 (Novagen). Sequence comparisons for GK and PFK-2/FBP-2 between human and rat liver show extensive identity and almost complete homology; therefore, the difference in species should not influence the results or conclusions.

### Labeling Proteins with Extrinsic Fluorophores

Only non-$H_6$ proteins were labeled with AlexaFluor (AF) amine-reactive fluorophores. Purified proteins were exchanged into the labeling buffer and reacted with either Alexa Fluor-488, or AF-594 according to the protocols supplied by the manufacturer (Molecular Probes). After the labeling reactions were complete, the unreacted dye and other small molecule reaction products were removed by desalting and dialysis into the storage and binding buffers. Dyes were chosen to support FRET with AF-488 as donor and AF-594 as acceptor ($R_0$ = 60). Because the fluorophores are well characterized and the extinction coefficients known, the amount of these in the protein samples can be determined and then compared with the amount of protein as determined by the Coomassie dye binding assay (Bio-Rad Laboratories, Inc., Hercules, CA).

### Affinity Resin Pull-Down Assays

The nickel chelating resin, Ni:NTA, was used to pull down proteins with the six histidine ($H_6$) affinity tag fused to the N terminus. All experiments were performed by adding samples containing the proteins to small vials containing wet Ni:NTA resin equilibrated in PBS (10 mM phosphate; 2.7 mM KCl; 137 mM NaCl, pH 7.4). To probe for binding of GK to the FBP-2 domain of PFK-2/FBP-2, the $H_6$ proteins were used as bait to pull the fluorescently labeled binding partners from solution. The results were revealed by assaying for the fluorescent protein after eluting the bait $H_6$-proteins with 500 mM imidazole in the M5 microplate reader by acquiring the pertinent emission spectrum of the AF dye used. $H_6$GK was used to pull down ND249*594 (ND249 tagged with AF-594) and to probe for reversibility by including a 5 molar excess of unlabeled ND249. Control experiments were conducted in exactly the same way, but substituted either TPI, HK1, or BSA for the unlabeled ND249. In a separate experiment, $H_6$PFK-2/FBP-2 was used to pull down either GK*594 or GK*488.

All pull-down experiments were conducted in PBS by the following protocol. First, 75 $\mu$l of a well-suspended, charged, 50% Ni:NTA resin slurry was dispensed into small vials. These were centrifuged very lightly, and the supernatant was quantitatively removed by pipet. The resin was equilibrated with fresh binding buffer (250 $\mu$l), which was removed by centrifugation and pipetting. The protein samples were premixed (total volume 250 $\mu$l) from concentrated protein stocks and binding buffer. Although the absolute amount of protein added varied from assay to assay, the molar ratio of GK to either PFK-2/FBP-2 or ND249 was typically 1, although in a few cases a 2–3 molar excess of GK was used. Protein concentrations ranged from 200 pM to 1.2 $\mu$M in the samples before they were added to the resin. Blanks were prepared using the dialysate from the $H_6$ protein samples in place of

the proteins themselves. After approximately 15 min incubation at room temperature, the mixed samples were added to the equilibrated resin, mixed gently, incubated for approximately 10 min, and centrifuged, and the supernatant was removed and placed in the wells of a 96-well microplate. The pellets were quickly washed with binding buffer (200 $\mu$l), and the wash was also placed in the microplate. The $H_6$ proteins were eluted from the Ni:NTA resin with 500 mM imidazole in the binding buffer, and the eluants were placed in the same microplate. The fluorescence in the samples was measured with a Microdynamics (Sunnyvale, CA) M5 plate-reader using excitation at 488 or 594 nm and emission at 519 nm and 617 nm, for AF-488 and AF-594, respectively. Samples were prepared in triplicate for each determination, and each plate was read three times.

**FRET Titration**

The ability of fluorophores to transfer fluorescence energy nonradiatively is a function of the spectral parameters of the fluorophores, their relative orientation, and the distance between them. The choice of amine-reactive AF-488 and AF-594 in these studies determines the first two of these, because the overlap constant is determined by the donor acceptor pair ($R_0$ = 60 in this case), and the attachment to amines, primarily lysine groups, implies that the fluorophores can move relative to the protein to which they are linked. The intensity of the transferred fluorescence at the emission wavelength for the acceptor is inversely proportional to the sixth power of the distance between the fluorophores (15). In the present report, because the multiple fluorophores are randomly attached to the proteins, detailed measurement of the putative FRET would not likely reveal much structural information.

Instead we used these labeled proteins to qualitatively determine the stoichiometry of the GK:PFK-2/FBP-2 complex. By acquiring the emission spectra at 1 nm resolution (excitation of AF-488 at 470 nm) from samples containing identical amounts of ND249*594 (acceptor) and increasing amounts of GK*488 (donor) while exciting at 470 nm (Microdynamics M5), we were, in effect, able to titrate ND249*594 with GK*488. We used 20 mM Tris, 100 mM NaCl, 2 mM dithiothreitol (pH 7.4) for the FRET titrations. On the same microplate we included samples of ND249*594 with the dialysate from GK*488 sample preparation added in place of GK*488 and GK*488 with dialysate from ND249*594 sample preparation in place of ND249*488. In practice we lowered the excitation wavelength to 470 nm to reduce direct excitation of AF-594. All samples were mixed in small vials, after which 200–250 $\mu$l was pipetted into the wells. The XSFI was calculated by subtracting the spectra according to Equation 1:

$$XSFI = GK*488w/ND249*594) - GK*488) - (ND249*594)$$

The XSFI 621 nm was then plotted as function of the GK*488 added. If the proteins form a discrete complex, then the XSFI at 621 nm is due to nonradiative transfer (FRET) and will be limited by complex formation producing a nonlinear plot that plateaus at high concentrations of GK*488. Any influence of inner-filter effects are nominal because the absorbance of the excitation light by the acceptor is identical in all samples because the concentration of ND249*594 is the same in every sample and is taken into account by Equation 1. Absorbance of the fluoresced light by the acceptor is precluded by the relative lack of absorbance for AF-488 at 621 nm.

Significantly, we designed these experiments so that the titration range covers the substoichiometric through GK molar excess based on our hypothesis of a 1:1 complex. Furthermore, we prepared samples in an array with the dimensions determined by the number of GK concentrations and the three conditions, *i.e.* GK*488 alone, ND*594 alone, and the mixed protein samples. In this way, we had multiple determinations of the ND249*594 in each assay, and these worked as internal controls that provide a measure of the inherent scatter in the data introduced by sample handling and inhomogeneities in the wells. All XSFI values reported, although small relative to the absolute FI measured, are much larger than the range of FI at 621 nm observed between samples of the fluorescently labeled proteins alone and so are significant changes in the emission of the acceptor (data not shown). Because we were able to prepare concentrated protein stock solutions of GK*488 and ND249*594, we could match the protein concentrations between FRET titrations. Based on the fluorescence emission spectra of the ND249*594 and the low population of the bound state due to the weak binding suggested by the pull-down assays and a plethora of negative evidence from biophysical methods for assessing protein-protein binding that are predicated on tight binding and predominant bound states, we used relatively high protein concentrations titrating 5 $\mu$M ND249*594 with GK*488. We tried to prepare fluorescently labeled PFK-2/FBP-2 but found very low labeling, likely due to the relative paucity of expressed protein (relative to GK and ND249) and the difficulty in preparing highly concentrated protein samples of PFK-2/FBP-2 for use in the labeling reactions.

**Liver Extracts**

Male C57BL mice (12 wk old) were obtained from the Jackson Laboratory (Bar Harbor, ME). After an overnight (16-h) fast, the mice were given an ip bolus injection of saline (fasted) or glucose solution at a dose of 2 g/kg body weight (fed) over 30 min. Mice were immediately euthanized, and the livers were collected, as described previously (25). Fresh liver extracts were prepared in homogenizing buffer containing 20 mM TES (pH 7.8), 1 mM dithiothreitol, 100 mM KCl, 5 mM EDTA, 5 mM EGTA, 1.2 mM phenylmethylsulfonylfluoride, and 2.5 mg/liter leupeptin. Total protein concentrations were quantified using the BCA reaction (Pierce Chemical Co., Rockford, IL), or Coomassie Dye Binding (Bio-Rad). Extracts were aliquoted, flash frozen, and kept at −70 C until use in the PFK-2 assays.

**Enzyme Kinetics**

The kinetic assays of GK and PFK-2/FBP-2 are well established and have long been the interest of the authors. Here, we have adapted the fluorometric FBP-2 assay of Tominaga *et al.* (33) to the use of microplates and the M5 plate reader, whereas the assays of GK and PFK-2 were conducted according to published methods (34, 35). $H_6$GK was incubated for 60 min without and with PFK-2/FBP-2 in a 1:2 and 1:10 ratio in GK assay buffer (20 mM HEPES; 125 mM KCl; 7.5 mM $MgCl_2$*$6H_2O$; 5 mM Na-ATP; 0.5 mM NADP; 0.5 U/ml glucose-6-phosphate-dehydrogenase). One unit of enzyme activity was defined as 1 mol glucose-6-phosphate formed from glucose and ATP per min at 37 C. Enzyme activity was expressed as units per mg cellular protein. The plate-based fluorometric FBP-2 assay was accomplished by adding a reaction mix (200 $\mu$l) containing (50 mM Tris, pH 7.5; 5 mM $MgCl_2$; 50 $\mu$M NADP; 15 $\mu$M F-2,6-$P_2$; 0.4 $\mu$g glucose-6-phosphate dehydrogenase; and PGI) to 10 $\mu$l of each sample predispensed into the wells of a microplate and subsequent reading of the FI due to reduced nicotinamide adenine dinucleotide (Ex = 354 nm; Em = 452 nm) produced as a monitor of substrate hydrolysis (33). The PFK-2 activity is determined by following the change in F-2,6-$P_2$ over time, where the F-2,6-$P_2$ is determined by the Van Shaftingen method using potato PPi:PFK-1 (36) prepared in house by the protocol of Wessberg *et al.* (37) adapted to the BioCAD and POROS resin. The PFK-2 reaction mix contains 20 mM Tris (pH 7.5), 5 mM ATP, 5 mM F-6-P, 10 mM $MgCl_2$, and 5 mM Pi. All activities are expressed as picomoles of F-2,6-$P_2$ produced per min per mg of total protein. Determination of the PFK-2 activity in

the mouse liver extracts was complicated by the presence of F-2,6-$P_2$ in the extracts, as well as that of any other compound that might influence PFK-2 activity over the 10–15 min needed to determined changes in F-2,6-$P_2$. Addition of GK, or the control proteins (TPI, HK1, BSA, and PKI), to the liver extracts was made from concentrated stock solutions. The final concentration of the added proteins was the same, because we compensated for less extract by addition of extraction buffer.

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### REFERENCES

1. Pilkis SJ, Walderhaug M, Murray K, Beth A, Venkataramu SD, Pilkis J, El-Maghrabi MR 1983 6-Phosphofructo-2-kinase/fructose 2,6-bisphosphatase from rat liver. J Biol Chem 258:6135–6141
2. Okar DA, Manzano A, Navarro-Sabate A, Riera L, Bartrons R, Lange AJ 2001 PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose-2,6-bisphosphate. Trends Biochem Sci 26:30–35
3. Okar DA, Wu C, Lange AJ 2004 Regulation of the regulatory enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Adv Enzyme Regul 44:123–154
4. Jin ES, Uyeda K, Kawaguchi T, Burgess SC, Malloy CR, Sherry AD 2003 Increased hepatic fructose 2,6-bisphosphate after an oral glucose load does not affect gluconeogenesis. J Biol Chem 278:28427–28433
5. Wu C, Kang JE, Peng LJ, Li H, Khan SA, Hillard CJ, Okar DA, Lange AJ 2005 Enhancing hepatic glycolysis reduces obesity: differential effects on lipogenesis depend on site of glycolytic modulation. Cell Metab 2:131–140
6. Baltrusch S, Lenzen S, Okar DA, Lange AJ, Tiedge M 2001 Characterization of glucokinase-binding protein epitopes by a phage-displayed peptide library. Identification of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase as a novel interaction partner. J Biol Chem 276:43915–43923
7. Payne VA, Arden C, Wu C, Lange AJ, Agius L 2005 Dual role of phosphofructokinase-2/fructose bisphosphatase-2 in regulating the compartmentation and expression of glucokinase in hepatocytes. Diabetes 54:1949–1957
8. Nishimura M, Uyeda K 1995 Purification and characterization of a novel xylulose 5-phosphate-activated protein phosphatase catalyzing dephosphorylation of fructose-6-phosphate,2-kinase:fructose-2,6-bisphosphatase. J Biol Chem 270:26341–26346
9. Nouspikel T, Iynedjian PB 1992 Insulin signalling and regulation of glucokinase gene expression in cultured hepatocytes. Eur J Biochem 210:365–373
10. El-Maghrabi MR, Claus TH, Pilkis J, Pilkis SJ 1982 Regulation of 6-phosphofructo-2-kinase activity by cyclic AMP-dependent phosphorylation. Proc Natl Acad Sci USA 79:315–319
11. Matschinsky F, Liang Y, Kesavan P, Wang L, Froguel P, Velho G, Cohen D, Permutt MA, Tanizawa Y, Jetton TL, Niswender K, Magnuson MA 1993 Glucokinase as pancreatic $\beta$ cell glucose sensor and diabetes gene. J Clin Invest 92:2092–2098
12. Thorens B 1996 Glucose transporters in the regulation of intestinal, renal, and liver glucose fluxes. Am J Physiol 270:G541–G553
13. Massa L, Baltrusch S, Okar DA, Lange AJ, Lenzen S, Tiedge M 2004 Interaction of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) with glucokinase activates glucose phosphorylation and glucose metabolism in insulin-producing cells. Diabetes 53:1020–1029
14. Garcia-Herrero CM, Galan M, Vincent O, Flandez B, Gargallo M, Delgado-Alvarez E, Blazquez E, Navas MA 2007 Functional analysis of human glucokinase gene mutations causing MODY2: exploring the regulatory mechanisms of glucokinase activity. Diabetologia 50:325–333
15. Lakowicz J 1999 Energy transfer. In: Principles of fluorescence spectroscopy, 2nd ed. New York: Plenum; 367–394
16. Shibata T, Fujimoto K 2005 Noisy signal amplification in ultrasensitive signal transduction. Proc Natl Acad Sci USA 102:331–336
17. Ferrell Jr JE 1997 How responses get more switch-like as you move down a protein kinase cascade. Trends Biochem Sci 22:288–289
18. Craciun G, Tang Y, Feinberg M 2006 Understanding bistability in complex enzyme-driven reaction networks. Proc Natl Acad Sci USA 103:8697–8702
19. Arkin A, Ross J 1994 Computational functions in biochemical reaction networks. Biophys J 67:560–578
20. Pilkis SJ, El-Maghrabi MR, Pilkis J, Claus TH, Cumming DA 1981 Fructose 2,6-bisphosphate. A new activator of phosphofructokinase. J Biol Chem 256:3171–3174
21. Pozuelo Rubio M, Peggie M, Wong BH, Morrice N, MacKintosh C 2003 14-3-3s Regulate fructose-2,6-bisphosphate levels by binding to PKB-phosphorylated cardiac fructose-2,6-bisphosphate kinase/phosphatase. EMBO J 22:3514–3523
22. Wu C, Okar DA, Newgard CB, Lange AJ 2001 Overexpression of 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase in mouse liver lowers blood glucose by suppressing hepatic glucose production. J Clin Invest 107:91–98
23. Choi IY, Wu C, Okar DA, Lange AJ, Gruetter R 2002 Elucidation of the role of fructose 2,6-bisphosphate in the regulation of glucose fluxes in mice using in vivo (13)C NMR measurements of hepatic carbohydrate metabolism. Eur J Biochem 269:4418–4426
24. Wu C, Okar DA, Newgard CB, Lange AJ 2002 Increasing fructose 2,6-bisphosphate overcomes hepatic insulin resistance of type 2 diabetes. Am J Physiol Endocrinol Metab 282:E38–E45
25. Wu C, Okar DA, Stoeckman AK, Peng LJ, Herrera AH, Herrera JE, Towle HC, Lange AJ 2004 A potential role for fructose-2,6-bisphosphate in the stimulation of hepatic glucokinase gene expression. Endocrinology 145:650–658
26. Nagradova N 2003 Interdomain communications in bifunctional enzymes: how are different activities coordinated? IUBMB Life 55:459–466
27. Ninfa AJ, Mayo AE 2004 Hysteresis vs. graded responses: the connections make all the difference. Sci STKE 2004:pe20
28. Hervagault JF, Canu S 1987 Bistability and irreversible transitions in a simple substrate cycle. J Theor Biol 127:439–449

29. Van Schaftingen E, Vandercammen A, Detheux M, Davies DR 1992 The regulatory protein of liver glucokinase. Adv Enzyme Regul 32:133–148
30. Lin K, Kurland I, Xu LZ, Lange AJ, Pilkis J, el-Maghrabi MR, Pilkis SJ 1990 Expression of mammalian liver glycolytic/gluconeogenic enzymes in *Escherichia coli*: recovery of active enzyme is strain and temperature dependent. Protein Expr Purif 1:169–176
31. Okar DA, Felicia ND, Gui L, Lange AJ 1997 Labeling of recombinant protein for NMR spectroscopy: global and specific labeling of the rat liver fructose 2,6-bisphosphatase domain. Protein Expr Purif 11:79–85
32. Tauler A, Lange AJ, el-Maghrabi MR, Pilkis SJ 1989 Expression of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and its kinase domain in *Escherichia coli*. Proc Natl Acad Sci USA 86:7316–7320
33. Tominaga N, Minami Y, Sakakibara R, Uyeda K 1993 Significance of the amino terminus of rat testis fructose-6-phosphate, 2-kinase:fructose-2,6-bisphosphatase. J Biol Chem 268:15951–15957
34. Lange AJ, Xu LZ, Van Poelwijk F, Lin K, Granner DK, Pilkis SJ 1991 Expression and site-directed mutagenesis of hepatic glucokinase. Biochem J 277:159–163
35. Lenzen S, Tiedge M, Panten U 1987 Glucokinase in pancreatic B-cells and its inhibition by alloxan. Acta Endocrinol (Copenh) 115:21–29
36. Van Schaftingen E, Lederer B, Bartrons R, Hers HG 1982 A kinetic study of pyrophosphate: fructose-6-phosphate phosphotransferase from potato tubers. Application to a microassay of fructose 2,6-bisphosphate. Eur J Biochem 129:191–195
37. Wessberg KL, Skolnick S, Xu J, Marciano-Cabral F, Kemp RG 1995 Cloning, sequencing and expression of the pyrophosphate-dependent phosphofructo-1-kinase from *Naegleria fowleri*. Biochem J 307:143–149

# Endogenous activation of glucokinase by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase is glucose dependent

Running title: Glucokinase-PFK-2/FBPase-2 interaction

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**Key words**: glucokinase; 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; glucokinase regulatory protein; pancreatic beta-cell; fluorescence microscopy

**Abbreviations:** AD, activation domain; BD, binding domain; DMEM, Dulbecco's modified Eagle's medium; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; FRET, fluorescence resonance energy transfer; FRETN, sensitized emission-based FRET efficiency; GK, glucokinase; GRP, glucokinase regulatory protein; MMTHS, mammalian two-hybrid system; PFK-2/FBPase-2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase

## Abstract

Glucokinase plays a crucial role as glucose sensor in glucose-induced insulin secretion in pancreatic beta-cells. The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) acts as an endogenous glucokinase activator. Therefore the goal of this study was the quantitative analysis of the glucokinase-PFK-2/FBPase-2 complex formation and its separation in living beta-cells. The interaction between glucokinase and PFK-2/FBPase-2 or liver glucokinase regulatory protein (GRP) was analyzed in insulin-secreting MIN6 cells and COS cells, respectively, with a fluorescence-based mammalian two-hybrid system and through labeling with the fluorescent proteins ECFP and EYFP. In contrast to the commonly used mammalian two-hybrid systems which require sampling prior to detection the system used allows monitoring of the effects of environmental changes on protein-protein interactions on the single cell level. At 25 mmol/l glucose a significant interaction between glucokinase and PFK-2/FBPase-2 was measured. Lowering of the glucose concentration in the cell culture medium abolished the interaction between the enzymes stepwise, but enforced the interaction between glucokinase and GRP. The fluorescent fusion proteins ECFP-glucokinase and EYFP-PFK-2/FBPase-2 were colocalized in a comparable manner to the endogenous proteins with an optimum at 25 mmol/l glucose. Furthermore, transfer of fluorescence energy from ECFP-GK to EYFP-PFK-2/FBPase-2 was detectable demonstrating a direct interaction between glucokinase and PFK-2/FBPase-2. Thus, binding and activation of glucokinase by PFK-2/FBPase-2 is clearly promoted by glucose. Furthermore the established mammalian two-hybrid system offers the possibility to monitor changes in the association of these two key regulatory beta-cell proteins in dependence on metabolic conditions such as the ones occurring in type 2 diabetes.

## Introduction

The glucose sensor enzyme glucokinase (GK) plays a pivotal role in coupling millimolar changes of the extracellular glucose concentration to metabolism both in pancreatic beta-cells and in the liver (1-4). In pancreatic beta-cells glucose phosphorylation by glucokinase is the rate-limiting step of glucose metabolism and thus of glucose-induced insulin secretion (5-7). Glucose is the most potent activator of glucokinase inducing a comprehensive conformational change. Furthermore, for short-term regulation of glucokinase activity posttranslational mechanisms including protein-protein interactions proved to be of particular importance. The liver-specific glucokinase regulatory protein (GRP), which competitively inhibits glucokinase activity, was the first glucokinase binding protein to be identified (8). The interaction between glucokinase and the GRP takes place at low glucose concentrations and is stimulated in the presence of fructose-6-phosphate (8, 9). Furthermore the complex is deprived of the cytoplasm by translocation to the nucleus (10-13). In previous experiments binding between glucokinase and the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) has been shown (14-17). PFK-2/FBPase-2 determines intracellular levels of fructose-2,6-bisphosphate by synthesizing and degrading this important regulator of carbohydrate metabolism (18-20). Yeast two-hybrid studies have shown that both the liver and the pancreatic beta-cell isoform of PFK-2/FBPase-2 are able to bind to the glucokinase protein (15). Overexpression of liver or beta-cell PFK-2/FBPase-2 results in a significant activation of glucokinase enzyme activity in insulin-producing cells (16). In pancreatic beta-cells the PFK-2/FBPase-2 acts not only as an endogenous activator of glucokinase enzyme activity but also significantly improves the coupling between glycolysis and oxidative flux (14). Importantly, the activation of glucokinase by PFK-2/FBPase-2 affects only the $V_{max}$ value of the enzyme, but not the $S_{0.5}$ for glucose (16). Recently discovered small chemical glucokinase activators allosterically activate the enzyme by lowering the glucose $S_{0.5}$ (21-29). Some of the compounds were able to additionally increase the $V_{max}$ (21, 22, 25) . Glucokinase activators proved to be able to enhance glucose-stimulated insulin release from pancreatic islets as well as glucose disposal by the liver (26, 28, 30). Further elucidation of the physiological glucokinase activation by PFK-2/FBPase-2 will help to advance the concept of glucokinase activation based on physiological needs for treatment of type 2 diabetes.

Since the detection of protein-protein interactions is a major challenge in proteome research, two-hybrid assays have been established and modified in various ways in the past decade (31-37). Thus, a two-hybrid system was further adapted in this study to analyze interactions in insulin-producing cells. Both in mammalian cells and in yeast these assays make use of the

fact that many eukaryotic transcriptional activators consist of two separable domains which only initiate reporter gene transcription when they are closely approximated (38-40). The required proximity is established through direct binding between the proteins whose interaction is studied. Solubility, folding and posttranslational modifications required for specific protein interactions of mammalian proteins are partially insufficient in yeast. Thus, only the mammalian system provides a natural environment to study interactions of mammalian proteins. In the mammalian two-hybrid systems used here the transcriptional activator domains are a herpes simplex virus VP16 transcriptional activation domain (AD) and the yeast GAL4 DNA-binding domain (BD) each of which is fused to one of the proteins of interest. Association of GAL4-DNA-BD and VP16-AD, driven by the interaction of the fused proteins of interest, results in binding to the promoter region and transcriptional activation of the reporter gene. In most mammalian two-hybrid experiments analyzing strong interactions of viral proteins, luciferase or chloramphenicol acetyltransferase activity was determined as a reporter signal (34, 35). However, in some studies fluorescent proteins, namely GFP and EYFP, have already been applied successfully as a reporter providing a higher resolution of signal detection (36, 41). The use of EYFP as a reporter gene together with ECFP expression under control of a constitutive promoter allows normalization for differences in transfection efficiency and enables quantitative analysis of protein-protein interactions on the single cell level (41).

The present study applied such a fluorescence based mammalian two-hybrid system in combination with a semi-automated microscopy approach to elucidate the regulatory principle of the interaction between glucokinase and its activator 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in insulin-secreting MIN6 cells. Moreover, this system was compared with the analysis of protein-protein interactions by the use of fluorescent fusion proteins and intracellular fluorescence resonance energy transfer (FRET).

## Results

### Glucose-dependent interaction of glucokinase and the glucokinase regulatory protein in the mammalian two-hybrid system

The well characterized interaction between glucokinase (GK) and the glucokinase regulatory protein (GRP) (42) could be displayed with the fluorescence based mammalian two-hybrid system. Transient transfection of COS cells with the glucokinase and GRP two-hybrid plasmids while culturing the cells in medium containing 25 mmol/l glucose resulted in a significant interaction signal, given as the ratio of EYFP over ECFP, that was 70% higher than in negative controls. As a two-hybrid signal is only produced by direct binding of the fusion proteins to each other this indicates complex formation of glucokinase and GRP. Incubation of the transfected cells with medium containing 5 mmol/l glucose resulted in a 50% higher EYFP/ECFP ratio than with 25 mmol/l glucose (Fig. 1). This is consistent with the role of GRP to bind glucokinase in hepatocytes at low glucose concentrations. Thus, the applied mammalian two-hybrid system is a suitable tool for analyzing protein-protein interactions in a quantitative way while different extracellular parameters can be set. In contrast, a mammalian two-hybrid system using secreted alkaline phosphatase as a reporter was not sufficient to detect interaction between glucokinase and GRP (data not shown). In this study two available mammalian two-hybrid systems were used in a modified way. A problem of both original systems particularly with regard to the detection of weak interactions might be the sampling of supernatant or of lysed cells, respectively, while the fluorescence-based system allows calculation of the interaction strength for single cells. By this means, false positive cells contributing to a high background level can be identified. Moreover, the system based on the secreted alkaline phosphatase reporter did not provide a second reporter for normalization of differences in transfection efficiency.

### Glucose-dependent binding of islet PFK-2/FBPase-2 to glucokinase

The two-hybrid system was established in insulin-secreting MIN6 cells to study the interaction between glucokinase and PFK-2/FBPase-2. The islet PFK-2/FBPase-2 isoform fused to the GAL4 DNA-binding domain showed a significant interaction with glucokinase fused to the VP16 activation domain when the cells were incubated with standard medium containing 25 mmol/l glucose. The EYFP/ECFP ratio was 2.3 times higher than the ratio of the negative controls. Thus the interaction of glucokinase with PFK-2/FBPase-2 is weaker than the interaction of glucokinase with its regulatory protein GRP, as the latter interaction produces a 3.3 times higher two-hybrid signal than the negative controls under binding conditions at low glucose. At a low glucose concentration of 3 mmol/l the EYFP expression as the reporter sig-

nal of interaction between glucokinase and PFK-2/FBPase-2 shown in relation to constitutively expressed ECFP was diminished to a value only slightly above the negative control (Fig. 2). This suggests that an even smaller fraction of glucokinase is bound in the complex with PFK-2/FBPase-2 when the glucose concentration is decreased.

**A semi-automated approach to analyze protein-protein interactions with the mammalian two-hybrid system**

The use of a semi-automated microscopy approach made it possible to simultaneously monitor the interaction strength of glucokinase binding to PFK-2/FBPase-2 at three different glucose concentrations over a time period of 40 hours. The signals were highly variable for ten hours after adjusting the different glucose concentrations. The number of cells expressing fluorescent protein was low during this 10-h time period. Then the values ranged around a plateau before the negative controls started to increase slightly from 50 h after transfection. Generally the negative controls showed less variability than the samples in which glucokinase and islet PFK-2/FBPase-2 were coexpressed. Noticeable, the EYFP/ECFP ratio exhibited an oscillating profile particularly in the samples and slightly in the negative controls possibly due to batch expression of the proteins (Fig. 3a). Therefore the values were averaged across the period from 36 to 48 hours post transfection for final analysis. Glucokinase and islet PFK-2/FBPase-2 showed a significant interaction signal when the cells were preincubated at 25 mmol/l glucose. Also at 10 mmol/l glucose the proteins showed a distinct interaction. At 3 mmol/l only a slightly higher EYFP/ECFP ratio than in the negative controls was detected. The interaction strength at 3 mmol/l glucose was significantly lower than at 25 mmol/l (Fig. 3b).

**Effect of low versus high glucose on colocalization between glucokinase and PFK-2/FBPase-2**

COS cells were cotransfected with expression vectors encoding the fluorescent fusion proteins ECFP-GK and EYFP-PFK-2/FBPase-2. After preincubation with medium containing 3 mmol/l or 25 mmol/l glucose, respectively, the overexpressed proteins showed a cellular distribution that was comparable with that of the endogenous proteins. Glucokinase and PFK-2/FBPase-2 exhibited a distinct colocalization at both glucose concentrations (Fig. 3). The degree of colocalization was 7.8% at 3 mmol/l glucose and 11.0% at 25 mmol/l glucose. However, this difference was not significant, suggesting that the enzymes are in relative spatial proximity even if there is no direct binding.

**Effect of low versus high glucose on FRET between glucokinase and GRP or PFK-2/FBPase-2**

Sensitized emission-based FRET efficiency (FRETN) was measured between ECFP and EYFP fused to each other to determine the FRETN detection range of the microscopy setup. FRETN was 9.1 and therefore by the factor of 31 higher than the negative control in which ECFP and EYFP were expressed separately. A low FRET rate was also detectable in the negative control because of a residual dimerization tendency of ECFP and EYFP as well as bleed-through that happens when the donor ECFP is excited by the acceptor EYFP excitation wavelength or vice versa (Fig. 5A).

ECFP-GK and EYFP-GRP showed a significant FRET signal both at 5.5 mmol/l and 25 mmol/l glucose. At low glucose concentration FRETN was 2.3 times higher than the negative control and 50% higher in comparison to cells incubated at 25 mmol/l. This is consistent with the results obtained with the mammalian two-hybrid system (Fig. 5B).

Although the fusion proteins ECFP-GK and EYFP-PFK-2/FBPase-2 showed only a slightly higher colocalization at 25 mmol/l glucose than at 3 mmol/l glucose when they were co-overexpressed in COS cells, the enzymes exhibited FRET exclusively at 25 mmol/l glucose. At 25 mmol/l glucose FRETN was, with 0.82. 2.5 times higher than the negative control. When the cells were preincubated with medium containing 3 mmol/l glucose no significant sensitized emission-based FRET efficiency between ECFP-GK and EYFP-PFK-2/FBPase-2 was detectable (Fig. 5C).

## Discussion

The glucose sensor enzyme glucokinase plays an essential role during stimulus-secretion coupling in pancreatic beta-cells. In contrast to the transcriptional regulation of liver glucokinase by insulin and glucagon the beta-cell isoenzyme protein levels are only marginally affected by the nutritional state (43, 44). Posttranslational regulation mechanisms of glucokinase including protein-protein interaction and compartmentalization processes therefore make an important contribution to the complex network for maintaining glucose homeostasis. In beta-cells the glucokinase protein is exclusively localized in the cytoplasm where it is sequestered by matrix proteins (44, 45) and insulin granules (46) at sub-stimulatory glucose concentrations. The cytosolic bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2), a key regulator of glucose metabolism, proved to be a binding partner of glucokinase (15, 17). Unlike the hepatic glucokinase regulatory protein, which competitively inhibits glucokinase activity, PFK-2/FBPase-2 turned out to be an activating glucokinase binding partner. Furthermore, PFK-2/FBPase-2 amplified the increase of intrinsic glucokinase activity by glucose while both the affinity of glucokinase for glucose and the positive cooperativity were unaffected (16). Thus, the endogenous glucokinase activator PFK-2/FBPase-2 complies with the requirements of a fine-tuning of glucokinase activity while preserving the glucose sensor function.

The objective of this study was to analyze the interaction of glucokinase with the beta-cell isoform of PFK-2/FBPase-2 with special emphasis on glucose dependency. For the purpose of detecting differences in the interaction of the enzymes in a physiological environment fusion proteins were generated to perform FRET and mammalian two-hybrid experiments. Both methods were similarly applied to the interaction of glucokinase to its regulatory protein (GRP) whose modulation by glucose has been described earlier (10-13). FRET efficiency strongly depends on the distance of the donor-acceptor pair of fluorophores but also on the relative orientation of the dipole moments of donor and acceptor. The glucokinase binding motif is located within the bisphosphatase domain of PFK-2/FBPase-2 (15) and there is strong evidence for a 1:1 stoichiometry of the complex (17). Though the performed sensitized emission-based FRET measurements have to be considered as semi-quantitative, the obtained values for the interaction of glucokinase both with PFK-2/FBPase-2 and the glucokinase regulatory protein in relation to the internal positive control support the classification of both interactions as weak interactions. However, it has to be taken into account that in the positive control ECFP and EYFP are directly fused to each other providing a 1:1 ratio of donor and acceptor, while an exact 1:1 stoichiometry can never be achieved by expression of proteins from

different vectors, in this case ECFP-GK and EYFP-GRP or EYFP-PFK-2/FBPase-2. While there is a significant interaction between glucokinase and PFK-2/FBPase-2 at high glucose, the binding to the GRP was minor, but not completely diminished. Crystal structure analysis of glucokinase protein revealed a closed conformation of catalytically active glucokinase and a super-opened conformation in which glucokinase is inactive (27). As on one hand PFK-2/FBPase-2 and glucokinase also exhibited colocalization at low glucose and on the other hand a residual interaction of GRP and glucokinase could be detected at high glucose, the data argue against binding of PFK-2/FBPase-2 or GRP exclusively to the closed or to the super-opened form, respectively. Furthermore such a regulation does not seem to be flexible enough for the role of the liver to provide a fast switch between glucose usage and disposal. From kinetic modelling there is strong evidence that up to three additional con-formational intermediates exist between the two already established ones (27, 47, 48). Transferring this multiple conformational state model to the situation in the liver this study provides evidence for interplay of the glucokinase activator PFK-2/FBPase-2 and the inhibitor GRP. Both glucokinase binding partners would, in opposite directions, promote equilibration of the glucokinase conformation with respect to the needs of the nutritional state of the cell. As the transition of the glucokinase:GRP complex into the nucleus was not taken into account here, because FRET was measured in the cytoplasm, this hypothesis has to be further tested in additional experiments.

Two-hybrid systems are designed to measure direct binary interactions. Implementation of the fluorescent protein EYFP as a reporter in the mammalian two-hybrid system allows detection of weak protein-protein interactions in living mammalian cells in a quantitative manner, not available through analysis of the reporter gene alkaline phosphatase in the supernatant medium of cultured cells. Outlier cells that would strongly influence the mean value of a data set can be easily identified by single-cell analysis. However, fluorescence microscopy based data acquisition as well as analysis is time-consuming. Thus, the quantity of analyzed cells within a manually performed two-hybrid experiment is limited. Furthermore protein-protein interactions could be monitored only in a narrow time period. To increase both the number of analyzed cells and the recorded time points an open fluorescence microscope scanning system was used in a semi-automated approach. In addition high content data analysis was performed including background correction, automated cell detection and cell tracking. Finally the EYFP/ECFP ratio was directly available on the single cell level. Cells with a low or high constitutive ECFP expression were excluded from the final analysis using gates in the scan$^{R}$ analysis software. This procedure has significantly reduced the standard error of mean. Thus,

this approach made it possible to determine the interaction between glucokinase and PFK-2/FBPase-2 at three different glucose concentrations in parallel for a time period of 40 h. Different vectors than with the manual approach were used. Indeed measurements are not in real-time because the detection of a reporter protein whose expression is to be induced is dependent upon its half-life. In addition the translocation into the nucleus has to be taken into account. However, both systems provided evidence for a very weak and thus not significant interaction at 3 mmol/l glucose. At 25 mmol/l both systems proved to be able to detect a significant interaction of glucokinase and PFK-2/FBPase-2. The data obtained with the semi-automated approach in fact suggest a stepwise amplification of interaction strength between the proteins with increasing glucose concentrations. Thus, it was shown for the first time that the glucokinase-PFK-2/FBPase-2 binding process itself is glucose-dependent. Furthermore, a viable method for the intracellular detection of a weak protein-protein interaction and its post-translational modification was established. In future studies this method might be helpful not only to analyze the effect of potential new drugs on the molecular level (37), but also to provide first information of the intracellular half-life of such compounds. Indeed it appears feasible to study the effects of the recently developed small synthetic glucokinase activators on the binding of glucokinase to PFK-2/FBPase-2 with respect to the action in the beta-cell and to both PFK-2/FBPase-2 and GRP with respect to the action in liver by this high-throughput technique.

Together with the previous finding that overexpression of beta-cell PFK-2/FBPase-2 resulted in a greater stimulatory effect of glucose on glucokinase enzyme activity when the glucose concentration was increased (14), the present study provides direct evidence for a shift of the glucokinase conformational equilibrium to its enzymatically active closed conformation by PFK-2/FBPase-2 binding. Complex formation would then prevent the slow conformational change of glucokinase into the super-opened conformation. Thus, the interaction would lead to an increase in glucokinase enzyme activity to support its role as a glucose sensor. Furthermore, the complex could be a player in the macromolecular channelling of glycolytic intermediates.

The present study further underlines the physiological importance of posttranslational glucokinase regulation by protein-protein interactions. As glucokinase activation has been described as a promising therapeutic strategy in the treatment of type 2 diabetes further elucidation of the endogenous glucokinase activation mechanisms will contribute to a better understanding of the metabolic coupling between glucokinase activation and glucose-stimulated insulin secretion.

## Materials and methods

### Materials

Restriction enzymes and modifying enzymes for the cloning procedures were from New England Biolabs (Beverly, MA) or Fermentas (St. Leo-Rot, Germany). Custom oligonucleotides were synthesized by MWG Biotech (Ebersberg, Germany). The reporter vector pHASH-3 was a kind gift of Dr. S. Herlitze (Department of Neurosciences, Case Western Reserve University, Cleveland, OH). All reagents of analytical grade were from Merck (Darmstadt, Germany). All tissue culture equipment was from Invitrogen (Karlsruhe, Germany) and Greiner-Bio One (Frickenhausen, Germany).

### Cell culture and transient transfection

COS cells and MIN6 cells were grown in DMEM supplemented with 25 mmol/l glucose, 10% (v/v) fetal calf serum, 10 U/ml penicillin, 10 µg/ml streptomycin and 2 mmol/l glutamine in a humidified atmosphere at 37 °C and 5% $CO_2$. For the mammalian two-hybrid experiments COS cells (passages 13-35) were seeded in six-well microplates at a density of 3 x $10^4$ cells and grown for one day. MIN6 cells (passages 37-44) were seeded in six-well microplates at a density of 8 x $10^4$ cells and grown for five days. Thereafter, cells were transfected with the mammalian two-hybrid vectors with jetPEI (Qbiogene, Montreal, QC, Canada) according to the manufacturer's instructions. For each transfection 0.4 pmol of the reporter vector pHASH-3 was used in combination with 0.1 pmol of the expression vector pM (negative control), pM-GRP or pM-PFK-2/FBPase-2 and 0.1 pmol of the expression vector pVP16 (negative control) or pVP16-GK. COS cells were incubated for 21 h in the presence of 5 mmol/l or 25 mmol/l glucose, respectively, before image acquisition. MIN6 cells were incubated for 20 h after transfection with standard DMEM containing 25 mmol/l glucose. Thereafter, cells were incubated in the presence of 3 mmol/l or 25 mmol/l glucose, respectively, for another 24 h before image acquisition. For the semi-automated ScanR approach MIN6 cells (passages 39-40) were seeded at a density of 1.5 x $10^5$ in 12-well microplates and grown for three days. Then the cells were transfected with equimolar amounts of the vectors pGL4.EYFP, pBIND.ECFP-PFK-2/FBPase-2, and pACT or pACT-GK with a total amount of 1 µg DNA and grown for 24 h in standard DMEM. Thereafter, the medium was replaced and cells were incubated in DMEM containing 3, 10 or 25 mmol/l glucose. Image acquisition was started one hour after medium replacement. For colocalization and FRET studies COS cells (passages 5-20) were seeded at a density of 2 x $10^4$ cells on 35-mm glass-bottom dishes (MatTek, Ashland, MA) and grown for one day. Thereafter, cells were transfected as described above with either 0.5 µg of pECFP or pECFP-GK plasmid and 0.5 µg pEYFP-GRP or 0.5 µg of pEYFP-PFK-

2/FBPase-2, or with 0.5 µg pECFP and 0.5 µg pEYFP, or with 1 µg pECFP-EYFP alone. One day after transfection the culture medium was replaced and the cells were incubated for another 24 h with medium containing 3, 5.5 or 25 mmol/l glucose, respectively, before image acquisition.

**Mammalian two-hybrid analysis**

Mammalian two-hybrid analysis was performed using the cloning vectors pM and pVP16 from the BD Matchmaker Mammalian Assay Kit 2 (Clontech, Palo Alto, CA) to create the GAL4 DNA-BD and VP16 AD fusion constructs, respectively. The cDNA sequences of rat beta-cell PFK-2/FBPase-2 and rat liver GRP were amplified by PCR and subcloned in frame (*Sal*I and *Hind*III restriction sites) to the GAL4-DNA-BD into pM. Human beta-cell glucokinase coding cDNA was amplified by PCR and subcloned in-frame (*Sal*I and *Hind*III restriction sites) to the VP16-AD into pVP16 (Clontech). The fluorescence based reporter plasmid pHASH-3 (41) was used for transfection in combination with the pM and pVP16 constructs. pHASH-3 contains the gene for the yellow fluorescent protein (EYFP) under control of an inducible promoter containing GAL4 binding sites. Furthermore, pHASH-3 carries the cyan fluorescent protein (ECFP) gene which is constitutively expressed. Both EYFP and ECFP carry a nuclear localization signal so that fluorescence is detectable in the nucleus of transfected cells. COS cells or MIN6 cells were transfected with the two-hybrid vectors for expression of the fusion proteins and the reporter vector.

For a semi-automated microscope approach of mammalian two-hybrid analysis vectors of the CheckMate/Flexi Vector Mammalian Two-Hybrid System (Promega, Madison, WI) were modified as follows. Human beta-cell glucokinase coding cDNA was amplified by PCR and subcloned in-frame (*Sgf*I and *Pme*I restriction sites) to the VP16-AD into pFN10A (ACT) Flexi Vector to generate pACT-GK. The cDNA sequence of rat beta-cell PFK-2/FBPase-2 was amplified by PCR and subcloned in-frame (*Sgf*I and *Pme*I restriction sites) to the GAL4-DNA-BD into pFN11A (BIND) Flexi Vector to generate pBIND-PFK-2/FBPase-2. To generate pECFP-Nuc the vectors pECFP-N1 and pEYFP-Nuc (Clontech) were digested with the restriction enzymes *Age*I and *BsrG*I and the EYFP was replaced by ECFP. The cDNA of ECFP-Nuc including the CMV promoter and SV40 early polyadenylation signal was amplified by PCR using composite primers to introduce *Pvu*II (5') and *Cla*I (3') restriction sites. *Renilla Luciferase* coding cDNA was removed from pBIND-PFK-2/FBPase-2 by digestion with *Pvu*II and *Cla*I and ECFP-Nuc was subcloned into the vector frame. The resulting vector was called pBIND.ECFP-PFK-2/FBPase-2. The cDNA sequence of EYFP-Nuc was amplified by PCR using composite primers to introduce *Hind*III (5') and *Fse*I (3') restriction sites. The

coding *luc2P* firefly luciferase cDNA was removed from the reporter vector pGL4.31 (Promega) by digestion with *Hind*III and *Fse*I and EYFP-Nuc was subcloned into the vector frame. The resulting reporter vector was called pGL4.EYFP. MIN6 cells were transfected with pGL4.EYFP, pBIND.ECFP-PFK-2/FBPase-2 and pACT (Promega) as negative control or with pACT-GK. For analysis of an interaction of the fusion proteins fluorescence intensities of EYFP and ECFP were determined in the nuclei of transfected cells. To quantify the interaction strength the ratio EYFP/ECFP was calculated for single cells from the background corrected average grey values of the EYFP and ECFP fluorescence intensities.

**Colocalization and FRET analyses**

The expression vectors pECFP-GK and pEYFP-GRP as described previously (10) were used for overexpression of fluorescent fusion proteins. Rat islet PFK-2/FBPase-2 coding cDNA was amplified by PCR and subcloned in frame (*Kpn*I and *BamH*I restriction sites) to the EYFP into pEYFP-C1 (Clontech, Palo Alto, CA). A plasmid encoding an ECFP-EYFP fusion protein with a two amino acid spacer between ECFP and EYFP was generated as described (49) and used as a positive control for FRET. COS cells were transfected with combinations of ECFP and EYFP plasmids. ECFP and EYFP fluorescence intensities were recorded in the cytoplasm of living cells by fluorescence microscopy. Colocalization of ECFP and EYFP was determined with the Imaris software (Bitplane, Zurich, Switzerland). The sensitized emission based FRET efficiency (FRETN) was calculated from the ECFP emission with excitation at 436 nm, EYFP emission with excitation at 436 nm and EYFP emission with excitation at 500 nm, based on the calculation of Vanderklish et al. (50).

**Fluorescence microscopy**

A cell$^{R}$/Olympus IX81 inverted microscope system equipped with a Cellcubator, as described previously (45), was used. D436/10-455DCLP-D480/40 and HQ500/20-530DCLP-D560/40 single-band filter sets were used for ECFP and EYFP, respectively (AHF Analysentechnik, Tübingen, Germany). For the mammalian two-hybrid analyses the cell culture plates with transfected, living cells were fixed on the microscope stage, and images were obtained with an UPLSAPO 20 × 0.75 numerical aperture objective (Olympus, Hamburg, Germany). For colocalization and fluorescence resonance energy transfer (FRET) studies glass-bottom dishes were fixed on the microscope stage, and ECFP and EYFP images were obtained with an UPLSAPO 60 × 1.35 numerical aperture oil-immersion objective (Olympus). For FRET measurements ECFP and EYFP emission was detected simultaneously using a DV-CC Dual View Simultaneous Imaging System (Optical Insights, LLC., Tucson, AZ) equipped with a 505 dcxr beam splitter and D465/30 and HQ535/30 emission filters (AHF Analysentechnik).

For multiple image analysis of 12-well microplates all motorized devices of the microscope system were synchronized by the scan$^R$ acquisition software (Olympus Soft Imaging Solutions, München, Germany). The image focus was automatically determined with a gradient method from transmitted light images. In a 40 h time-lapse experiment 20 fluorescence images of each well were taken hourly with an UPLSAPO 20 × 0.75 numerical aperture objective (Olympus, Hamburg, Germany). Analysis parameters were specified and image processing was carried out with the Scan$^R$ analysis software. After background correction individual cells were detected using an intensity threshold method and tracked over time. By setting gates only cells with a mean ECFP fluorescence were finally considered to exclude artefacts. Thereafter the ECFP/EYFP ratio of each single cell was available over time.

**Statistical analyses**

Data are expressed as means ± SEM. Statistical analyses were performed by ANOVA followed by Bonferroni's test for multiple comparison or Student's *t* test using the Prism analysis program (Graphpad, San Diego, CA, USA).

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## References


1. Baltrusch S, Tiedge M 2006 Glucokinase Regulatory Network in Pancreatic {beta}-Cells and Liver. Diabetes 55 Suppl 2:S55-64
2. Lenzen S, Panten U 1988 Signal recognition by pancreatic B-cells. Biochem Pharmacol 37:371-378
3. Matschinsky F, Liang Y, Kesavan P, Wang L, Froguel P, Velho G, Cohen D, Permutt MA, Tanizawa Y, Jetton TL, et al. 1993 Glucokinase as pancreatic beta cell glucose sensor and diabetes gene. The Journal of clinical investigation 92:2092-2098
4. Meglasson MD, Matschinsky FM 1984 New perspectives on pancreatic islet glucokinase. The American journal of physiology 246:E1-13
5. Matschinsky FM 1996 Banting Lecture 1995. A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. Diabetes 45:223-241
6. Matschinsky FM 2002 Regulation of pancreatic beta-cell glucokinase: from basics to therapeutics. Diabetes 51 Suppl 3:S394-404
7. Matschinsky FM, Glaser B, Magnuson MA 1998 Pancreatic beta-cell glucokinase: closing the gap between theoretical concepts and experimental realities. Diabetes 47:307-315
8. Van Schaftingen E 1989 A protein from rat liver confers to glucokinase the property of being antagonistically regulated by fructose 6-phosphate and fructose 1-phosphate. European journal of biochemistry / FEBS 179:179-184
9. Vandercammen A, Detheux M, Van Schaftingen E 1992 Binding of sorbitol 6-phosphate and of fructose 1-phosphate to the regulatory protein of liver glucokinase. Biochem J 286 ( Pt 1):253-256
10. Baltrusch S, Francini F, Lenzen S, Tiedge M 2005 Interaction of glucokinase with the liver regulatory protein is conferred by leucine-asparagine motifs of the enzyme. Diabetes 54:2829-2837
11. Bosco D, Meda P, Iynedjian PB 2000 Glucokinase and glucokinase regulatory protein: mutual dependence for nuclear localization. Biochem J 348 Pt 1:215-222
12. de la Iglesia N, Veiga-da-Cunha M, Van Schaftingen E, Guinovart JJ, Ferrer JC 1999 Glucokinase regulatory protein is essential for the proper subcellular localisation of liver glucokinase. FEBS Lett 456:332-338
13. Shiota C, Coffey J, Grimsby J, Grippo JF, Magnuson MA 1999 Nuclear import of hepatic glucokinase depends upon glucokinase regulatory protein, whereas export is due to a nuclear export signal sequence in glucokinase. J Biol Chem 274:37125-37130
14. Baltrusch S, Langer S, Massa L, Tiedge M, Lenzen S 2006 Improved metabolic stimulus for glucose-induced insulin secretion through GK and PFK-2/FBPase-2 coexpression in insulin-producing RINm5F cells. Endocrinology 147:5768-5776
15. Baltrusch S, Lenzen S, Okar DA, Lange AJ, Tiedge M 2001 Characterization of glucokinase-binding protein epitopes by a phage-displayed peptide library. Identification of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase as a novel interaction partner. J Biol Chem 276:43915-43923
16. Massa L, Baltrusch S, Okar DA, Lange AJ, Lenzen S, Tiedge M 2004 Interaction of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) with glucokinase activates glucose phosphorylation and glucose metabolism in insulin-producing cells. Diabetes 53:1020-1029
17. Smith WE, Langer S, Wu C, Baltrusch S, Okar DA 2007 Molecular coordination of hepatic glucose metabolism by the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase:glucokinase complex. Mol Endocrinol 21:1478-1487

18. Claus TH, El-Maghrabi MR, Regen DM, Stewart HB, McGrane M, Kountz PD, Nyfeler F, Pilkis J, Pilkis SJ 1984 The role of fructose 2,6-bisphosphate in the regulation of carbohydrate metabolism. Curr Top Cell Regul 23:57-86
19. Okar DA, Manzano A, Navarro-Sabate A, Riera L, Bartrons R, Lange AJ 2001 PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose-2,6-bisphosphate. Trends Biochem Sci 26:30-35
20. Pilkis SJ, Claus TH, Kurland IJ, Lange AJ 1995 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase: a metabolic signaling enzyme. Annu Rev Biochem 64:799-835
21. Brocklehurst KJ, Payne VA, Davies RA, Carroll D, Vertigan HL, Wightman HJ, Aiston S, Waddell ID, Leighton B, Coghlan MP, Agius L 2004 Stimulation of hepatocyte glucose metabolism by novel small molecule glucokinase activators. Diabetes 53:535-541
22. Efanov AM, Barrett DG, Brenner MB, Briggs SL, Delaunois A, Durbin JD, Giese U, Guo H, Radloff M, Gil GS, Sewing S, Wang Y, Weichert A, Zaliani A, Gromada J 2005 A novel glucokinase activator modulates pancreatic islet and hepatocyte function. Endocrinology 146:3696-3701
23. Futamura M, Hosaka H, Kadotani A, Shimazaki H, Sasaki K, Ohyama S, Nishimura T, Eiki J, Nagata Y 2006 An allosteric activator of glucokinase impairs the interaction of glucokinase and glucokinase regulatory protein and regulates glucose metabolism. J Biol Chem 281:37668-37674
24. Fyfe MC, White JR, Taylor A, Chatfield R, Wargent E, Printz RL, Sulpice T, McCormack JG, Procter MJ, Reynet C, Widdowson PS, Wong-Kai-In P 2007 Glucokinase activator PSN-GK1 displays enhanced antihyperglycaemic and insulinotropic actions. Diabetologia 50:1277-1287
25. Grimsby J, Sarabu R, Corbett WL, Haynes NE, Bizzarro FT, Coffey JW, Guertin KR, Hilliard DW, Kester RF, Mahaney PE, Marcus L, Qi L, Spence CL, Tengi J, Magnuson MA, Chu CA, Dvorozniak MT, Matschinsky FM, Grippo JF 2003 Allosteric activators of glucokinase: potential role in diabetes therapy. Science 301:370-373
26. Guertin KR, Grimsby J 2006 Small molecule glucokinase activators as glucose lowering agents: a new paradigm for diabetes therapy. Current medicinal chemistry 13:1839-1843
27. Kamata K, Mitsuya M, Nishimura T, Eiki J, Nagata Y 2004 Structural basis for allosteric regulation of the monomeric allosteric enzyme human glucokinase. Structure 12:429-438
28. Leighton B, Atkinson A, Coghlan MP 2005 Small molecule glucokinase activators as novel anti-diabetic agents. Biochem Soc Trans 33:371-374
29. Matschinsky FM 2009 Assessing the potential of glucokinase activators in diabetes therapy. Nat Rev Drug Discov 8:399-416
30. Printz RL, Granner DK 2005 Tweaking the glucose sensor: adjusting glucokinase activity with activator compounds. Endocrinology 146:3693-3695
31. Hoat TX, Bertin N, Ninomiya N, Fukuda S, Usui K, Kawai J, Hayashizaki Y, Suzuki H 2009 Development of a high-throughput method for the systematic identification of human proteins nuclear translocation potential. BMC Cell Biol 10:69
32. Ikeda M, Inoue S, Muramatsu M, Minatogawa Y 2007 Characterization and identification of a steroid receptor-binding protein, SRB-RGS. Biol Pharm Bull 30:1056-1064
33. Massoud TF, Paulmurugan R, De A, Ray P, Gambhir SS 2007 Reporter gene imaging of protein-protein interactions in living subjects. Curr Opin Biotechnol 18:31-37
34. Murakami Y, Fukazawa H, Kobatake T, Yamagoe S, Takebe Y, Tobiume M, Matsuda M, Uehara Y 2002 A mammalian two-hybrid screening system for inhibitors of

interaction between HIV Nef and the cellular tyrosine kinase Hck. Antiviral Res 55:161-168
35. Pan J, Peng X, Gao Y, Li Z, Lu X, Chen Y, Ishaq M, Liu D, Dediego ML, Enjuanes L, Guo D 2008 Genome-wide analysis of protein-protein interactions and involvement of viral proteins in SARS-CoV replication. PLoS One 3:e3299
36. Shioda T, Andriole S, Yahata T, Isselbacher KJ 2000 A green fluorescent protein-reporter mammalian two-hybrid system with extrachromosomal maintenance of a prey expression plasmid: application to interaction screening. Proc Natl Acad Sci U S A 97:5220-5224
37. Zhao HF, Kiyota T, Chowdhury S, Purisima E, Banville D, Konishi Y, Shen SH 2004 A mammalian genetic system to screen for small molecules capable of disrupting protein-protein interactions. Anal Chem 76:2922-2927
38. Bartel P, Chien CT, Sternglanz R, Fields S 1993 Elimination of false positives that arise in using the two-hybrid system. Biotechniques 14:920-924
39. Chien CT, Bartel PL, Sternglanz R, Fields S 1991 The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc Natl Acad Sci U S A 88:9578-9582
40. Fields S, Song O 1989 A novel genetic system to detect protein-protein interactions. Nature 340:245-246
41. Hummer A, Delzeith O, Gomez SR, Moreno RL, Mark MD, Herlitze S 2003 Competitive and synergistic interactions of G protein beta(2) and Ca(2+) channel beta(1b) subunits with Ca(v)2.1 channels, revealed by mammalian two-hybrid and fluorescence resonance energy transfer measurements. J Biol Chem 278:49386-49400
42. van Schaftingen E, Veiga-da-Cunha M, Niculescu L 1997 The regulatory protein of glucokinase. Biochem Soc Trans 25:136-140
43. Iynedjian PB, Pilot PR, Nouspikel T, Milburn JL, Quaade C, Hughes S, Ucla C, Newgard CB 1989 Differential expression and regulation of the glucokinase gene in liver and islets of Langerhans. Proc Natl Acad Sci U S A 86:7838-7842
44. Tiedge M, Steffeck H, Elsner M, Lenzen S 1999 Metabolic regulation, activity state, and intracellular binding of glucokinase in insulin-secreting cells. Diabetes 48:514-523
45. Baltrusch S, Lenzen S 2007 Novel insights into the regulation of the bound and diffusible glucokinase in MIN6 beta-cells. Diabetes 56:1305-1315
46. Rizzo MA, Magnuson MA, Drain PF, Piston DW 2002 A functional link between glucokinase binding to insulin granules and conformational alterations in response to glucose and insulin. J Biol Chem 277:34168-34175
47. Antoine M, Boutin JA, Ferry G 2009 Binding kinetics of glucose and allosteric activators to human glucokinase reveal multiple conformational states. Biochemistry 48:5466-5482
48. Zhang J, Li C, Chen K, Zhu W, Shen X, Jiang H 2006 Conformational transition pathway in the allosteric process of human glucokinase. Proc Natl Acad Sci U S A 103:13368-13373
49. He L, Bradrick TD, Karpova TS, Wu X, Fox MH, Fischer R, McNally JG, Knutson JR, Grammer AC, Lipsky PE 2003 Flow cytometric measurement of fluorescence (Forster) resonance energy transfer from cyan fluorescent protein to yellow fluorescent protein using single-laser excitation at 458 nm. Cytometry A 53:39-54
50. Vanderklish PW, Krushel LA, Holst BH, Gally JA, Crossin KL, Edelman GM 2000 Marking synaptic activity in dendritic spines with a calpain substrate exhibiting fluorescence resonance energy transfer. Proc Natl Acad Sci U S A 97:2253-2258

## Figure legends

**FIG. 1.** Glucose dependent modulation of glucokinase binding to the glucokinase regulatory protein. The interaction strength of the fusion proteins DNA-BD-GRP and AD-GK was measured in a fluorescence based mammalian two-hybrid assay. DNA-BD and AD-GK (white bars) or DNA-BD-GRP and AD (dashed bars) or DNA-BD-GRP and AD-GK (black bars) were coexpressed in COS cells. The cells were preincubated in the presence of 5 mmol/l or 25 mmol/l glucose. Thereafter, the fluorescence intensities of ECFP and EYFP were determined. Data are expressed as means ± SEM of three to five individual experiments with a total of 142 to 266 nuclei analyzed. **, $p < 0.01$; ***, $p < 0.001$ compared with negative controls; ##, $p < 0.01$ compared with cells incubated with 5 mmol/l glucose (ANOVA/Bonferroni's test).

**FIG. 2.** Glucose promoted binding of islet PFK-2/FBPase-2 to glucokinase. The interaction strength of the fusion proteins DNA-BD-PFK-2/FBPase-2 and AD-GK was measured in a fluorescence based mammalian two-hybrid assay in insulin-secreting MIN6 cells. DNA-BD-PFK-2/FBPase-2 and AD (white bars) or DNA-BD-PFK-2/FBPase-2 and AD-GK (black bars) were coexpressed in MIN6 cells and preincubated with 3 mmol/l or 25 mmol/l glucose. Thereafter, the fluorescence intensities for ECFP and EYFP were determined. Data are expressed as means ± SEM of three individual experiments with a total of 60 to 70 nuclei analyzed. **, $p < 0.01$ compared with negative control (ANOVA/Bonferroni's test).

**FIG. 3.** The interaction strength between glucokinase and PFK-2/FBPase-2 rises with increasing glucose concentrations. The interaction of the fusion proteins DNA-BD-PFK-2/FBPase-2 and AD-GK was measured in a semi-automated fluorescence based mammalian two-hybrid assay. DNA-BD-PFK-2/FBPase-2 and AD (dashed line in A and white bars in B) or DNA-BD-PFK-2/FBPase-2 and AD-GK (solid line in A and black bars in B) were coexpressed in insulin-secreting MIN6 cells. In cells cultured at 3, 10 or 25 mmol/l glucose, respectively, the degree of interaction between DNA-BD and AD proteins was analyzed every hour as the mean value for the nuclear EYFP/ECFP ratio. (A) Images were taken hourly over a period of 40 h. The time lapse of the calculated EYFP/ECFP ratio is exemplary shown for a representative single experiment for the interaction of glucokinase with islet PFK-2/FBPase-2 at 25 mmol/l glucose (solid line) together with the respective negative control at 25 mmol/l glucose (dashed line). Data are expressed as means ± SEM of 2-120 nuclei analyzed per time point. (B) The mean nuclear EYFP/ECFP ratios obtained between 36 and 48 hours post transfection were determined together with the respective SEM for 341 to 1369 cells per sample.

The means ± SEM of the EYFP/ECFP ratio are shown for three to five independent experiments with a total of 1331 to 5905 nuclei analyzed. *, $p < 0.05$ compared with negative control, #, $p < 0.05$ compared with cells incubated with 3 mmol/l glucose (ANOVA/Bonferroni's test).

**FIG. 4.** Effect of low versus high glucose on colocalization between glucokinase and islet PFK-2/FBPase-2. ECFP-GK and EYFP-PFK-2/FBPase-2 fusion proteins were co-overexpressed in COS cells and precultured in medium containing 3 mmol/l or 25 mmol/l glucose, respectively. Fluorescence images were taken and the degree of colocalization was calculated with the Imaris software (Bitplane). (A, B) A typical image overlay of one cell incubated at 3 mmol/l glucose (A) and one at 25 mmol/l glucose (B) is shown with the associated correlation diagram. ECFP-GK is depicted in green and EYFP-PFK-2/FBPase-2 in red. (C) Colocalization of ECFP-GK and EYFP-PFK-2/FBPase-2 is expressed as means ± SEM of three individual experiments with a total of 6 to 8 cells analyzed.

**FIG. 5.** Effect of low versus high glucose on FRET between glucokinase and its binding partners GRP and islet PFK-2/FBPase-2. Fluorescent proteins were coexpressed in COS cells and precultured with the indicated glucose concentrations. Thereafter, fluorescence images were taken in living cells and the sensitized emission based FRET efficiency (FRETN) was calculated from the ECFP and EYFP emission intensities. (A) Difference in FRETN between separately expressed ECFP and EYFP (white bars) or the fluorescent fusion protein ECFP-EYFP (black bars). (B) FRETN between EYFP-GRP and ECFP (white bars) or ECFP-GK (black bars). (C) FRETN between EYFP-PFK-2/FBPase-2 and ECFP (white bars) or ECFP-GK (black bars). Data are expressed as means ± SEM of three individual experiments with a total of 9 to 22 cells analyzed. *, $p < 0.05$; ***, $p < 0.001$ compared with the negative control; ###, $p < 0.001$ compared with cells incubated with 3 mmol/l or 5.5 mmol/l glucose, respectively (A, student's *t* test; B and C, ANOVA/Bonferroni's test).

**FIG. 1.**

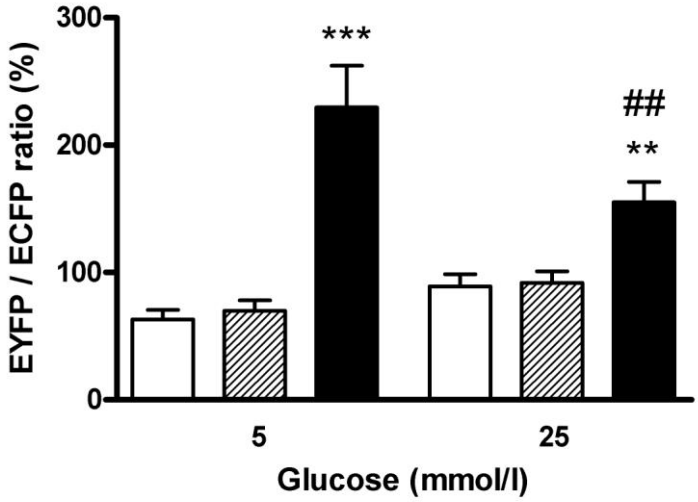


**FIG. 2.**

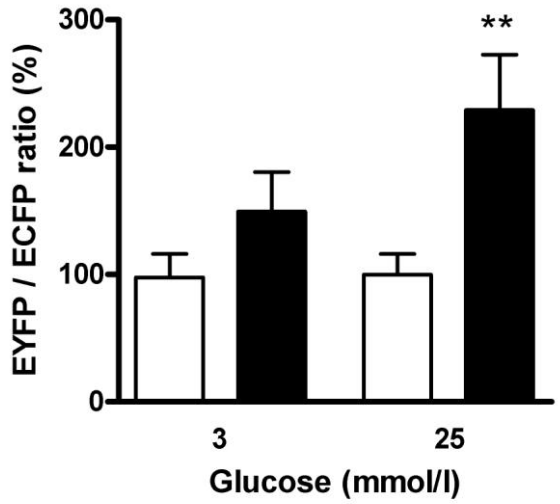


**FIG. 3.**

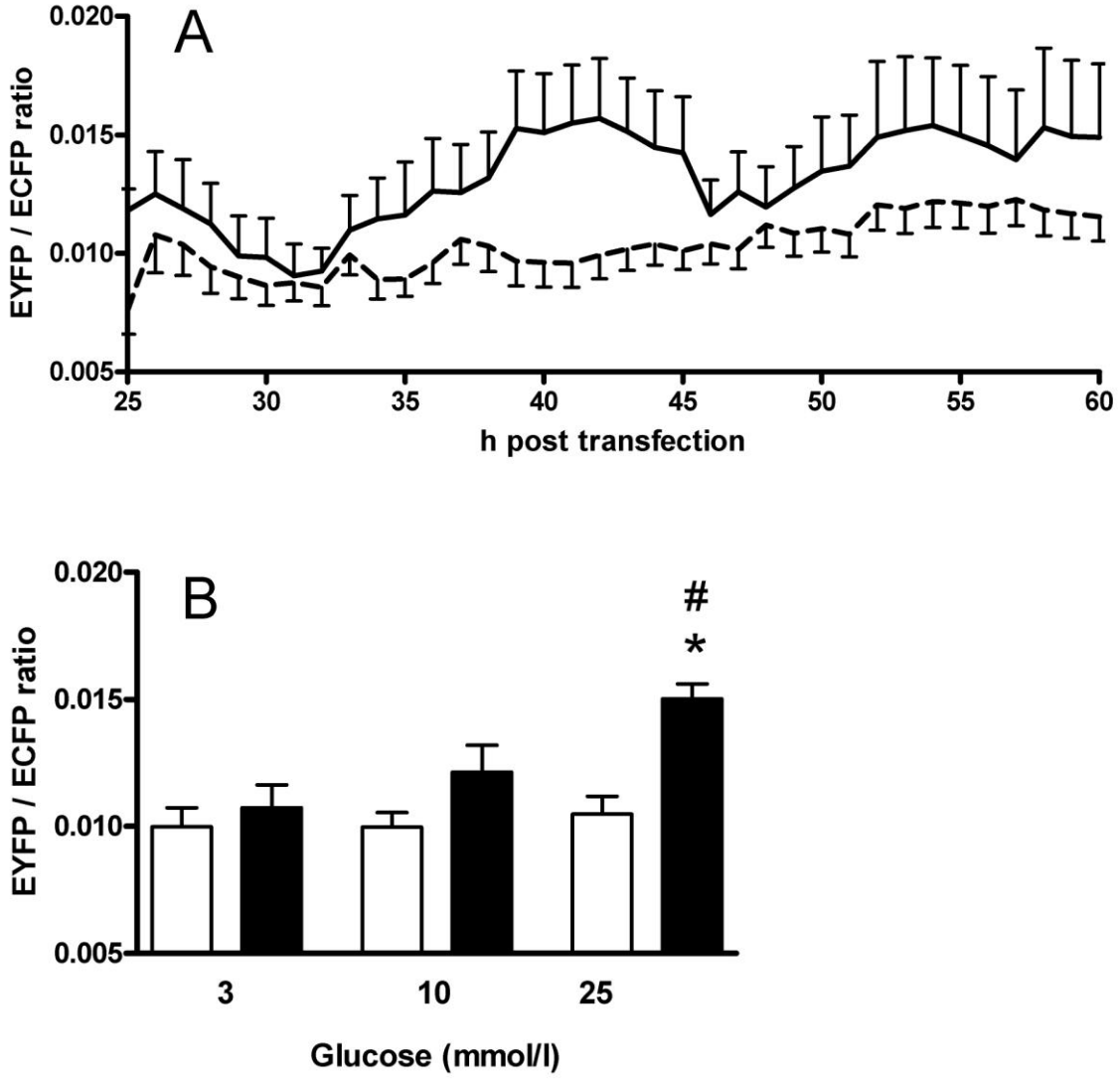

A
0.020
0.015
0.010
0.005
EYFP / ECFP ratio
25
30
35
40
45
50
55
60
h post transfection
B
#
*
0.020
0.015
0.010
0.005
EYFP / ECFP ratio
3
10
25
Glucose (mmol/l)

**FIG. 4.**

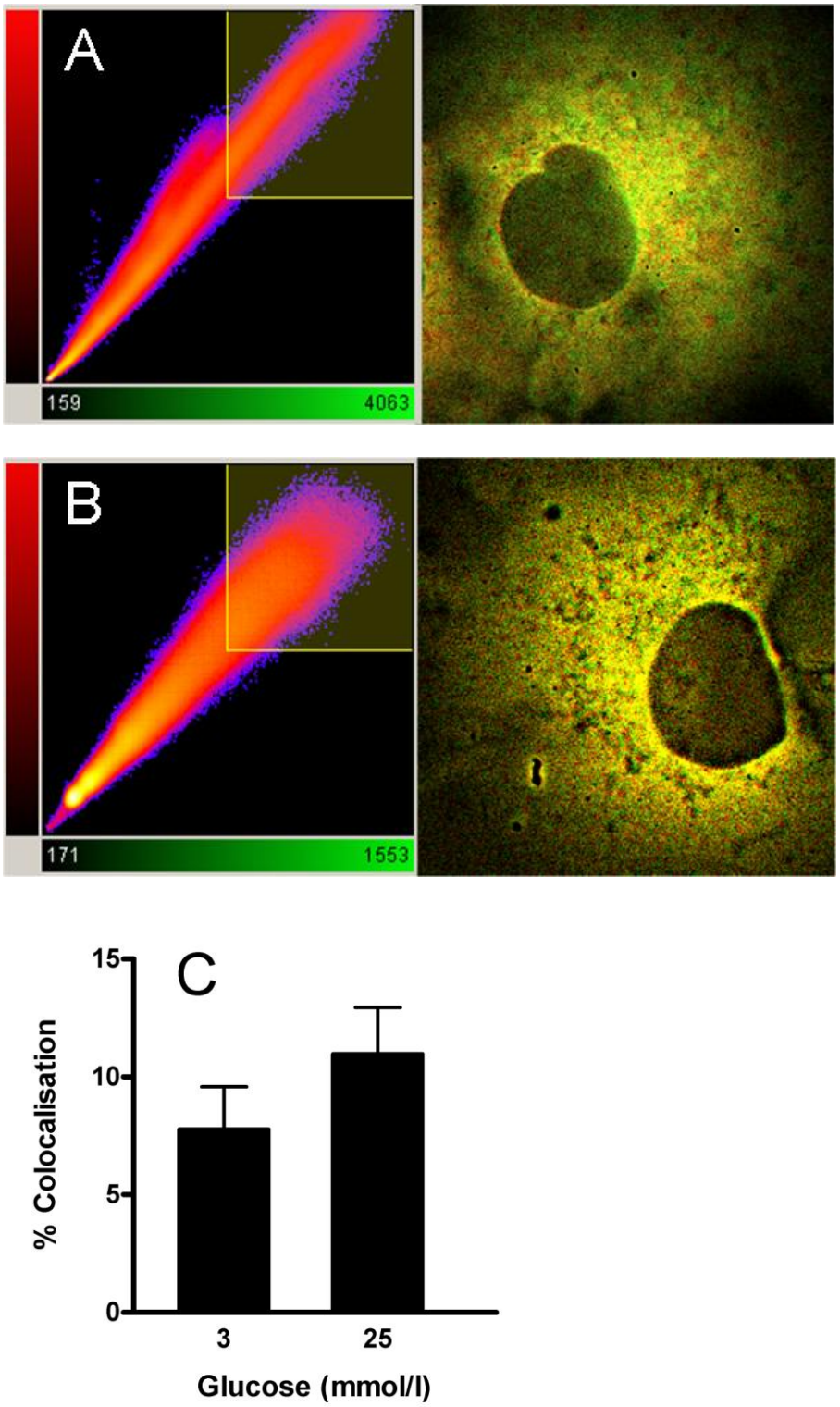
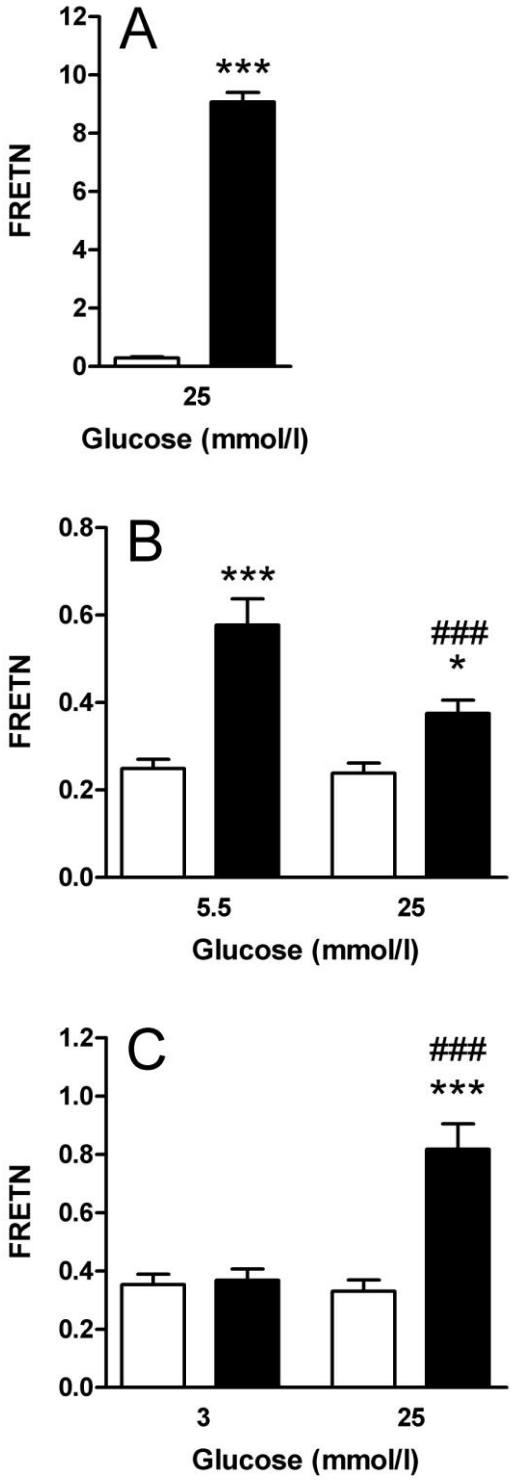

A
159
4063
B
171
1553
C
15
10
5
0
% Colocalisation
3
25
Glucose (mmol/l)

**FIG. 5.**


A
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FRETN
12
10
8
6
4
2
0
25
Glucose (mmol/l)
B
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*
FRETN
0.8
0.6
0.4
0.2
0.0
5.5
25
Glucose (mmol/l)
C
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FRETN
1.2
1.0
0.8
0.6
0.4
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0.0
3
25
Glucose (mmol/l)

# Dimer interface rearrangement of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase rat liver isoenzyme by cAMP-dependent Ser-32 phosphorylation

Short title: PFK-2/FBPase-2 dimerization

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Abbreviations used: AD, activation domain; BD, binding domain; DMEM, Dulbecco's modified Eagle's medium; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; FRET, fluorescence resonance energy transfer; PFK-2/FBPase-2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase

## Abstract

The bifunctional enzyme 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (PFK-2/FBPase-2) determines the cellular fructose 2,6-bisphosphate content and thus, is a key regulator of carbohydrate metabolism in liver. Ser-32 phosphorylation reciprocally modulates the activities of the liver bifunctional enzyme. Although the molecular mechanism has not been fully defined, conformational changes in quaternary and tertiary structures have been implicated. Therefore the goal of this study was to elucidate the regulatory role of Ser-32 phosphorylation on the kinase domain mediated dimerization of PFK-2/FBPase-2 and potential rearrangement of the quaternary structure. Forskolin, mimicking the effect of glucagon, increased the cellular cAMP content by 60% in COS cells and induced a five-fold increase in Ser-32 phosphorylation in the wild-type but not in a S32A/H258A double mutant enzyme. The use of a fluorescence-based mammalian two-hybrid system revealed clear evidence of preferential binding within homodimers in contrast to heterodimers, namely binding of two Ser-32 phosphorylated wild-type proteins or two S32A/H258A double mutant proteins. Using FRET a close proximity of two PFK-2/FBPase-2 monomers was only detectable in the phosphorylated wild-type enzyme dimer, thus, suggesting a flexible kinase interaction mode enabling at least two PFK-2/FBPase-2 dimer conformations. Furthermore the obtained data support the hypothesis that the conformational switch within the PFK-2/FBPase-2 dimer plays an important role in coupling of hormonal and posttranslational enzyme regulation to the metabolic response. Reduction in the Ser-32 phosphorylation concomitantly increases the kinase:bisphosphatase activity ratio and switches the liver metabolism to glycolysis. Dephosphorylation of Ser-32 relaxes the PFK-2/FBPase-2 dimer thereby promoting binding and activation of the glucose-sensing enzyme glucokinase. Interaction of glucokinase and PFK-2/FBPase-2 provides coordinated regulation of glucose phosphorylation and the committing step to glycolysis in the liver. The results of this study also suggest a novel quaternary structure for a PFK-2/FBPase-2 dimer within living cells, the heterodimer.

## Introduction

Fructose 2,6-bisphosphate is an important regulatory metabolite of the mammalian carbohydrate metabolism that has been found in virtually every eukaryotic tissue or cell [1]. Fructose 2,6-bisphosphate is the most potent allosteric activator of the enzyme 6-phosphofructo-1-kinase that catalyzes the rate-limiting step of glycolysis [2]. The activation of 6-phosphofructo-1-kinase by increased fructose 2,6-bisphosphate has been confirmed in rat liver, while inhibition of fructose 1,6-bisphosphatase was not detected upon glucose gavage [3]. 6-Phosphofructo-2-kinase/fructose 2,6-bisphosphatase (PFK-2/FBPase-2) provides a fast switch that along with glucokinase co-ordinately upregulates hepatic glucose disposal [2]. In mammals this regulatory enzyme with bifunctional activity is responsible for determining the levels of fructose 2,6-bisphosphate by synthesizing and degrading this compound at distinct active sites.

Four genes, designated PFKFB1-PFKFB4, encode the mammalian bifunctional isoenzymes, and differential splicing gives rise to a number of isoforms that share the catalytic core of the parent isoenzyme, but differ in flanking sequences [4]. The N-terminal kinase (EC 2.7.1.105) of PFK-2/FBPase-2 synthesizes fructose 2,6-bisphosphate from fructose 6-phosphate and ATP, whereas the C-terminal bisphosphatase (EC 3.1.3.46) of PFK-2/FBPase-2 catalyzes the hydrolysis of fructose 2,6-bisphosphate to fructose 6-phosphate and inorganic phosphate. As neither the kinase reaction nor the bisphosphatase reaction is freely reversible the steady state concentration of fructose 2,6-bisphosphate is determined by the kinase:bisphosphatase activity ratio [5]. This ratio is modulated by allosteric and hormonal regulation as well as dimerization of PFK-2/FBPase-2 and binding of glucokinase. The PFK-2/FBPase-2 protein exists as a dimer that is stabilized by protein-protein interactions between the kinase domains [6, 7]. Tissue-specific hormonal regulation of the enzyme activities is mediated by phosphorylation sites that are located in the terminal regions of both, the kinase and the bisphosphatase domain of PFK-2/FBPase-2. The terminal regions have also been shown to mediate the effect of Ser-32 phosphorylation to both active sites which are located in opposite ends of the monomer [8].

The complex regulation of PFK-2/FBPase-2 through metabolic and hormonal signals is best understood in the case of the liver isoenzyme which is encoded by the PFKFB1 gene [5, 9]. The hepatic PFK-2/FBPase-2 is posttranslationally regulated by cAMP-dependent phosphorylation by protein kinase A in response to glucagon at the amino acid residue Ser-32 [10, 11]. Phosphorylation of Ser-32 causes both a reduction of the kinase activity and an enhancement of the bisphosphatase activity resulting in a decrease in the kinase:bisphosphatase activity

ratio and subsequently in increased hepatic glucose production [5]. Molecular rearrangements in the PFK-2/FBPase-2 dimer have been shown to be involved in the regulation of the enzyme activities of the kinase and the bisphosphatase [8, 12, 13].

In liver the glucose phosphorylating and glucose-sensing enzyme glucokinase (Hexokinase Typ IV; EC 2.7.1.1) has an important role in regulating fuel metabolism. Interestingly a regulatory nexus between glucokinase and PFK-2/FBPase-2 by two different mechanisms has been conclusively demonstrated. First, a rise in intracellular fructose 2,6-bisphosphate stimulating gene expression of glucokinase [14]; second, the posttranslational regulation of PFK-2/FBPase-2 binding to, and activating, glucokinase [15-18]. The glucokinase binding motif is located in the bisphosphatase domain of PFK-2/FBPase-2 and highly conserved between the isoenzymes [7], suggesting an even wider regulatory role of the bifunctional enzyme in carbohydrate metabolism [19].

In the present study the effect of cAMP-dependent Ser-32 phosphorylation on homodimerization of the rat liver PFK-2/FBPase-2 enzyme was investigated. The interaction between two PFK-2/FBPase-2 monomers was analyzed by a mammalian two-hybrid system and fluorescence resonance energy transfer (FRET) for the liver wild-type enzyme and compared to a S32A/H258A double mutant of the bifunctional enzyme.

## Experimental

### Materials

Restriction enzymes and modifying enzymes for the cloning procedures were from Fermentas (St. Leo-Rot, Germany) or New England Biolabs (Beverly, MA, USA). Custom oligonucleotides were synthesized by MWG Biotech (Ebersberg, Germany) or Invitrogen (Karlsruhe, Germany). The reporter vector pHASH-3 was a kind gift of Dr. S. Herlitze (Department of Neurosciences, Case Western Reserve University, Cleveland, OH). Forskolin was from ICN Biomedicals (Irvine, CA). Immobilon-P polyvinylidine difluoride membranes were from Millipore (Bedford, MA). All reagents of analytical grade were from Merck (Darmstadt, Germany). All tissue culture equipment was from Invitrogen and Greiner-Bio One (Frickenhausen, Germany).

### Cell culture and transient transfection

COS cells were grown in DMEM supplemented with 25 mmol/l glucose, 10% (v/v) fetal calf serum, 10 U/ml penicillin, 10 µg/ml streptomycin and 2 mmol/l glutamine in a humidified atmosphere at 37 °C and 5% $CO_2$. For the mammalian two-hybrid experiments COS cells (passages 13-29) were seeded in six-well microplates at a density of 2 x $10^4$ cells and grown for one day. Thereafter, cells were transfected with the mammalian two-hybrid vectors with jetPEI (Qbiogene, Montreal, QC, Canada) according to the manufacturer's instructions. For each transfection 0.4 pmol of the reporter vector pHASH-3 was used in combination with 0.1 pmol of the expression vector pM (negative control), pM-PFK-2/FBPase-2 wild-type or pM-PFK-2/FBPase-2(S32A/H258A) and 0.1 pmol of the expression vector pVP16 (negative control), pVP16-PFK-2/FBPase-2 wild-type or pVP16-PFK-2/FBPase-2(S32A/H258A). After incubation for 20 h the cells were incubated for further 2 h in DMEM with or without 10 µmol/l forskolin before data acquisition. For determination of the cellular cAMP content COS cells (passages 26-31) were seeded in six-well microplates at a density of 1 x $10^4$ cells and grown for four days. Thereafter, cells were transfected with the mammalian two-hybrid vectors as described above and cultured for another 20-24 h. For Western blot analysis of PFK-2/FBPase-2 Ser-32 phosphorylation COS cells (passages 15-21) were seeded on 10-cm tissue culture dishes at a density of 5 x $10^5$ cells and grown for one day. Then the cells were transfected with 5 µg of the previously described [17] expression vectors pcDNA3zeo-PFK-2/FBPase-2 wild-type or pcDNA3zeo-PFK-2/FBPase-2(S32A/H258A) mutant with jetPEI (Qbiogene) and were cultured for one further day. For the isolation of Dendra2 fusion protein COS cells (passages 12-28) were seeded on 14.5-cm tissue culture dishes at a density of 5 x $10^5$ cells and grown for one day. Thereafter, cells were transfected, three dishes each for pro-

tein isolation, with 14.5 µg of the expression vectors pDendra2-GK, pDendra2-PFK-2/FBPase-2 wild-type, or pDendra2-PFK-2/FBPase-2(S32A/H258A) mutant with jetPEI (Qbiogene). One day after transfection the culture medium was replaced and the cells were incubated for one further day before the overexpressed fusion proteins were isolated.

**Mammalian two-hybrid analysis**

The cDNAs of rat liver PFK-2/FBPase-2 wild-type and S32A/H258A double mutant were amplified by PCR and subcloned in frame (*Sal*I and *Hind*III restriction sites) to both the GAL4-DNA-BD into pM and the VP16-AD into pVP16 (Clontech, Palo Alto, CA). The fluorescence-based reporter plasmid pHASH-3 [20] was used for transfection of COS cells in combination with the pM and pVP16 constructs. pHASH-3 contains the gene for the yellow fluorescent protein (EYFP) under control of an inducible promoter containing GAL4 binding sites. Furthermore, pHASH-3 carries the cyan fluorescent protein (ECFP) gene which is constitutively expressed. Both EYFP and ECFP carry a nuclear localization signal. For analysis of an interaction of the fusion proteins fluorescence intensities of EYFP and ECFP were determined in the nuclei of transfected cells. To quantify the interaction strength the ratio EYFP/ECFP was calculated from the background corrected average grey values of the EYFP and ECFP fluorescence intensities.

**Determination of cAMP**

Transfected COS cells were washed twice with Krebs-Ringer solution (115 mmol/l NaCl, 4.7 mmol/l KCl, 2.6 mmol/l $CaCl_2$, 1.2 mmol/l $KH_2PO_4$, 1.2 mmol/l $MgSO_4$, 10 mmol/l Hepes, 20 mmol/l $NaHCO_3$, 25 mmol/l glucose, pH 7.2) and incubated for 15 min in Krebs-Ringer solution with or without 100 µmol/l forskolin. Analysis of cAMP was performed with the cAMP-Glo™ Assay (Promega, Mannheim, Germany) according to the manufacturer's instructions. The assay is based on the principle that cAMP stimulates protein kinase A holoenzyme activity, decreasing available ATP and leading to decreased light production in a coupled luciferase reaction. Light emission was recorded as a 1-sec integral using a Victor$^2$ Multilabel Counter (Wallac, Freiburg, Germany). cAMP values were calculated in relation to cAMP standard values.

**Western blot analysis**

Untransfected COS cells and COS cells overexpressing PFK-2/FBPase-2 wild-type or S32A/H258A mutant were washed with PBS (pH 7.4) and incubated for 10 min in PBS with or without 100 µmol/l forskolin. Immediately after incubation the cells were homogenized by sonication in PBS, and insoluble material was pelleted by centrifugation. FBPase-2 and

phospho-Ser-32-PFK-2 Western blot analyses were performed from identical samples to allow determination of the Ser-32 phosphorylated subfraction of PFK-2/FBPase-2. Immunoreactivity to FBPase-2 was determined by Western blotting from 15 µg total cellular protein using a previously described antibody against rat liver FBPase-2 raised in chicken [17] and to phospho-Ser-32-PFK-2 from 150 µg total cellular protein using a phospho-specific antibody raised against the peptide LQRRRG[p]SSIPQF (Eurogentec, Seraing, Belgium). The specific protein bands were visualized using the enhanced chemiluminescence detection system (GE Healthcare, Amersham, Buckinghamshire, UK) in the ChemiLux Imager (Intas, Göttingen, Germany) and quantified by densitometry using the Gel-Pro Analyser software (MediaCybernetics, Silver Spring, MD, USA).

**Measurement of FRET from purified Dendra2 fusion proteins.**

The pDendra2-C vector (Evrogen, Moscow, Russia) was used for expression of the green to red photoconvertible monomeric fluorescent protein Dendra2. The cDNA encoding rat liver PFK-2/FBPase-2 wild-type or S32A/H258A double mutant or human beta-cell glucokinase was amplified by PCR and subcloned in frame (*Kpn*I and *BamH*I or *ApaI* and *BamH*I restriction sites, respectively) to the Dendra2 into pDendra2-C. The fusion proteins were overexpressed in COS cells. The cells from three 14.5-cm culture dishes of the respective protein were combined, homogenized in PBS (pH 7.4), and insoluble material was pelleted by centrifugation. Dendra2 fusion protein was isolated using Dynabeads Protein G (Invitrogen, Karlsruhe, Germany) with anti-Dendra2-IgG antibody (Evrogen, Moscow, Russia) at 4 °C according to manufacturer's instructions. One fraction of purified protein of each preparation was immediately used for FRET measurements. The remaining fraction was incubated at -20 °C for at least 24 h before the measurement was performed. The red fluorescence (filter setup F572/28, AHF Analysentechnik, Tübingen, Germany) of 0.2 µmol/l purified protein in 100 µl 20 mmol/l Tris, 100 mmol/l NaCl and 2 mmol/l dithiothreitol (pH 7.4) was recorded in a black microplate as a 0.1-sec integral in a Victor$^2$ Multilabel Counter (Wallac, Freiburg, Germany) while the green form of the fluorescent protein was excited (filter setup F485/14, AHF Analysentechnik, Tübingen, Germany). Then the sample was irradiated with a 405 nm UV diode at 12 V for 15 min to induce photoconversion of Dendra2 [21, 22] and the fluorescence measurement was repeated. The increase of red fluorescence with F485/14 excitation after photoconversion was calculated.

**Fluorescence microscopy**

A cell$^{R}$/Olympus IX81 inverted microscope system equipped with a Cellcubator, as described previously [23], was used. The six-well plates were fixed on the microscope stage, and images were obtained with an UPLSAPO 20 × 0.75 NA objective (Olympus, Hamburg, Germany). D436/10-455DCLP-D480/40 and HQ500/20-530DCLP-D560/40 filter sets were used for ECFP and EYFP, respectively (AHF Analysentechnik, Tübingen, Germany).

**Statistical analyses**

The data are expressed as means ± SEM. Statistical analyses were performed by ANOVA followed by Bonferroni's test for multiple comparison or Student's *t* test using the Prism analysis program (GraphPad, San Diego, CA, USA).

## Results

### Mammalian two-hybrid interaction within the liver PFK-2/FBPase-2 protein homodimer

Using a yeast two-hybrid system it was previously shown, that the kinase domain mediates PFK-2/FBPase-2 dimerization [7]. Two-hybrid assays in mammalian cells are based on the same principles as in yeast cells. However, the use of mammalian cells opens the possibility to study the effect of physiologically relevant posttranslational modifications on protein interactions. The interaction of the fusion proteins DNA-BD-PFK-2 and AD-PFK-2 was determined in a fluorescence-based mammalian two-hybrid assay. Binding of the two kinase domains, namely DNA-BD-PFK-2 and AD-PFK-2 was significant in COS cells (data not shown). Hepatic PFK-2/FBPase-2 phosphorylation in COS cells was induced with forskolin mimicking the effect of glucagon. The interaction strength of the fusion proteins DNA-BD-PFK-2/FBPase-2 and AD-PFK-2/FBPase-2 was measured for the liver wild-type enzyme (Fig. 1A) and for the S32A/H258A double mutant (Fig. 1B) with or without forskolin. The dimeric organization of both the wild-type and the mutant enzyme could clearly be demonstrated. The EYFP/ECFP ratio in untreated cells was six times higher than the negative controls for the wild-type PFK-2/FBPase-2 and eight times higher than the negative controls for the mutant enzyme, indicating a stronger interaction. When the cells were cultured in the presence of forskolin for 2 h before data acquisition the two-hybrid signal was unchanged for the wild-type enzyme. Unexpectedly, in case of the S32A/H258A mutant forskolin treatment resulted in an almost 50% increase of the EYFP/ECFP ratio. Indeed forskolin-induced Ser-32 phosphorylation is impossible due to the S32A mutation in the PFK-2/FBPase-2 protein. However, the liver-type PFK-2/FBPase-2 is endogenously expressed in COS cells. If this is taken into account, the observed phenomenon can be explained by an intramolecular quenching effect on the two-hybrid fusion proteins (Fig. 2).

### Effects of forskolin on the cAMP content in COS cells

COS cells were transfected with the two-hybrid vectors to constitute the same cellular conditions as used in the two-hybrid measurements. Without forskolin the cellular cAMP content was only slightly higher in cells overexpressing PFK-2/FBPase-2 fusion proteins than in cells expressing solely the DNA-BD and AD. Forskolin incubation induced a 50-65% increase of the cAMP content irrespective of the overexpressed PFK-2/FBPase-2 proteins (Fig. 3).

**Effects of forskolin on Ser-32 phosphorylation of PFK-2/FBPase-2.**

Untransfected COS control cells and COS cells overexpressing PFK-2/FBPase-2 wild-type or S32A/H258A double mutant were analyzed by Western blotting. Expression of the wild-type and mutant enzyme was comparable (Fig. 4A). Ser-32 phosphorylation could only be detected for wild-type PFK-2/FBPase-2 (Fig. 4B). The degree of phosphorylated protein was five-fold higher after forskolin treatment (Fig. 5C).

**Interaction of PFK-2/FBPase-2 kinase domains within the enzyme homodimer.**

Fusion proteins of Dendra2 and the liver PFK-2/FBPase-2 wild-type, the PFK-2/FBPase-2 S32A/H258A double mutant and glucokinase were expressed in COS cells and isolated. Dendra2 is a green-to-red photoactivatable fluorescent protein. The UV light-induced red fluorescence of Dendra2 is stable [22]. Thus, the two fluorescent states can act as a donor-acceptor pair in FRET experiments, and Dendra2 can be used to detect close spatial proximities of the proteins fused to it. As the N-terminal kinase domain of PFK-2/FBPase-2 mediates dimerization of the PFK-2/FBPase-2 homodimer FRET is supposed to occur only when two kinase domains are very close to each other. Glucokinase is a monomeric protein [24] and was used in control experiments as a Dendra2 fusion protein to determine the amount of bleed-through of green light that is detected by the red emission channel due to unavoidable transmission of the fluorescence filter. A significant FRET signal was detected between wild-type PFK-2/FBPase-2 proteins immediately used after isolation, but not between S32A/H258A double mutant proteins (Fig. 5). Incubation at -20 °C generally diminishes protein phosphorylation, and thus phosphate binding at Ser-32 of PFK-2/FBPase-2. Proteins from batches previously freshly measured were incubated at -20 °C. Thereafter no FRET signal was observed between the wild-type PFK-2/FBPase-2 proteins (Fig. 5). This provides clear evidence that phosphorylation leads to a stronger binding of the PFK-2/FBPase-2 kinase domains within the enzyme homodimer (Fig. 6).

## Discussion

The enzyme 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (PFK-2/FBPase-2) plays a pivotal role in the regulation of hepatic carbohydrate metabolism. As the enzyme is bifunctional, thus able to synthesize and degrade fructose 2,6-bisphosphate, it has to be regulated in a comprehensive manner [25]. An important regulatory principle of the liver PFK-2/FBPase-2 isoenzyme is the glucagon-mediated Ser-32 phosphorylation which causes high bisphosphatase activity and concomitantly increases net production of glucose [10, 11, 26]. Reciprocally, diminishing phosphorylation provokes a fast switch of PFK-2/FBPase-2 to a high kinase:bisphosphatase activity ratio assuring effective glycolysis upon cellular glucose uptake. While it has been shown by different experimental approaches that dimerization of the bifunctional enzyme is mediated by the N-terminal kinase domain [6, 7, 27], the potential role of Ser-32 phosphorylation on enzyme dimerization is so far poorly understood. This is critical because interaction of the C- and N-terminal regions with the active sites has been suggested to provide the means for reciprocal modulation of the activities in response to de/phosphorylation of Ser-32 [8, 12].

The yeast two-hybrid system is a well established and an excellent technique to determine protein-protein interactions. Implementing the two-hybrid assays in mammalian cells paves the way to study the effect of physiologically relevant posttranslational modifications on protein interactions. Thus, this system was applied to characterize the role of Ser-32 phosphorylation on PFK-2/FBPase-2 dimerization. However, certain aspects should be taken into account interpreting the mammalian two-hybrid data. First, the quantification of a dimerization via a two-hybrid signal will always lead to an underestimation of the binding strength. Two monomers fused to the same two-hybrid domain can dimerize, namely two AD or two DNA-BD fusion proteins. In this case, even though there is an interaction between the proteins the complex is not able to induce the reporter protein. Second, the PFK-2/FBPase-2 enzyme is expressed in virtually every mammalian tissue and as well in COS cells. Recently it was shown by real-time reverse transcription-PCR for determination of PFKFB1-3 expression, that in rat and mouse liver, heart, brain and islet tissue there is typically one isoenzyme predominantly expressed. However, the expression of the remaining isoenzymes was also detected in significant quantities [15]. Thus, interaction of the two-hybrid fusion proteins with endogenous PFK-2/FBPase-2 will further reduce the signal for the dimerization.

The present study allows the conclusion that an unphosphorylated endogenous PFK-2/FBPase-2 monomer could bind to every unphosphorylated exogenous monomer, regardless of the S32A mutation in the two-hybrid fusion protein. Dephosphorylation of Ser-32 prior to

addition of forskolin is assured by glucose level in media [10]. This results in “mixed dimer” formation, as indicated with a circle in the scheme (Fig. 2). “Mixed dimers” clearly exert a quenching effect on the two-hybrid signal because the involved two-hybrid fusion proteins are not able to contribute to the induction of the reporter gene. It can be assumed that such a formation of a “mixed dimer” is adverse because it contains a phosphorylated and a dephosphorylated monomer.

To prove this hypothesis the Ser-32 phosphorylation state of PFK-2/FBPase-2 in COS cells was specifically enhanced with forskolin as the fluorescent signals were assayed. It could be demonstrated that forskolin significantly increased the intracellular cAMP content and that expression of the two-hybrid vectors did not affect the cAMP content in COS cells. Finally cAMP-dependent Ser-32 phosphorylation of PFK-2/FBPase-2 was demonstrated with a specific phospho-Ser-32-PFK-2 antibody for the wild-type PFK-2/FBPase-2 protein in contrast to the S32A/H258A mutant. Thus, there is conclusive evidence that forskolin enhanced Ser-32 phosphorylation of PFK-2/FBPase-2 via the cAMP-dependent protein kinase A pathway. An effect on other intracellular regulatory phosphorylation cascades, namely AMP-activated protein kinase [28], possibly mediating PFK-2/FBPase-2 dimerization is not preluded, but seems to be less likely from the presented data.

Referring to the scheme (Fig. 2) forskolin treatment increased Ser-32 phosphorylation within the endogenous PFK-2/FBPase-2 pool and within the wild-type PFK-2/FBPase-2 two-hybrid fusion protein pool. Thus, following the hypothesis that the formation of a “mixed dimer” consisting of a phosphorylated and an unphosphorylated monomer would be kinetically not preferential, in the case of the S32A/H258A mutant PFK-2/FBPase-2 more two-hybrid fusion proteins were able to augment reporter gene expression. In contrast, the PFK-2/FBPase-2 wild-type enzyme in the two-hybrid fusion protein would not be distinguishable from the endogenous enzyme with respect to the phosphorylation state. Therefore, “mixed dimers” would form to the same extent before and after forskolin treatment, exactly as observed in the mammalian two-hybrid experiments. Thus, cAMP-dependent phosphorylation of endogenous PFK-2/FBPase-2 accounted for the unexpected observation that forskolin treatment had an apparent effect on the dimerization strength exclusively in case of the mutant PFK-2/FBPase-2 two-hybrid fusion protein which cannot be phosphorylated at Ser-32.

The preferential homodimerization of PFK-2/FBPase-2 that was revealed by the two-hybrid experiments raised the question of whether de/phosphorylation changed the binding between monomers. To address this FRET analysis was performed using the photoactivatable fluorescent protein Dendra2 fused to the N-terminus of the PFK-2/FBPase-2 wild-type or

S32A/H258A mutant enzyme. While green Dendra2 being the FRET donor, the FRET acceptor, namely the red fluorescent form was produced by restricted UV light exposure. Interestingly, FRET, indicating close spatial proximity, was detected only in the partially phosphorylated wild-type enzyme fusion protein. Loss of Ser-32 phosphorylation diminished the FRET signal, providing clear evidence for a stronger interaction of the monomers in the phosphorylated state. In combination with the results from the two-hybrid experiments the data revealed a higher binding strength within the Ser-32-phosphorylated PFK-2/FBPase-2 dimer. Phosphorylation of Ser-32 inhibits the PFK-2 activity and activates FPBase-2 in addition to disfavouring the binding to glucokinase.

The effects on activities are mediated by the 22 amino acids in the N-terminus and the 30 amino acids in the C-terminus [8, 12] in a poorly understood mechanism. The crystal structure indicates that the kinase domains form a continuous β-sheet structure across the dimer interface. The "tightening" of the quaternary structure observed upon phosphorylation of Ser-32 suggests either increased interaction between the kinase domains, closer association of FBPase-2 domains, or both. The latter is consistent with the proposed molecular mechanism for activation of FBPase-2 by Ser-32 phosphorylation [2]. The glucokinase-binding site is located in the FBPase-2 domain [7]. Thus, a conformational change in the quaternary structure indicated by the present study suggests that in phospho-Ser-32-PFK-2/FBPase-2 the GK-binding sites may be sequestered.

Interestingly, the obtained results strengthen the proposed molecular coordination of hepatic carbohydrate metabolism by PFK-2/FBPase-2 and the glucose-sensor enzyme glucokinase. In previous studies it was shown that glucokinase enzyme activity is increased upon co-overexpression of the PFK-2/FBPase-2 liver isoenzyme [17]. Furthermore glucokinase activation through the interaction with the liver wild-type PFK-2/FBPase-2, but not the S32A/H258A mutant PFK-2/FBPase-2, could be counteracted by forskolin indicating binding of glucokinase mainly to the dephosphorylated PFK-2/FBPase-2 [17]. In hepatocytes overexpression of both the wild-type and the S32A/H258A mutant PFK-2/FBPase-2 promoted the cytoplasmic localization of glucokinase in the absence of glucagon [29]. However, only the S32A/H258A mutated bifunctional enzyme was able to counteract the glucagon-induced reduction of cellular fructose 2,6-bisphosphate, inhibition of glycolysis and reduction of the freely diffusible glucokinase fraction [29]. It could also be demonstrated with recombinant purified proteins that glucokinase:PFK-2/FBPase-2 complex formation increases both the glucokinase and the PFK-2 activity whereas FBP-2 activity is unchanged [18]. Therefore a glucose rise in liver not only increases the glucokinase enzyme activity and the

kinase:bisphosphatase activity ratio but furthermore activates both enzymes by promoting formation of a $GK_2$(PFK-2/FBPase-2)$_2$ complex [18]. Thus the present study contributes to the hypothesis that glucokinase binding to the PFK-2/FBPase-2 bisphosphatase domain is unfavourable upon Ser-32 phosphorylation of the bifunctional enzyme and opens new perspectives for further experiments.

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## References

1 Okar, D. A. and Lange, A. J. (1999) Fructose-2,6-bisphosphate and control of carbohydrate metabolism in eukaryotes. Biofactors. **10**, 1-14
2 Okar, D. A., Wu, C. and Lange, A. J. (2004) Regulation of the regulatory enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Adv Enzyme Regul. **44**, 123-154
3 Jin, E. S., Uyeda, K., Kawaguchi, T., Burgess, S. C., Malloy, C. R. and Sherry, A. D. (2003) Increased hepatic fructose 2,6-bisphosphate after an oral glucose load does not affect gluconeogenesis. J Biol Chem. **278**, 28427-28433
4 Rider, M. H., Bertrand, L., Vertommen, D., Michels, P. A., Rousseau, G. G. and Hue, L. (2004) 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: head-to-head with a bifunctional enzyme that controls glycolysis. Biochem J. **381**, 561-579
5 Okar, D. A., Manzano, A., Navarro-Sabate, A., Riera, L., Bartrons, R. and Lange, A. J. (2001) PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose-2,6-bisphosphate. Trends Biochem Sci. **26**, 30-35
6 Hasemann, C. A., Istvan, E. S., Uyeda, K. and Deisenhofer, J. (1996) The crystal structure of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase reveals distinct domain homologies. Structure. **4**, 1017-1029
7 Baltrusch, S., Lenzen, S., Okar, D. A., Lange, A. J. and Tiedge, M. (2001) Characterization of glucokinase-binding protein epitopes by a phage-displayed peptide library. Identification of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase as a novel interaction partner. J Biol Chem. **276**, 43915-43923
8 Lin, K., Kurland, I. J., Li, L., Lee, Y. H., Okar, D., Marecek, J. F. and Pilkis, S. J. (1994) Evidence for NH2- and COOH-terminal interactions in rat 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. J Biol Chem. **269**, 16953-16960
9 Pilkis, S. J., Claus, T. H., Kurland, I. J. and Lange, A. J. (1995) 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase: a metabolic signaling enzyme. Annu Rev Biochem. **64**, 799-835
10 El-Maghrabi, M. R., Fox, E., Pilkis, J. and Pilkis, S. J. (1982) Cyclic AMP-dependent phosphorylation of rat liver 6-phosphofructo 2-kinase, fructose 2,6-bisphosphatase. Biochem Biophys Res Commun. **106**, 794-802
11 Murray, K. J., El-Maghrabi, M. R., Kountz, P. D., Lukas, T. J., Soderling, T. R. and Pilkis, S. J. (1984) Amino acid sequence of the phosphorylation site of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. J Biol Chem. **259**, 7673-7681
12 Kurland, I. J., Li, L., Lange, A. J., Correia, J. J., el-Maghrabi, M. R. and Pilkis, S. J. (1993) Regulation of rat 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Role of the NH2-terminal region. J Biol Chem. **268**, 14056-14064
13 Zhu, Z., Ling, S., Yang, Q. H. and Li, L. (2001) Involvement of the chicken liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase sequence His444-Arg-Glu-Arg in modulation of the bisphosphatase activity by its kinase domain. Biochem J. **357**, 513-520
14 Wu, C., Okar, D. A., Stoeckman, A. K., Peng, L. J., Herrera, A. H., Herrera, J. E., Towle, H. C. and Lange, A. J. (2004) A potential role for fructose-2,6-bisphosphate in the stimulation of hepatic glucokinase gene expression. Endocrinology. **145**, 650-658
15 Arden, C., Hampson, L. J., Huang, G. C., Shaw, J. A., Aldibbiat, A., Holliman, G., Manas, D., Khan, S., Lange, A. J. and Agius, L. (2008) A role for PFK-2/FBPase-2, as distinct from fructose 2,6-bisphosphate, in regulation of insulin secretion in pancreatic beta-cells. Biochem J. **411**, 41-51
16 Baltrusch, S., Langer, S., Massa, L., Tiedge, M. and Lenzen, S. (2006) Improved metabolic stimulus for glucose-induced insulin secretion through GK and PFK-2/FBPase-2 coexpression in insulin-producing RINm5F cells. Endocrinology. **147**, 5768-5776

17 Massa, L., Baltrusch, S., Okar, D. A., Lange, A. J., Lenzen, S. and Tiedge, M. (2004) Interaction of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) with glucokinase activates glucose phosphorylation and glucose metabolism in insulin-producing cells. Diabetes. **53**, 1020-1029
18 Smith, W. E., Langer, S., Wu, C., Baltrusch, S. and Okar, D. A. (2007) Molecular coordination of hepatic glucose metabolism by the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase:glucokinase complex. Mol Endocrinol. **21**, 1478-1487
19 Okar, D. A., Lange, A. J. and Wu, C. (2009) Interaction with PFK-/FBP-2 is essential to glucokinase molecular physiology. Cell Mol Life Sci. **66**, 731-732
20 Hummer, A., Delzeith, O., Gomez, S. R., Moreno, R. L., Mark, M. D. and Herlitze, S. (2003) Competitive and synergistic interactions of G protein beta(2) and Ca(2+) channel beta(1b) subunits with Ca(v)2.1 channels, revealed by mammalian two-hybrid and fluorescence resonance energy transfer measurements. J Biol Chem. **278**, 49386-49400
21 Baltrusch, S. and Lenzen, S. (2008) Monitoring of glucose-regulated single insulin secretory granule movement by selective photoactivation. Diabetologia. **51**, 989-996
22 Gurskaya, N. G., Verkhusha, V. V., Shcheglov, A. S., Staroverov, D. B., Chepurnykh, T. V., Fradkov, A. F., Lukyanov, S. and Lukyanov, K. A. (2006) Engineering of a monomeric green-to-red photoactivatable fluorescent protein induced by blue light. Nat Biotechnol. **24**, 461-465
23 Baltrusch, S. and Lenzen, S. (2007) Novel insights into the regulation of the bound and diffusible glucokinase in MIN6 beta-cells. Diabetes. **56**, 1305-1315
24 Kamata, K., Mitsuya, M., Nishimura, T., Eiki, J. and Nagata, Y. (2004) Structural basis for allosteric regulation of the monomeric allosteric enzyme human glucokinase. Structure. **12**, 429-438
25 Pilkis, S. J. and Claus, T. H. (1991) Hepatic gluconeogenesis/glycolysis: regulation and structure/function relationships of substrate cycle enzymes. Annu Rev Nutr. **11**, 465-515
26 el-Maghrabi, M. R., Claus, T. H., Pilkis, J. and Pilkis, S. J. (1982) Regulation of 6-phosphofructo-2-kinase activity by cyclic AMP-dependent phosphorylation. Proc Natl Acad Sci U S A. **79**, 315-319
27 Okar, D. A., Kakalis, L. T., Narula, S. S., Armitage, I. M. and Pilkis, S. J. (1995) Identification of transient intermediates in the bisphosphatase reaction of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase by 31P-NMR spectroscopy. Biochem J. **308 ( Pt 1)**, 189-195
28 Hurley, R. L., Barre, L. K., Wood, S. D., Anderson, K. A., Kemp, B. E., Means, A. R. and Witters, L. A. (2006) Regulation of AMP-activated protein kinase by multisite phosphorylation in response to agents that elevate cellular cAMP. J Biol Chem. **281**, 36662-36672
29 Payne, V. A., Arden, C., Wu, C., Lange, A. J. and Agius, L. (2005) Dual role of phosphofructokinase-2/fructose bisphosphatase-2 in regulating the compartmentation and expression of glucokinase in hepatocytes. Diabetes. **54**, 1949-1957

## Figure legends

**FIGURE 1**

**Effect of Ser-32 phosphorylation on dimerization strength of PFK-2/FBPase-2 wild-type and S32A/H258A mutant two-hybrid fusion proteins**

The interaction strength of the fusion proteins DNA-BD-PFK-2/FBPase-2 and AD-PFK-2/FBPase-2 was measured in a fluorescence based mammalian two-hybrid assay for wild-type enzyme (A) and for S32A/H258A double mutant (B). DNA-BD and AD (white bars), DNA-BD-PFK-2/FBPase-2 and AD (striped bars), DNA-BD and AD-PFK-2/FBPase-2 (checked bars) or DNA-BD-PFK-2/FBPase-2 and AD-PFK-2/FBPase-2 (black bars) were over-expressed in COS cells and preincubated with or without forskolin. Thereafter, the fluorescence intensities for ECFP and EYFP were determined. Data are expressed as means ± SEM from three individual experiments with a total of 82 to 379 nuclei analyzed. ***, $p < 0.001$ compared with negative controls; ##, $p < 0.01$ compared with the respective untreated cells (ANOVA/Bonferroni's test).

**FIGURE 2**

**Model of the binding behaviour of endogenous PFK-2/FBPase-2 and the mutant PFK-2/FBPase-2 mammalian two-hybrid fusion proteins**

The endogenous PFK-2/FBPase-2 is depicted in light grey. The PFK-2/FBPase-2 S32A/H258A double mutant fused to the indicated two-hybrid domains (DNA-BD and AD, respectively) is shown in dark grey. The kinase and bisphosphatase domains of PFK-2/FBPase-2 are designated as K and B, respectively. The letter size represents the activity status of the domain. Phospho-Ser-32 residues are indicated as grey circles.

**FIGURE 3**

**Effects of forskolin on the cAMP content in COS cells transfected with the two-hybrid vectors**

COS cells were transfected with pM + pVP16 + pHASH-3 (white columns), pM-PFK-2/FBPase-2 wild-type + pVP16-PFK-2/FBPase-2 wild-type + pHASH-3 (black columns) or pM-PFK-2/FBPase-2(S32A/H258A) + pVP16-PFK-2/FBPase-2(S32A/H258A) + pHASH-3 (grey columns). One day after transfection the cells were incubated for 15 minutes in the absence or presence of 100 µM forskolin. Then the cellular cAMP content was measured by a luminometric assay. Data are expressed as percentage of untreated COS cells. Shown are means ± SEM from four individual experiments. ***, $p < 0.001$ compared with the respective untreated cells (ANOVA/Bonferroni's test).

**FIGURE 4**

**Effects of forskolin on Ser-32 phosphorylation of PFK-2/FBPase-2**

COS cells were transfected with pcDNA3zeo-PFK-2/FBPase-2 wild-type (lanes 1, 2) or S32A/H258A double mutant (lanes 3, 4) or were left untransfected (lanes 5, 6). One day after transfection the cells were incubated for ten minutes in the absence or presence of 100 µM forskolin. Thereafter cells were harvested, homogenized and used for Western blot analysis. (A) For analysis with a specific antibody against FBPase-2 15 µg cellular protein were analyzed per lane. (B) For analysis with a specific antibody against phospho-Ser-32-PFK-2 150 µg cellular protein were analyzed per lane. Shown are representative blots from four individual experiments. (C) Quantitative analysis of the PFK-2/FBPase-2 wild-type immunoblot bands was performed and the Ser-32 phosphorylated portion of PFK-2/FBPase-2 was calculated. Ser-32 phosphorylated PFK-2/FBPase-2 is expressed as percentage of total PFK-2/FBPase-2 protein. Shown are means ± SEM from four individual experiments. **, $p < 0.01$ compared with untreated cells (Student's *t* test).

**FIGURE 5**

**Analysis of interaction of PFK-2/FBPase-2 kinase domains within the enzyme homodimer**

Fusion proteins of the green to red photoconvertible fluorescent protein Dendra2 and the liver PFK-2/FBPase-2 wild-type (black bars), the S32A/H258A double mutant (grey bars) or the enzyme glucokinase (white bars) were expressed in COS cells and purified. To determine the degree of FRET occurring between a green and a red Dendra2 fusion protein the red fluorescence intensity was measured upon excitation of the green Dendra2. Measurements of fluorescence intensities were performed before and after converting a defined fraction of Dendra2 to the red form by radiation with UV-light. The increase in FRET is shown for proteins immediately used after purification and for proteins from the same purification batch but incubated at -20 °C before the measurement (grey background). Data are expressed as means ± SEM from three individual experiments. *, $p < 0.05$ compared to Dendra2-GK; #, $p < 0.05$ compared to freshly purified Dendra2-PFK-2/FBPase-2 mutant; $, $p < 0.05$ compared to Dendra2-PFK-2/FBPase-2 wild-type incubated at -20 °C.

**FIGURE 6**

**Scheme of FRET within a dimer of Dendra2-PFK-2/FBPase-2 wild-type**

PFK-2/FBPase-2 is depicted in light grey, the kinase and bisphosphatase domains are designated as K and B, respectively. The letter size represents the activity status of the domain. Green fluorescent Dendra2 is shown as a fading globe with white centre. Red fluorescent Dendra2 is shown as a fading globe with dark centre. Phosphorylation of Ser-32 is indicated by grey circles. The dashed arrow indicates radiationless transfer of excitation energy from the donor (green Dendra2) to the acceptor (red Dendra2).

**FIGURE 1**

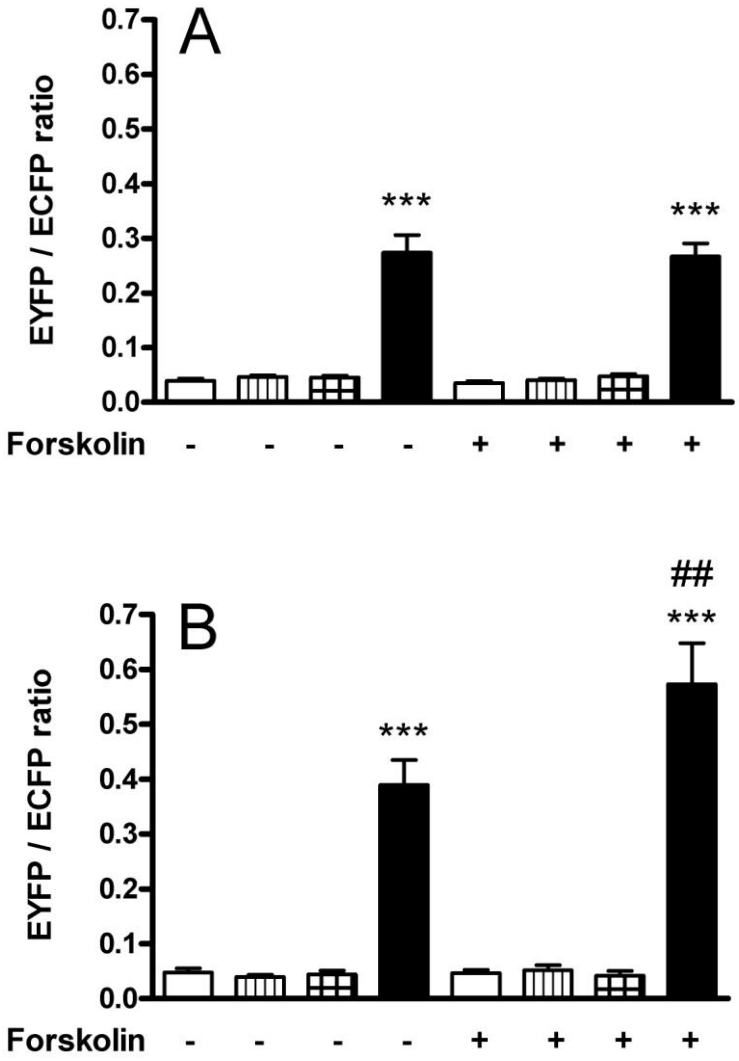

A
EYFP / ECFP ratio
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
***
Forskolin - - - - + + + +
B
EYFP / ECFP ratio
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
##
***
Forskolin - - - - + + + +

**FIGURE 2**

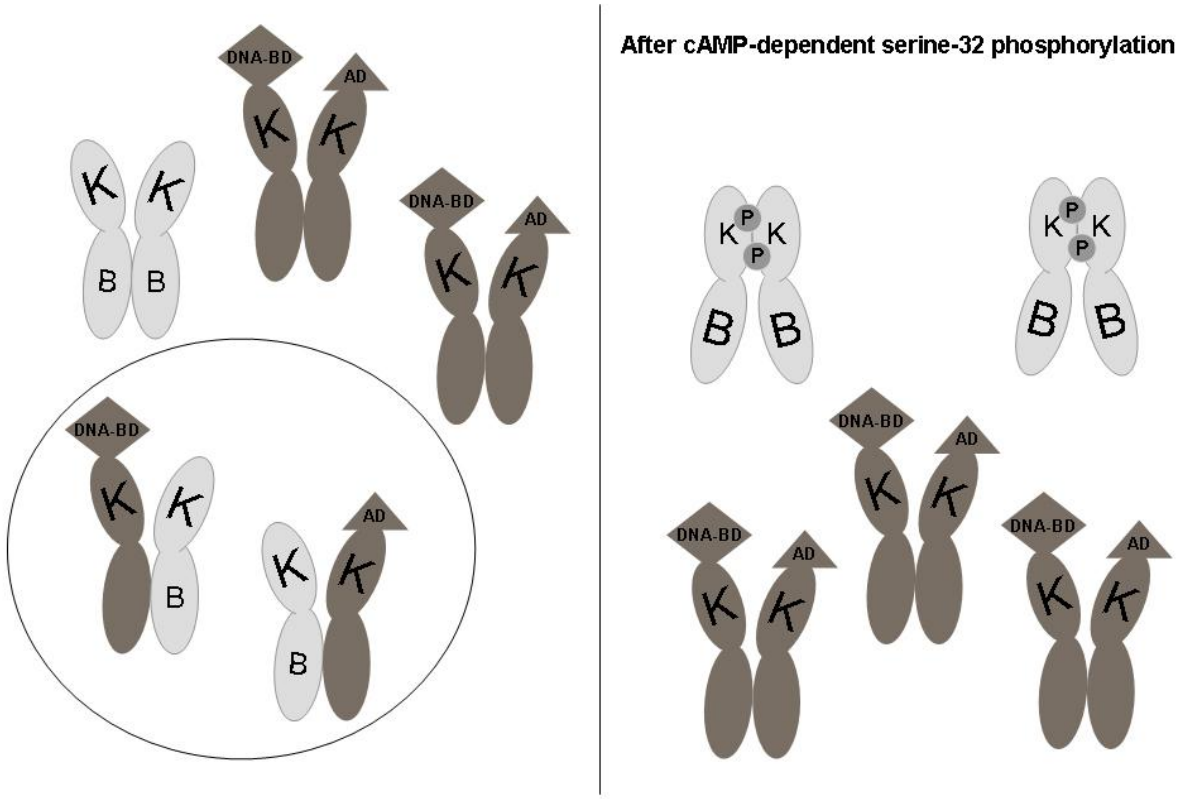

DNA-BD
AD
K
B
After cAMP-dependent serine-32 phosphorylation
P

**FIGURE 3**

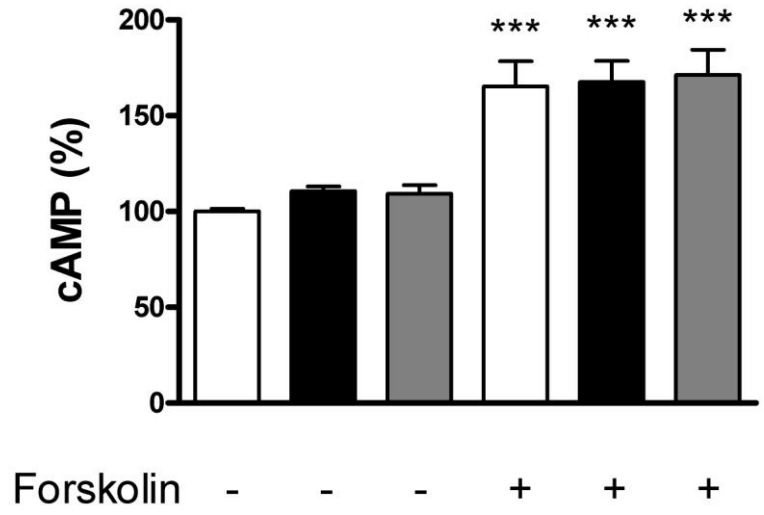


**FIGURE 4**

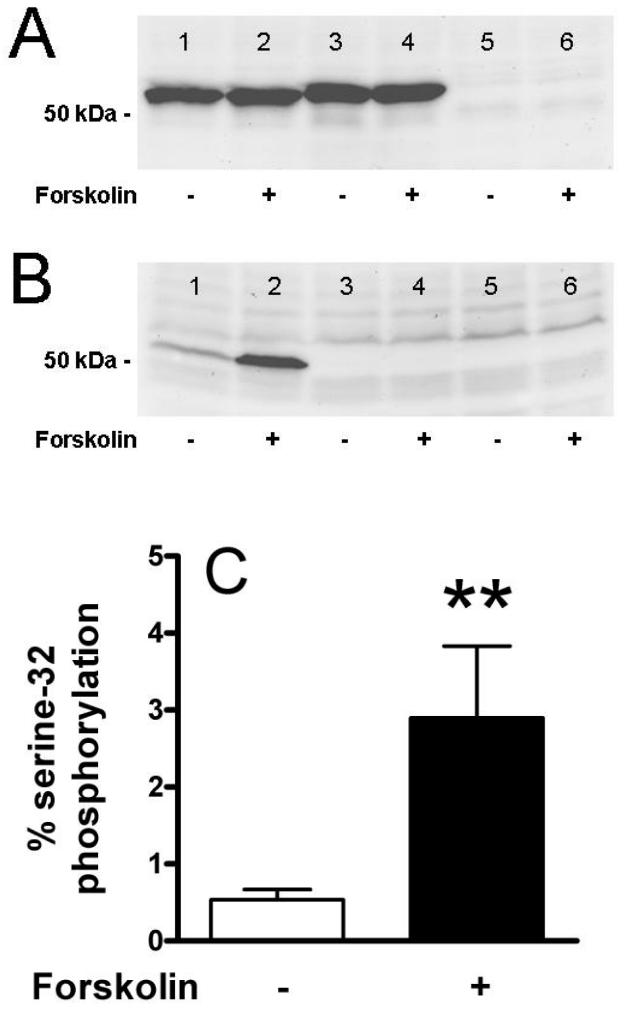

A
1 2 3 4 5 6
50 kDa -
Forskolin - + - + - +
B
1 2 3 4 5 6
50 kDa -
Forskolin - + - + - +
C
% serine-32 phosphorylation
0 1 2 3 4 5
**
Forskolin - +

**FIGURE 5**

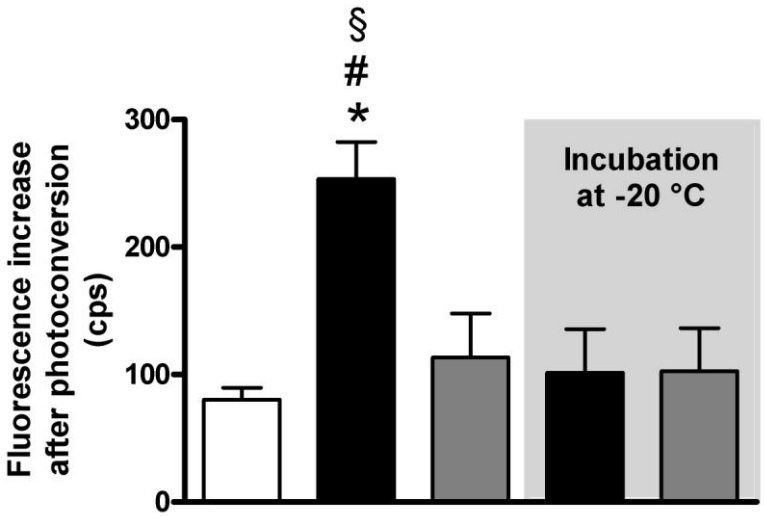
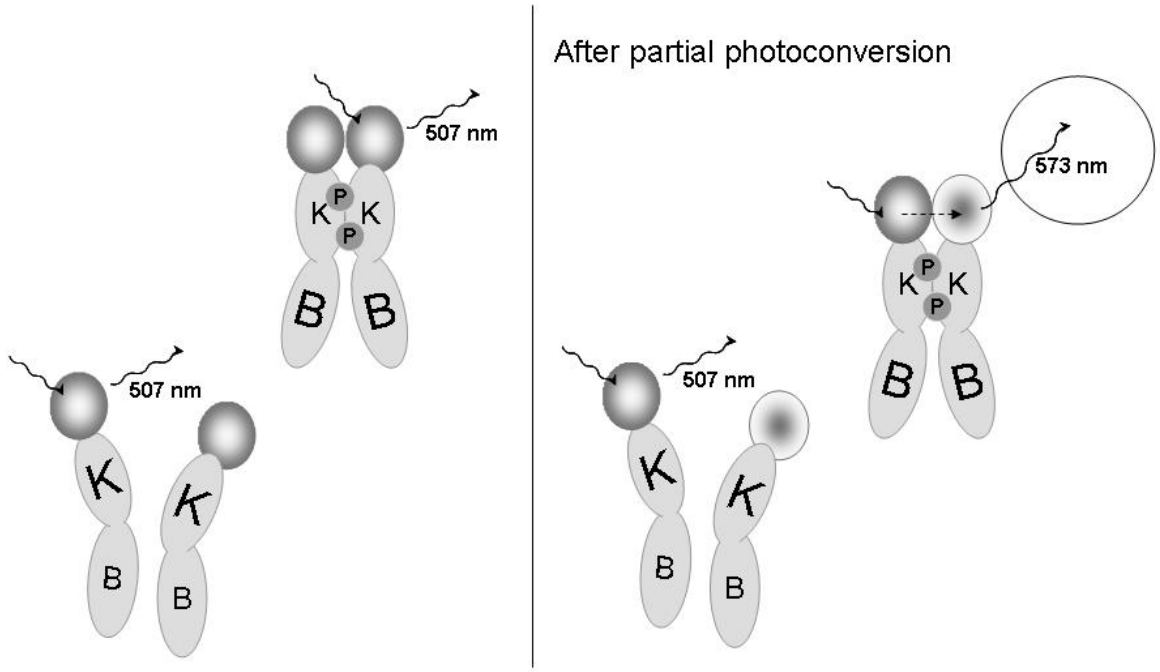

§
#
*
300
200
100
0
Fluorescence increase after photoconversion (cps)
Incubation at -20 °C

**FIGURE 6**


507 nm
P
K
K
P
B
B
507 nm
K
K
B
B
After partial photoconversion
573 nm
P
K
K
P
B
B
507 nm
K
K
B
B

# Activation of glucokinase by the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and the chemical compound LY2121260

Running title: Glucokinase activation

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GK, glucokinase; PFK-2/FBPase-2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase;

## Abstract

**OBJECTIVE** Metabolic stimulus-secretion coupling in pancreatic beta cells is dependent on the glucose phosphorylating enzyme glucokinase. Activation of glucokinase is endogenously mediated by interaction with the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and can be achieved also by a new class of compounds that might be useful for type 2 diabetes therapy. In this study, we examined whether the regulation of glucokinase enzyme conformation and activation by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in beta-cells influences the response to a chemical glucokinase activator.

**RESEARCH DESIGN AND METHODS** Enzyme activities of recombinant glucokinase protein and protein extracts isolated from insulin-producing RINm5F cells were studied in dependence upon 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, the glucokinase activator LY2121260, the sugar mannoheptulose and the sugar alcohol perseitol.

**RESULTS** While activation of glucokinase by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase resulted exclusively in a significant increase of the enzymatic phosphorylating capacity, LY2121260 increased in addition the affinity of glucokinase to glucose. In cellular extracts from glucokinase plus 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase over-expressing RINm5F cells and by use of recombinant proteins an additive glucokinase activating effect of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and LY2121260 was observed, highest at glucose concentrations below 12.5 mmol/l. 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase partly antagonized competitive glucokinase inhibition by mannoheptulose. But while LY2121260 did not counteract inhibition by mannoheptulose, perseitol could be identified as a new glucokinase inhibitor, whose action was significantly antagonized by LY2121260.

**CONCLUSIONS** We demonstrated that LY2121260 binds to glucokinase in the presence of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Furthermore this study provided evidence that 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase facilitates the glucose effect on the intracellular glucokinase conformational equilibrium, while LY2121260 enforces the closed conformation independently of glucose, thus, being unable to counteract competitive inhibition.

## Introduction

The glucose phosphorylating enzyme glucokinase has a flux controlling role in glycolysis (1; 2). In contrast to the other members of the hexokinase family glucokinase shows a sigmoidal saturation curve and a low affinity for its substrate glucose (2-6). Thus, glucokinase is well suited to play the crucial role of the glucose sensor in the pancreatic beta cell thereby controlling glucose-induced insulin secretion (1; 2; 4; 6; 7). Recent studies indicate that the enzyme acts in a tissue-specific manner also in endocrine cells of the gut, pituitary gland, and brain (1). In liver, with a high expression level, glucokinase is the main regulator of glucose metabolism (8).

Glucose binding induces a global conformational transition of glucokinase (9). This is the explanation for the positive cooperativity of glucokinase although the enzyme is clearly monomeric (10). Kinetic models, namely the mnemonical and the slow-transition model have been suggested long time ago to illustrate the glucokinase positive cooperativity (11-13). In 2004, two glucokinase conformations were characterized by its crystal structure, a super-opened, catalytically inactive glucokinase conformation and a closed, catalytically active one (14). But there is convincing evidence that further conformational states exist during the transition between the closed and super-opened conformation (14; 15). Three intermediates on that pathway have been recently assumed from fluorescence spectroscopy measurements indicating intracellular equilibration of glucokinase conformations in dependence upon the glucose concentration (16).

This glucose-mediated regulation of glucokinase is modulated on the posttranslational level by interaction with different proteins (1; 17). In liver, glucokinase is inhibited by its glucokinase regulatory protein, which binds in a fructose-6-phosphate dependent manner and shuttles glucokinase to the nucleus (18-21). The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) was established as an activating glucokinase binding partner, both in liver and in pancreatic beta cells (2; 17; 22-26). It was shown, that interaction between glucokinase and the bifunctional enzyme is mediated by a motive, which is present in all isoforms in the bisphosphatase domain (23). The corresponding binding site within the glucokinase protein is yet not identified. Activation of glucokinase through PFK-2/FBPase-2 increases the $V_{max}$ value of the enzyme, while the $S_{0.5}$ for glucose remained unchanged (17; 24). In beta cells glucokinase activation by PFK-2/FBPase-2 potentiates glucose-induced insulin secretion (17).

An increase of insulin secretion to an extent that induces hypoglycaemia was identified in patients carrying activating glucokinase mutations (27). The activating mutations, which are located in the hinge domain opposite and spatially remote from the glucose binding site significantly reduce the $S_{0.5}$ of glucokinase for glucose (27-32). The region, which accommodates the activating mutations (29; 31) is the allosteric binding site of small molecules, which were recently discovered to act as glucokinase activators (14; 33-38). Their use as antihyperglycemic agents is an attractive therapeutic concept (39-42). Currently glucokinase activators are a fast growing heterogeneous class of chemical compounds differing both in structure and function (35; 36; 42). The action of the available compounds obviously varies in their activating potency and by the way how they modify maximal enzyme velocity ($V_{max}$) and affinity to glucose ($S_{0.5}$) (14; 33-37). The recently discovered glucokinase activator LY2121260 raises both the $V_{max}$ and the $S_{0.5}$ value (35). It has been demonstrated that LY2121260 increases insulin secretion in isolated rat pancreatic islets and stimulates glucose uptake in cultured rat hepatocytes. Furthermore, an improved glucose tolerance after an oral glucose challenge was demonstrated in healthy rats (35).

To date the interplay between the endogenous activator PFK-2/FBPase-2 and small molecule activators like LY2121260 on glucokinase enzyme activity is unknown. The aim of this study was to analyse in a comprehensive manner the molecular mechanisms of glucokinase activation by PFK-2/FBPase-2 and LY2121260.

## Research design and methods

**Materials.** Mannoheptulose and perseitol were from Glycoteam (Hamburg, Germany) and sedoheptulose from Sigma-Aldrich (Steinheim, Germany). The glucokinase activator LY2121260 (2-(S)-cyclohexyl-1-(R)-(4-methanesulfonyl-phenyl)-cyclopropanecarboxylic acid thiazol-2-ylamide) (35) used in this study was kindly provided by Eli Lilly (Lilly Research Laboratories, Hamburg, Germany). All tissue culture equipment was from Invitrogen (Karlsruhe, Germany) and Greiner-Bio One (Frickenhausen, Germany).

**RINm5F cell culture.** Insulin-producing RINm5F cells overexpressing glucokinase (RINm5F-GK cells) were generated by stable transfection of the human beta cell glucokinase cDNA as described previously (43). RINm5F-GK cells overexpressing PFK-2/FBPase-2 were generated by a second stable transfection of the cDNA for rat islets (RINm5F-GK-PFK-2/FBPase-2 I 4) as described (24). Cells were grown in RPMI 1640 medium supplemented with 10 mmol/l glucose, 10% (v/v) fetal calf serum (FCS), penicillin and streptomycin in a humidified atmosphere at 37°C and 5% $CO_2$. The medium for RINm5F-GK cells was additionally supplemented with 250 µg/ml G418 and the medium for RINm5F-GK-PFK-2/FBPase-2 cells with 250 µg/ml G418 and 250 µg/ml Zeocin$^{TM}$. For glucokinase activity measurements cells were homogenized in phosphate buffered saline (pH 7.4) and insoluble material was pelleted by centrifugation. The protein concentration was quantified by a Bio-Rad protein assay.

**Recombinant glucokinase and FBPase-2 protein.** Recombinant beta cell glucokinase was expressed and purified as $His_6$-tag protein by the pQE30 vector system as described previously (44).The bisphosphatase domain of rat liver PFK-2/FBPase-2 (amino acid residues 250–470) was expressed and purified as GST-tag protein by the pGEX-6P-1 vector system. The cleavage of the GST-tag was achieved with PreScission protease as described (21).

**Glucokinase enzyme activity.** Glucose phosphorylating activity was measured at different glucose concentrations (1, 1.56, 3.12, 6.25, 12.5, 25 and 100 mmol/l) in recombinant protein solutions or soluble cellular fractions of RINm5F-GK and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells by an enzyme-coupled photometric assay as described previously (45). Pretreatment was performed as indicated. One unit of enzyme activity was defined as 1 µmol glucose-6-phosphate formed from glucose and ATP per minute at 37°C. Enzyme activities were expressed as units per mg glucokinase protein or cellular protein, respectively. $V_{max}$ values were estimated by sigmoidal curve fitting. $S_{0.5}$ and Hill coefficients of glucokinase were calculated from Hill plots.

**Native protein separation and Dot Blotting.** Cells were homogenized in phosphate buffered saline (pH 7.4) and insoluble material was pelleted by centrifugation. The supernatant was incubated for 1 h with 25 mmol/l glucose alone or in addition with 10 µM LY2121260. Thereafter for gel filtration chromatography the lysate was loaded on a HiLoad 16/60 Superdex 200 column and eluted with 1 ml/min using ÄKTA® PrimePlus system (Amersham Biosciences, Freiburg, Germany). Resolution of protein separation in the molecular range was calibrated using recombinant glucokinase, bovine serum albumin (70 kDa), aldolase (158 kDa) and catalase (232 kDa). 15 µl of each fraction was electroblotted to equilibrated polyvinylidine difluoride (PVDF) membranes using a 96-well Dot-Blot system (Roth, Karlsruhe, Germany). Nonspecific binding sites of the membranes were blocked by non-fat dry milk overnight at 4°C. Glucokinase and PFK-2/FBPase-2 immunodetection was performed as described (24).

**Statistical analyses.** Data are expressed as means ± SEM. Statistical analyses were performed by ANOVA followed by Bonferroni's test for multiple comparisons using the Prism analysis program (Graphpad Inc., San Diego, CA).

## Results

**Glucokinase inhibition by mannoheptulose and perseitol.** Mannoheptulose and sedoheptulose are epimers. The C7 sugars differ only in the location of the hydroxyl group at the 4th carbon atom (Fig. 1). Glucokinase was significantly inhibited by mannoheptulose in a concentration-dependent manner (Fig. 2). This inhibition was sugar conformation selective as sedoheptulose did not affect glucokinase enzyme activity (Fig. 2). However, the C7 sugar alcohol perseitol with a mannoheptulose corresponding structure at the 4th carbon atom (Fig. 1) showed a significant inhibition of glucokinase (Fig. 2). Mannoheptulose and perseitol increased the $S_{0.5}$ value of glucokinase for glucose (control, 7.26 ± 0.46 mmol/l) to 17.9 ± 1.57 mmol/l and 15.1 ± 2.53 mmol/l, respectively indicating competitive inhibition (Table 1).

**Activation of glucokinase enzyme activity by LY2121260 in dependence upon inhibition through mannoheptulose and perseitol.** Treatment of glucokinase with LY2121260 resulted both in an increase in the maximal enzyme velocity ($V_{max}$ 2.85 ± 0.11 U/mg versus 4.18 ± 0.06 U/mg) and an increase in the affinity to its substrate glucose ($S_{0.5}$ 7.26 ± 0.46 mmol/l versus 2.51 ± 0.06 mmol/l) (Table 1, Fig. 3). Glucokinase activation by LY2121260 was accompanied by a slight decrease in the enzyme cooperativity ($n_{Hill}$ 1.79 ± 0.09 vs 1.59 ± 0.12). Sedoheptulose did not counteract glucokinase activation by LY2121260 (Fig. 3), while mannoheptulose and perseitol showed different actions. Interestingly the chemical compound LY2121260 was not able to activate glucokinase in the presence of mannoheptulose. In contrast the affinity of glucokinase to glucose significantly decreased further ($S_{0.5}$ 17.9 ± 1.57 mmol/l versus 29.9 ± 2.92 mmol/l) (Table 1, Fig. 3). LY2121260 increased at least in part glucokinase enzyme activity in the presence of perseitol and evoked a significant increase in both the maximal enzyme velocity ($V_{max}$ 1.48 ± 0.13 U/mg versus 2.53 ± 0.10 U/mg) and the affinity to its substrate glucose ($S_{0.5}$ 15.1 ± 2.53 mmol/l versus 3.55 ± 0.43 mmol/l) (Table 1, Fig. 3).

**Comparison of glucokinase activation by the chemical compound LY2121260 and the protein FBPase-2.** Glucokinase interaction with the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) is mediated by the bisphosphatase domain (FBPase-2) (23). Incubation of glucokinase with FBPase-2 in a 1:5 molar ratio significantly increased the maximal enzyme velocity ($V_{max}$ 2.85 ± 0.11 U/mg versus 4.98 ± 0.72 U/mg), but did not change the affinity to glucose ($S_{0.5}$ 7.26 ± 0.46 mmol/l versus 7.09 ± 0.27 mmol/l) (Table 2, Fig. 4). Thus, FBPase-2 provoked glucokinase activation by a mechanism different from that of the chemical compound LY2121260, although both LY2121260

and FBPase-2 were able to increase the glucokinase $V_{max}$ value by 47% and 75%, respectively (Table 2). Activation of glucokinase by LY2121260 in the presence of FBPase-2 resulted in a further increase of the glucokinase enzyme velocity by 30% (Table 2, Fig. 4). However, a significant decrease in the $S_{0.5}$ value of glucokinase for glucose was caused also by activation through LY2121260 plus FBPase-2 in comparison to FBPase-2 alone ($S_{0.5}$ 1.92 ± 0.16 mmol/l versus 7.09 ± 0.27 mmol/l) (Table 2).

**Characterization of glucokinase interaction, activation and inhibition in RINm5F cells**. The stable insulin-producing cell lines RINm5F-GK and RINm5F-GK-PFK-2/FBPase-2 express the glucokinase protein on the same level, but show a significant difference in their glucokinase enzyme activity as previously demonstrated (17; 24). Overexpression of PFK-2/FBPase-2 resulted in an increase in glucokinase enzyme activity at 100 mmol/l glucose by 40% (Fig. 5). Treatment of RINm5F-GK cells with LY2121260 increased the glucokinase enzyme activity at 100 mmol/l glucose by 27% and increased the glucokinase enzyme activity in RINm5F-GK-PFK-2/FBPase-2 cells by 17% (Fig. 5 and 6). Indeed glucokinase activation through interaction with the endogenous activator PFK-2/FBPase-2 was highest at glucose concentrations greater than 6.25 mmol/l. In agreement with our results observed using recombinant proteins, an additional increase in the glucokinase enzyme activity was achieved by LY2121260 treatment (Fig. 5 and 6). However, both glucokinase activation through LY2121260 in RINm5F-GK cells and PFK-2/FBPase-2 overexpressing RINm5F-GK-PFK-2/FBPase-2 cells was highest below 6.25 mmol/l glucose (Fig. 6). Glucokinase-PFK-2/FBPase-2 complex formation in RINm5F-GK-PFK-2/FBPase-2 was detectable as a higher molecular weight fraction by gel filtration chromatography in comparison to RINm5F-GK cells (Fig. 7). Glucokinase was verifiable by Dot-Blot immunodetection analysis in fractions between 50 – 70 kDa and additionally within a molecular range between 130 – 230 kDa. The first peak (50 – 70 kDa) corresponded to the elution of separated recombinant glucokinase protein in control experiments (data not shown). In fractions within the peak at higher molecular weight (130 – 230 kDa) PFK-2/FBPase-2 was identified by Dot-Blot immunodetection analysis with a specific antibody raised against the FBPase-2 domain (data not shown) (24). Indeed, the glucokinase-PFK-2/FBPase-2 complex was not abolished by treatment with LY2121260 (Fig. 7). Mannoheptulose inhibited glucokinase enzyme activity in both RINm5F-GK and RINm5F-GK-PFK-2/FBPase-2 cells (Fig. 5). However, in RINm5F-GK-PFK-2/FBPase-2 cells glucokinase enzyme activity at 100 mmol/l glucose was not reduced to the control value observed in RINm5F-GK (Fig. 5). Thus, at least in part PFK-2/FBPase-2 was able to counteract glucokinase inhibition by mannoheptulose especially at glucose con-

centrations greater than 12.5 mmol/l (Fig. 6). In contrast, LY2121260 was not able to activate glucokinase in the presence of mannoheptulose (Fig. 5 and 6).

## Discussion

Glucokinase is the glucose sensor in pancreatic beta cells and the key regulator of glucose metabolism in liver (1; 2; 4; 6; 7; 46). Therapeutically activation of glucokinase is a promising idea to reduce hyperglycemia in type 2 diabetic individuals, thus several chemical glucokinase activators have been recently developed (14; 33-38). While the blood glucose lowering effect of many of these compounds have been demonstrated in studies with healthy and diabetic animals, to date only few have been applied in humans (39-42). Orally administered activators have been shown to increase insulin secretion from the pancreatic beta-cells and glucose usage in liver (33-35).

The glucokinase activators bind to an allosteric site of glucokinase thereby increasing enzyme activity. However, little is known how the activators interfere with the important posttranslational glucokinase regulation in both beta cells and liver. The glucokinase inhibition through the glucokinase regulatory protein that takes place in liver has been shown to be counteracted by most of the glucokinase activators (47; 48). In this study glucokinase activation by the endogenous interaction partner PFK-2/FBPase-2 and the chemical compound LY2121260 has been elucidated. In agreement with previous studies (35) LY2121260 has increased the affinity of glucokinase to glucose. In contrast, the low affinity for glucose was maintained by endogenous activation through the bifunctional enzyme PFK-2/FBPase-2. Both activators were able to increase the maximal velocity of the enzyme; PFK-2/FBPase-2 to a greater extent than LY2121260. This feature of LY2121260 cannot be generally attributed to the class of glucokinase activators. Other activators have been demonstrated to solely increase the $S_{0.5}$ (36-38). It remains open, by which mechanism LY2121260 is able to increase the $V_{max}$, since activating mutations which are located in this allosteric binding region of the glucokinase activator did not significantly affect the $V_{max}$ (27-32).

Activation of glucokinase enzyme activity by PFK-2/FBPase-2 in cellular extracts from insulin-secreting RINm5F-GK-PFK-2/FBPase-2 cells has been elucidated before (24). Furthermore this activation has been shown with purified recombinant proteins after one hour preincubation (26). The protein-protein interaction between glucokinase and the single FBPase-2 domain of the bifunctional enzyme, which contains the glucokinase binding site, has been already established (23; 26). The present study demonstrates that the recombinantly expressed FBPase-2 domain alone was able to activate glucokinase. Altogether this indicates that the glucokinase:FBPase-2 complex alone causes glucokinase activation. However, glucokinase activation was higher in cellular extracts compared to recombinant proteins and a 1:5 molecu-

lar ratio of glucokinase:FBPase-2 was necessary to evoke activation using recombinant proteins. Thus, it is likely that first, the PFK-2 domain has a regulatory role and second, intracellularly available components promote complex formation and stabilize the interaction. Previous studies have provided considerable evidence for glucose promoted complex formation (17; 26).

In this study it was demonstrated for the first time, that the glucokinase:PFK-2/FBPase-2 complex was not diminished by the glucokinase activator LY2121260. Interestingly it has been shown that LY2121260 is able to further activate glucokinase in the presence of PFK-2/FBPase-2. The combined beneficial effect of both activators on the maximal velocity is of particular interest and might be unique for the glucokinase activator LY2121260. An increase in the $V_{max}$ will result in higher glucokinase activity only at stimulatory glucose concentrations, thus keeping glucose-induced insulin secretion vital. The $S_{0.5}$ value was significantly decreased through LY2121260 also in the presence of PFK-2/FBPase-2, which will result in an active enzyme already at sub-stimulatory glucose concentrations, therefore increasing the likelihood of hypoglycemia.

Several studies support in addition to the two crystallographically confirmed conformations, namely the closed active and super-opened inactive conformation, the existence of additional intermediate conformations of glucokinase (14-16). For a comprehensive explanation of the positive cooperativity of the enzyme five glucokinase conformations in a glucose dependent intracellular equilibrium have been proposed in a recent study (16). With respect to such a "multiple conformational state model" it can be presumed, that LY2121260 freezes the closed glucokinase conformation and thus prevents the glucose dependent equilibration of glucokinase conformations resulting in a decrease of $S_{0.5}$. Rather, the endogenous activation by PFK-2/FBPase-2 appears to be highly flexible and thus works together with glucose towards equilibration of glucokinase conformations and maintains the $S_{0.5}$.

To proof this hypothesis and to further elucidate the molecular mechanisms of glucokinase activation the competitive inhibitor mannoheptulose has been applied in this study (44; 49). Mannoheptulose stabilizes the closed glucokinase conformation (50; 51). So far it was demonstrated by two different studies that glucokinase could not be activated by a chemical activator in the presence of mannoheptulose (36; 38). This seems to be a unique feature of the glucokinase activators, since in this study it has been shown that mannoheptulose also completely counteracted glucokinase activation through LY2121260. In contrast, PFK-2/FBPase-2 has achieved activation in the presence of mannoheptulose, but only at higher glucose con-

centrations. This can be convincingly explained by the fact, that glucose displaced the competitive inhibitor mannoheptulose from the catalytic site at higher concentrations. For this process the conformation of glucokinase has to be changed, as it is possible in the presence of PFK-2/FBPase-2, but not in the presence of LY2121260.

The heptose mannoheptulose has been shown to have a high affinity to the catalytic center of glucokinase. To proof the hypothesis, that the position of the hydroxyl group at the 4th carbon atom in the heptose is mandatory for binding to glucokinase, the effect of the epimer of mannoheptulose, namely sedoheptulose was determined. Indeed sedoheptulose did not inhibit glucokinase. Furthermore the action of perseitol on glucokinase, the sugar alcohol of mannoheptulose (52), holding the same orientation of the hydroxyl group at the 4th carbon atom was analyzed. Interestingly perseitol has been elucidated in this study as a new inhibitor of glucokinase. It could be clearly demonstrated that perseitol is a competitive inhibitor in the same way as mannoheptulose. Furthermore it was shown that LY2121260 was at least in part able to counteract inhibition by perseitol. Thus, the sugar alcohol seems to have a lower affinity to the catalytic center of glucokinase compared to mannoheptulose.

Notably, the analysis of intracellular inhibition of glucokinase by perseitol was not feasible, as neither insulin-secreting RINm5F and MIN6 cells nor COS cells took up the sugar alcohol. Thus, it can be assumed that neither the GLUT 1 glucose transporter, mainly expressed in COS cells (53), nor the GLUT 2 glucose transporter mainly expressed in beta-cells (54) can channel perseitol in contrast to mannoheptulose. It has been proposed that intestinal uptake of sugar alcohols is mediated by the fructose transporter GLUT 5 (53). However, so far experimental studies are only available in sparrows and furthermore the GLUT 5 transporter is not expressed in pancreatic beta-cells (55).

In conclusion, this study provides evidence that PFK-2/FBPase-2 through complex formation with glucokinase facilitates the glucose effect on the conformational equilibrium, while LY2121260 enforces the closed glucokinase conformation in which the enzyme is active independently of glucose.

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**References**

1. Matschinsky FM, Magnuson MA, Zelent D, Jetton TL, Doliba N, Han Y, Taub R, Grimsby J: The network of glucokinase-expressing cells in glucose homeostasis and the potential of glucokinase activators for diabetes therapy. *Diabetes* 55:1-12, 2006

2. Baltrusch S, Tiedge M: Glucokinase Regulatory Network in Pancreatic {beta}-Cells and Liver. *Diabetes* 55 Suppl 2:S55-64, 2006

3. Iynedjian PB: Mammalian glucokinase and its gene. *Biochem J* 293 ( Pt 1):1-13, 1993

4. Lenzen S: Glucokinase: signal recognition enzyme for glucose-induced insulin secretion. In *Nutrient regulation of insulin secretion* Flatt PR, Ed. London and Chapel Hill, Portland Press, 1992, p. 101-125

5. Lenzen S, Freytag S, Panten U, Flatt PR, Bailey CJ: Alloxan and ninhydrin inhibition of hexokinase from pancreatic islets and tumoural insulin-secreting cells. *Pharmacol Toxicol* 66:157-162, 1990

6. Matschinsky FM: Glucokinase as glucose sensor and metabolic signal generator in pancreatic beta-cells and hepatocytes. *Diabetes* 39:647-652, 1990

7. Matschinsky FM: Banting Lecture 1995. A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. *Diabetes* 45:223-241, 1996

8. Agius L: Glucokinase and molecular aspects of liver glycogen metabolism. *Biochem J* 414:1-18, 2008

9. St Charles R, Harrison RW, Bell GI, Pilkis SJ, Weber IT: Molecular model of human beta-cell glucokinase built by analogy to the crystal structure of yeast hexokinase B. *Diabetes* 43:784-791, 1994

10. Koranyi LI, Tanizawa Y, Welling CM, Rabin DU, Permutt MA: Human islet glucokinase gene. Isolation and sequence analysis of full-length cDNA. *Diabetes* 41:807-811, 1992

11. Lin SX, Neet KE: Demonstration of a slow conformational change in liver glucokinase by fluorescence spectroscopy. *J Biol Chem* 265:9670-9675, 1990

12. Cardenas ML: Kinetic behaviour of vertebrate hexokinases with emphasis on hexokinase D (IV). *Biochem Soc Trans* 25:131-135, 1997

13. Cornish-Bowden A, Storer AC: Mechanistic origin of the sigmoidal rate behaviour of rat liver hexokinase D ('glucokinase'). *Biochem J* 240:293-296, 1986

14. Kamata K, Mitsuya M, Nishimura T, Eiki J, Nagata Y: Structural basis for allosteric regulation of the monomeric allosteric enzyme human glucokinase. *Structure* 12:429-438, 2004

15. Zhang J, Li C, Chen K, Zhu W, Shen X, Jiang H: Conformational transition pathway in the allosteric process of human glucokinase. *Proc Natl Acad Sci U S A* 103:13368-13373, 2006

16. Antoine M, Boutin JA, Ferry G: Binding kinetics of glucose and allosteric activators to human glucokinase reveal multiple conformational states. *Biochemistry* 48:5466-5482, 2009

17. Baltrusch S, Langer S, Massa L, Tiedge M, Lenzen S: Improved metabolic stimulus for glucose-induced insulin secretion through GK and PFK-2/FBPase-2 coexpression in insulin-producing RINm5F cells. *Endocrinology* 147:5768-5776, 2006

18. van Schaftingen E, Vandercammen A, Detheux M, Davies DR: The regulatory protein of liver glucokinase. *Adv Enzyme Regul* 32:133-148, 1992

19. van Schaftingen E, Veiga-da-Cunha M, Niculescu L: The regulatory protein of glucokinase. *Biochem Soc Trans* 25:136-140, 1997

20. Vandercammen A, Van Schaftingen E: Competitive inhibition of liver glucokinase by its regulatory protein. *Eur J Biochem* 200:545-551, 1991

21. Baltrusch S, Francini F, Lenzen S, Tiedge M: Interaction of glucokinase with the liver regulatory protein is conferred by leucine-asparagine motifs of the enzyme. *Diabetes* 54:2829-2837, 2005

22. Arden C, Hampson LJ, Huang GC, Shaw JA, Aldibbiat A, Holliman G, Manas D, Khan S, Lange AJ, Agius L: A role for PFK-2/FBPase-2, as distinct from fructose 2,6-bisphosphate, in regulation of insulin secretion in pancreatic beta-cells. *Biochem J* 411:41-51, 2008

23. Baltrusch S, Lenzen S, Okar DA, Lange AJ, Tiedge M: Characterization of glucokinase-binding protein epitopes by a phage-displayed peptide library. Identification of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase as a novel interaction partner. *J Biol Chem* 276:43915-43923, 2001

24. Massa L, Baltrusch S, Okar DA, Lange AJ, Lenzen S, Tiedge M: Interaction of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) with glucokinase activates glucose phosphorylation and glucose metabolism in insulin-producing cells. *Diabetes* 53:1020-1029, 2004

25. Payne VA, Arden C, Wu C, Lange AJ, Agius L: Dual role of phosphofructokinase-2/fructose bisphosphatase-2 in regulating the compartmentation and expression of glucokinase in hepatocytes. *Diabetes* 54:1949-1957, 2005

26. Smith WE, Langer S, Wu C, Baltrusch S, Okar DA: Molecular coordination of hepatic glucose metabolism by the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase:glucokinase complex. *Mol Endocrinol* 21:1478-1487, 2007

27. Glaser B, Kesavan P, Heyman M, Davis E, Cuesta A, Buchs A, Stanley CA, Thornton PS, Permutt MA, Matschinsky FM, Herold KC: Familial hyperinsulinism caused by an activating glucokinase mutation. *N Engl J Med* 338:226-230, 1998

28. Christesen HB, Brusgaard K, Beck Nielsen H, Brock Jacobsen B: Non-insulinoma persistent hyperinsulinaemic hypoglycaemia caused by an activating glucokinase mutation: hypoglycaemia unawareness and attacks. *Clin Endocrinol (Oxf)* 68:747-755, 2008

29. Christesen HB, Jacobsen BB, Odili S, Buettger C, Cuesta-Munoz A, Hansen T, Brusgaard K, Massa O, Magnuson MA, Shiota C, Matschinsky FM, Barbetti F: The second activating glucokinase mutation (A456V): implications for glucose homeostasis and diabetes therapy. *Diabetes* 51:1240-1246, 2002

30. Cuesta-Munoz AL, Huopio H, Otonkoski T, Gomez-Zumaquero JM, Nanto-Salonen K, Rahier J, Lopez-Enriquez S, Garcia-Gimeno MA, Sanz P, Soriguer FC, Laakso M: Severe persistent hyperinsulinemic hypoglycemia due to a de novo glucokinase mutation. *Diabetes* 53:2164-2168, 2004

31. Gloyn AL, Noordam K, Willemsen MA, Ellard S, Lam WW, Campbell IW, Midgley P, Shiota C, Buettger C, Magnuson MA, Matschinsky FM, Hattersley AT: Insights into the biochemical and genetic basis of glucokinase activation from naturally occurring hypoglycemia mutations. *Diabetes* 52:2433-2440, 2003

32. Wabitsch M, Lahr G, Van de Bunt M, Marchant C, Lindner M, von Puttkamer J, Fenneberg A, Debatin KM, Klein R, Ellard S, Clark A, Gloyn AL: Heterogeneity in disease severity in a family with a novel G68V GCK activating mutation causing persistent hyperinsulinaemic hypoglycaemia of infancy. *Diabet Med* 24:1393-1399, 2007

33. Grimsby J, Matschinsky FM, Grippo JF: Discovery and action of glucokinase activators. In *Glucokinase and glycemic disease* Matschinsky FM, Magnuson MA, Eds. Basel, Karger, 2004, p. 360-378

34. Grimsby J, Sarabu R, Corbett WL, Haynes NE, Bizzarro FT, Coffey JW, Guertin KR, Hilliard DW, Kester RF, Mahaney PE, Marcus L, Qi L, Spence CL, Tengi J, Magnuson MA, Chu CA, Dvorozniak MT, Matschinsky FM, Grippo JF: Allosteric activators of glucokinase: potential role in diabetes therapy. *Science* 301:370-373, 2003

35. Efanov AM, Barrett DG, Brenner MB, Briggs SL, Delaunois A, Durbin JD, Giese U, Guo H, Radloff M, Gil GS, Sewing S, Wang Y, Weichert A, Zaliani A, Gromada J: A novel glucokinase activator modulates pancreatic islet and hepatocyte function. *Endocrinology* 146:3696-3701, 2005

36. Brocklehurst KJ, Payne VA, Davies RA, Carroll D, Vertigan HL, Wightman HJ, Aiston S, Waddell ID, Leighton B, Coghlan MP, Agius L: Stimulation of hepatocyte glucose metabolism by novel small molecule glucokinase activators. *Diabetes* 53:535-541, 2004

37. Fyfe MC, White JR, Taylor A, Chatfield R, Wargent E, Printz RL, Sulpice T, McCormack JG, Procter MJ, Reynet C, Widdowson PS, Wong-Kai-In P: Glucokinase activator PSN-GK1 displays enhanced antihyperglycaemic and insulinotropic actions. *Diabetologia* 50:1277-1287, 2007

38. Johnson D, Shepherd RM, Gill D, Gorman T, Smith DM, Dunne MJ: Glucose-dependent modulation of insulin secretion and intracellular calcium ions by GKA50, a glucokinase activator. *Diabetes* 56:1694-1702, 2007

39. Guertin KR, Grimsby J: Small molecule glucokinase activators as glucose lowering agents: a new paradigm for diabetes therapy. *Curr Med Chem* 13:1839-1843, 2006

40. Leighton B, Atkinson A, Coghlan MP: Small molecule glucokinase activators as novel anti-diabetic agents. *Biochem Soc Trans* 33:371-374, 2005

41. Printz RL, Granner DK: Tweaking the glucose sensor: adjusting glucokinase activity with activator compounds. *Endocrinology* 146:3693-3695, 2005

42. Matschinsky FM: Assessing the potential of glucokinase activators in diabetes therapy. *Nat Rev Drug Discov* 8:399-416, 2009

43. Tiedge M, Elsner M, McClenaghan NH, Hedrich HJ, Grube D, Klempnauer J, Lenzen S: Engineering of a glucose-responsive surrogate cell for insulin replacement therapy of experimental insulin-dependent diabetes. *Hum Gene Ther* 11:403-414, 2000

44. Tiedge M, Krug U, Lenzen S: Modulation of human glucokinase intrinsic activity by SH reagents mirrors post-translational regulation of enzyme activity. *Biochim Biophys Acta* 1337:175-190, 1997

45. Lenzen S, Tiedge M, Panten U: Glucokinase in pancreatic B-cells and its inhibition by alloxan. *Acta Endocrinol (Copenh)* 115:21-29, 1987

46. Lenzen S, Panten U: Signal recognition by pancreatic B-cells. *Biochem Pharmacol* 37:371-378, 1988

47. Ralph EC, Thomson J, Almaden J, Sun S: Glucose modulation of glucokinase activation by small molecules. *Biochemistry* 47:5028-5036, 2008

48. Futamura M, Hosaka H, Kadotani A, Shimazaki H, Sasaki K, Ohyama S, Nishimura T, Eiki J, Nagata Y: An allosteric activator of glucokinase impairs the interaction of glucokinase and glucokinase regulatory protein and regulates glucose metabolism. *J Biol Chem* 281:37668-37674, 2006

49. Lenzen S, Mirzaie-Petri M: Inhibition of aconitase by alloxan and the differential modes of protection of glucose, 3-O-methylglucose, and mannoheptulose. *Naunyn Schmiedebergs Arch Pharmacol* 346:532-536, 1992

50. Zelent D, Najafi H, Odili S, Buettger C, Weik-Collins H, Li C, Doliba N, Grimsby J, Matschinsky FM: Glucokinase and glucose homeostasis: proven concepts and new ideas. *Biochem Soc Trans* 33:306-310, 2005

51. Baltrusch S, Lenzen S: Novel insights into the regulation of the bound and diffusible glucokinase in MIN6 beta-cells. *Diabetes* 56:1305-1315, 2007

52. Kanters JA, Schouten A, van Bommel M: Structure of lactitol (4-O-beta-D-galactopyranosyl-D-glucitol) monohydrate: and artificial sweetener. *Acta Crystallogr C* 46 ( Pt 12):2408-2411, 1990

53. Uldry M, Thorens B: The SLC2 family of facilitated hexose and polyol transporters. *Pflugers Arch* 447:480-489, 2004

54. Thorens B: Molecular and cellular physiology of GLUT-2, a high-Km facilitated diffusion glucose transporter. *Int Rev Cytol* 137:209-238, 1992

55. Chediack JG, Caviedes-Vidal E, Fasulo V, Yamin LJ, Karasov WH: Intestinal passive absorption of water-soluble compounds by sparrows: effect of molecular size and luminal nutrients. *J Comp Physiol B* 173:187-197, 2003

| | $V_{max}$ (U/mg) | $S_{0.5}$ (mmol/l) | $n_{Hill}$ |
|---|---|---|---|
| GK (control) | 2.85 ± 0.11 | 7.26 ± 0.46 | 1.79 ± 0.09 |
| GK + LY2121260 | 4.18 ± 0.06$^{***###}$ | 2.51 ± 0.06 | 1.59 ± 0.12 |
| GK + Mannoheptulose | 1.64 ± 0.09$^{***}$ | 17.9 ± 1.57$^{**}$ | 1.82 ± 0.20 |
| GK + Mannoheptulose + LY2121260 | 1.80 ± 0.11$^{***}$ | 29.9 ± 2.92$^{***###}$ | 1.40 ± 0.05 |
| GK + Perseitol | 1.48 ± 0.13$^{***}$ | 15.1 ± 2.53 | 1.87 ± 0.16 |
| GK + Perseitol + LY2121260 | 2.53 ± 0.10$^{###}$ | 3.55 ± 0.43$^{##}$ | 1.39 ± 0.11 |
| GK + Sedoheptulose | 2.35 ± 0.05 | 7.46 ± 0.29 | 1.85 ± 0.09 |
| GK + Sedoheptulose + LY2121260 | 4.15 ± 0.13$^{***###}$ | 2.57 ± 0.16 | 1.41 ± 0.08 |

**Table 1** Glucokinase kinetic parameters with respect to mannoheptulose, perseitol, sedoheptulose and LY2121260. Data represent means ± SEM calculated from enzyme activities shown in Fig. 3. ***$p<0.001$ compared to control; $^{##}p<0.01$; $^{###}p<0.001$ compared without LY2121260 (ANOVA/ Bonferroni's test).

| | $V_{max}$ (U/mg) | $S_{0.5}$ (mmol/l) | $n_{Hill}$ |
|---|---|---|---|
| GK (control) | 2.85 ± 0.11 | 7.26 ± 0.46 | 1.79 ± 0.09 |
| GK + LY2121260 | 4.18 ± 0.06 | 2.51 ± 0.06$^{***}$ | 1.59 ± 0.12 |
| GK + FBPase-2 | 4.98 ± 0.72$^{*}$ | 7.09 ± 0.27 | 1.49 ± 0.37 |
| GK + FBPase-2 + LY2121260 | 6.49 ± 0.33$^{**\S}$ | 1.92 ± 0.16$^{***\#\#\#}$ | 0.35 ± 0.15$^{**\#\S}$ |

**Table 2** Glucokinase kinetic parameters with respect to FBPase-2 and LY2121260. Data represent means ± SEM calculated from measurements shown in Fig. 4. *p<0.05; **p<0.01; ***p<0.001 compared to control; $^{\#}$p<0.05; $^{\#\#\#}$p<0.001 compared without LY2121260; $^{\S}$p<0.05 compared without FBPase-2 (ANOVA/ Bonferroni's test).

## Figure legends

**Fig. 1.** Structural comparison between mannoheptulose, perseitol and sedoheptulose. Chemical structure of mannoheptulose (A), perseitol (B) and sedoheptulose (C).

**Fig. 2.** Inhibition of glucokinase enzyme activities by mannoheptulose, perseitol and sedoheptulose. Recombinant beta cell glucokinase was incubated for 5 min with 10, 25, 50 and 100 mmol/l mannoheptulose (A), perseitol (B) and sedoheptulose (C) and after addition of 100 mmol/l glucose enzyme activities were measured spectrophotometrically. Shown are means ± SEM in U/mg protein from 3 independent experiments. ***$p<0.001$ compared without compound (ANOVA/ Bonferroni's test).

**Fig. 3.** Effect of the glucokinase activator LY2121260 on glucokinase enzyme activities in the presence of mannoheptulose, perseitol and sedoheptulose. Recombinant beta cell glucokinase was incubated for 5 min without (black circles, solid line), in the presence of 10 µmol/l LY2121260 (black circles, dashed line), in the presence of 10 mmol/l mannoheptulose (A), perseitol (B) or sedoheptulose (C) (black squares, solid line) or in the presence of both 10 µmol/l LY2121260 and 10 mmol/l mannoheptulose (A), perseitol (B) or sedoheptulose (C) (black squares, dashed line). After addition of the indicated glucose concentration enzyme activities were measured spectrophotometrically. Shown are means ± SEM in U/mg protein from 3 independent experiments. (D) Comparison of glucokinase enzyme activities in the presence of LY2121260 (white bars) and mannoheptulose, perseitol or sedoheptulose (black bars) measured at 100 mmol/l glucose. $^{###}p<0.001$ compared to control; ***$p<0.001$ compared without LY2121260 (ANOVA/ Bonferroni's test).

**Fig. 4.** Effect of the glucokinase activator LY2121260 on glucokinase enzyme activities in the presence of FBPase-2. Recombinant beta cell glucokinase was incubated for 5 min without (black circles, solid line) or in the presence of 10 µmol/l LY2121260 (black circles, dashed line). Recombinant beta cell glucokinase was incubated for 20 min with FBPase-2 in a molar ratio of 1:5 without (black squares, solid line) or in the presence of 10 µmol/l LY2121260 (black squares, dashed line). After addition of the indicated glucose concentration enzyme activities were measured spectrophotometrically. Shown are means ± SEM in U/mg protein from 3 independent experiments. (B) Comparison of glucokinase enzyme activities without (white bar) and in the presence of FBPase-2 (black bar) or LY2121260 (grey bar), and both FBPase-2 and LY2121260 (black striped grey bar) measured at 100 mmol/l glucose. ***$p<0.001$ compared to control; $^{#}p<0.05$; $^{###}p<0.001$ compared without LY2121260; $^{§}p<0.05$; $^{§§§}p<0.001$ compared without FBPase-2 (ANOVA/ Bonferroni's test).

**Fig. 5.** Effects of the glucokinase activator LY2121260 and mannoheptulose on glucokinase enzyme activities in cell extracts of RINm5F-GK and RINm5F-GK-PFK-2/FBPase-2 cells. Glucokinase enzyme activities were measured spectrophotometrically after sonication of RINm5F-GK cells (A) and RINm5F-GK-PFK-2/FBPase-2 cells (B) and 5 min incubation without (black circles, solid line), in the presence of 10 µmol/l LY2121260 (black circles, dashed line), in the presence of 10 mmol/l mannoheptulose (black squares, solid line), or in the presence of both 10 µmol/l LY2121260 and 10 mmol/l mannoheptulose (black squares, dashed line). Shown are means ± SEM in mU/mg cellular protein from 4 independent experiments. (C) Comparison of glucokinase enzyme activities in cell extracts of RINm5F-GK (white bars) and RINm5F-GK-PFK-2/FBPase-2 (black bars) cells in the presence of LY2121260 and mannoheptulose measured at 100 mmol/l glucose. $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ compared to control RINm5F-GK cells; $^{\S}p<0.05$; $^{\S\S\S}p<0.001$ compared to control RINm5F-GK-PFK-2/FBPase-2 cells; ***p<0.001 compared with RINm5F-GK cells (ANOVA/ Bonferroni's test).

**Fig. 6.** Comparison of glucokinase activation by PFK-2/FBPase-2 and LY2121260 and with respect to inhibition through mannoheptulose. (A) Increase in glucokinase enzyme activity resulted by comparison of RINm5F-GK cells with RINm5F-GK cells in the presence of 10 µmol/l LY2121260 (grey bars), RINm5F-GK cells with RINm5F-GK-PFK-2/FBPase-2 cells (black bars) and RINm5F-GK-PFK-2/FBPase-2 cells with RINm5F-GK-PFK-2/FBPase-2 cells in the presence of 10 µmol/l LY2121260 (black striped grey bars). (B) Decrease in glucokinase enzyme activity resulted by comparison of RINm5F-GK cells with RINm5F-GK cells in the presence of 10 mmol/l mannoheptulose (white bars), RINm5F-GK cells in the presence of both 10 mmol/l mannoheptulose and 10 µmol/l LY2121260 (grey bars), RINm5F-GK-PFK-2/FBPase-2 cells in the presence of 10 µmol/l LY2121260 (black bars) and RINm5F-GK-PFK-2/FBPase-2 cells in the presence of both 10 mmol/l mannoheptulose and 10 µmol/l LY2121260 (black striped grey bars). Calculation based on data presented in Fig. 4.

**Fig. 7.** Glucokinase-PFK-2/FBPase-2 complex formation in RINm5F cells in dependence upon LY2121260. Protein extracts of RINm5F-GK cells (A) and RINm5F-GK-PFK-2/FBPase-2 cells (B, C) were incubated for 1 h with 25 mmol/l glucose alone (A, B) or together with 10 µM LY2121260 (C) and separated by gel filtration chromatography. Glucokinase appearance within the molecular weight fractions was visualized by immunoreactivity and quantified as percentage of total glucokinase protein. Shown are mean values from 3 independent experiments.

**Fig. 1.**

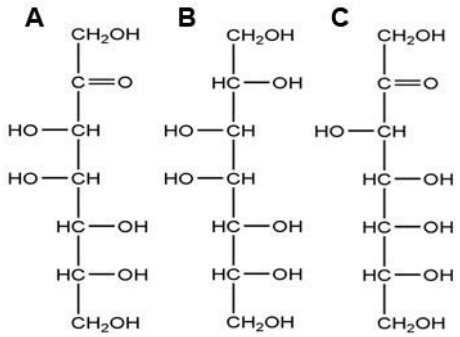


**Fig. 2.**

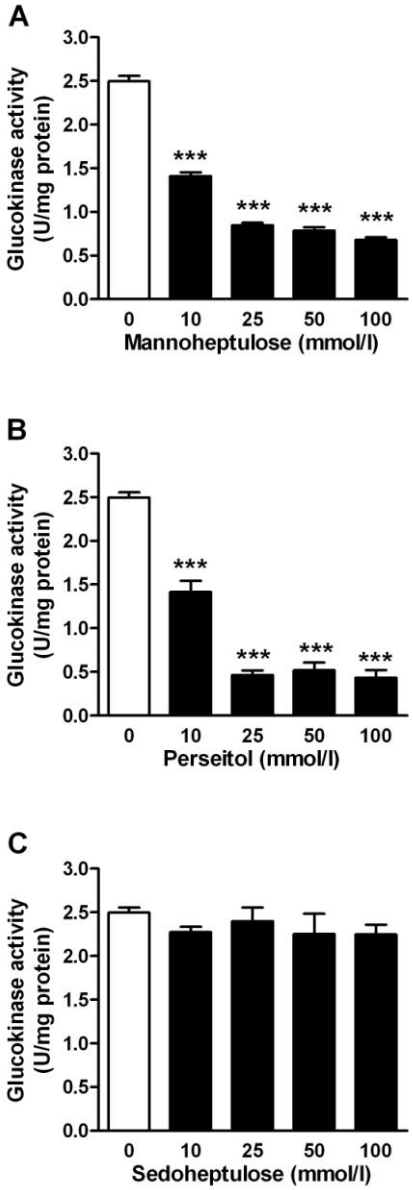

A
Glucokinase activity (U/mg protein)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
***
***
0
10
25
50
100
Mannoheptulose (mmol/l)
B
Glucokinase activity (U/mg protein)
***
***
***
***
Perseitol (mmol/l)
C
Glucokinase activity (U/mg protein)
Sedoheptulose (mmol/l)

**Fig. 3.**

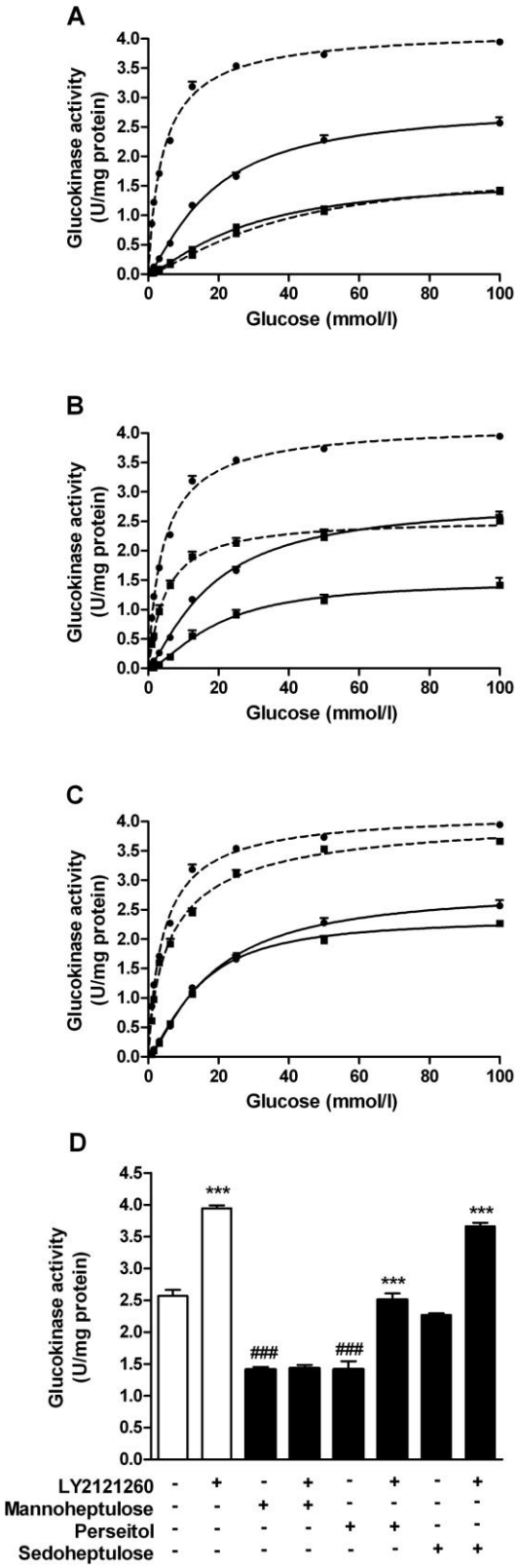

A
B
C
D
Glucokinase activity (U/mg protein)
Glucose (mmol/l)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
0
20
40
60
80
100
***
###
LY2121260
Mannoheptulose
Perseitol
Sedoheptulose

**Fig. 4.**

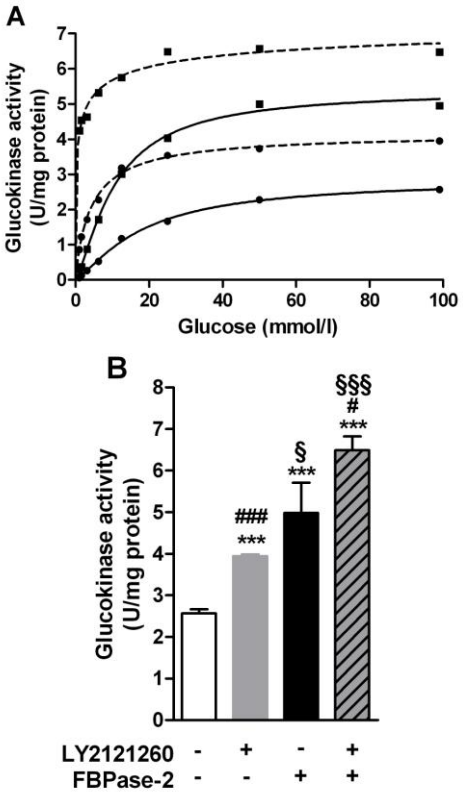

A
Glucokinase activity (U/mg protein)
Glucose (mmol/l)
B
Glucokinase activity (U/mg protein)
###
***
§
***
§§§
#
***
LY2121260 - + - +
FBPase-2 - - + +

**Fig. 5.**

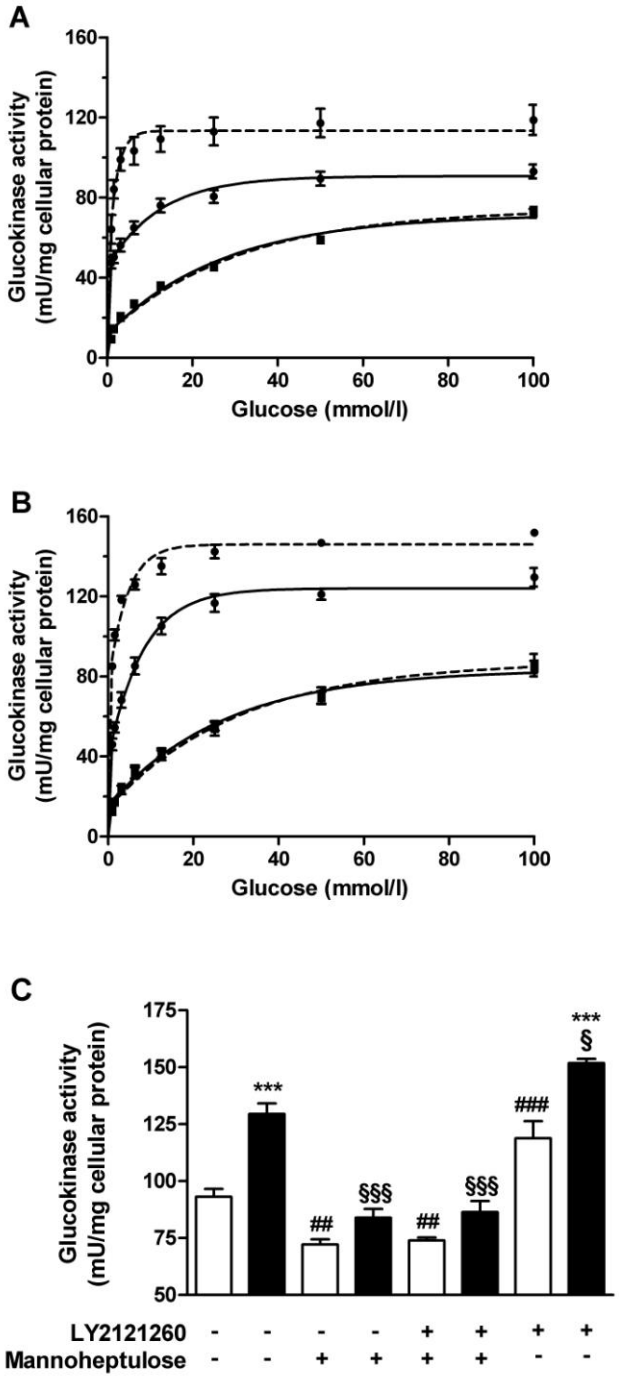

A
Glucokinase activity (mU/mg cellular protein)
160
120
80
40
0
0
20
40
60
80
100
Glucose (mmol/l)
B
Glucokinase activity (mU/mg cellular protein)
160
120
80
40
0
0
20
40
60
80
100
Glucose (mmol/l)
C
Glucokinase activity (mU/mg cellular protein)
175
150
125
100
75
50
***
##
§§§
##
§§§
###
***
§
LY2121260 - - - - + + + +
Mannoheptulose - - + + + + - -

**Fig. 6.**

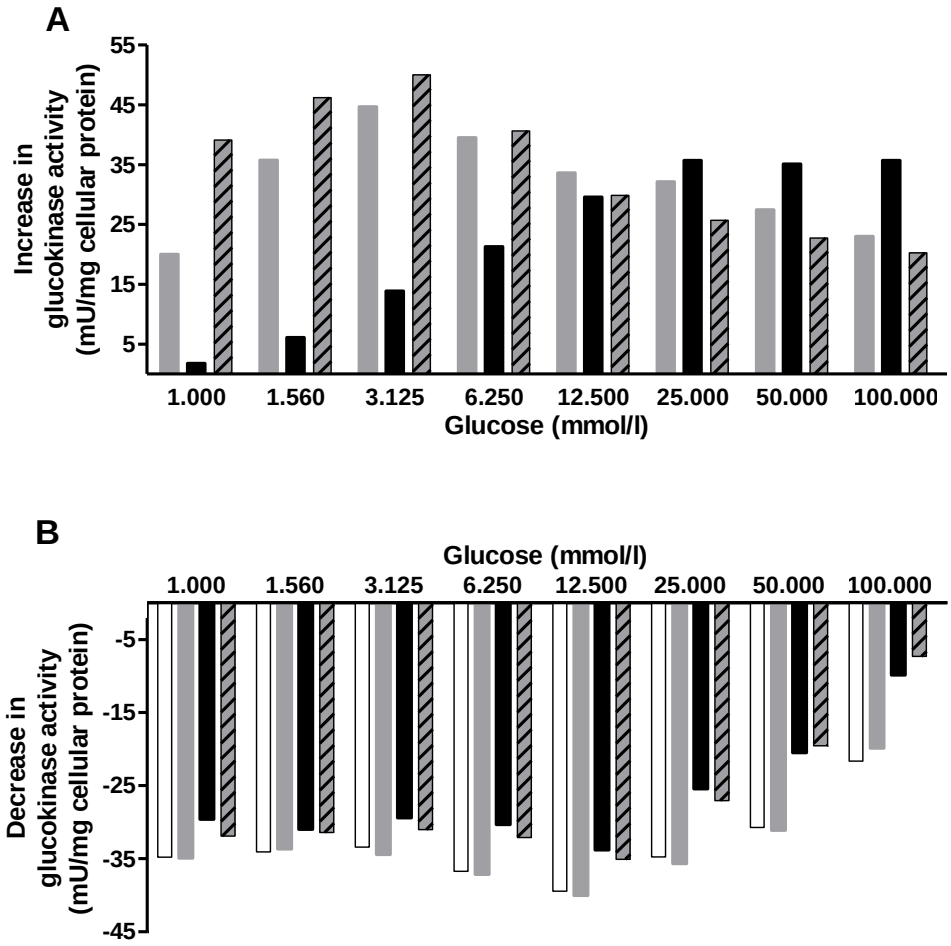
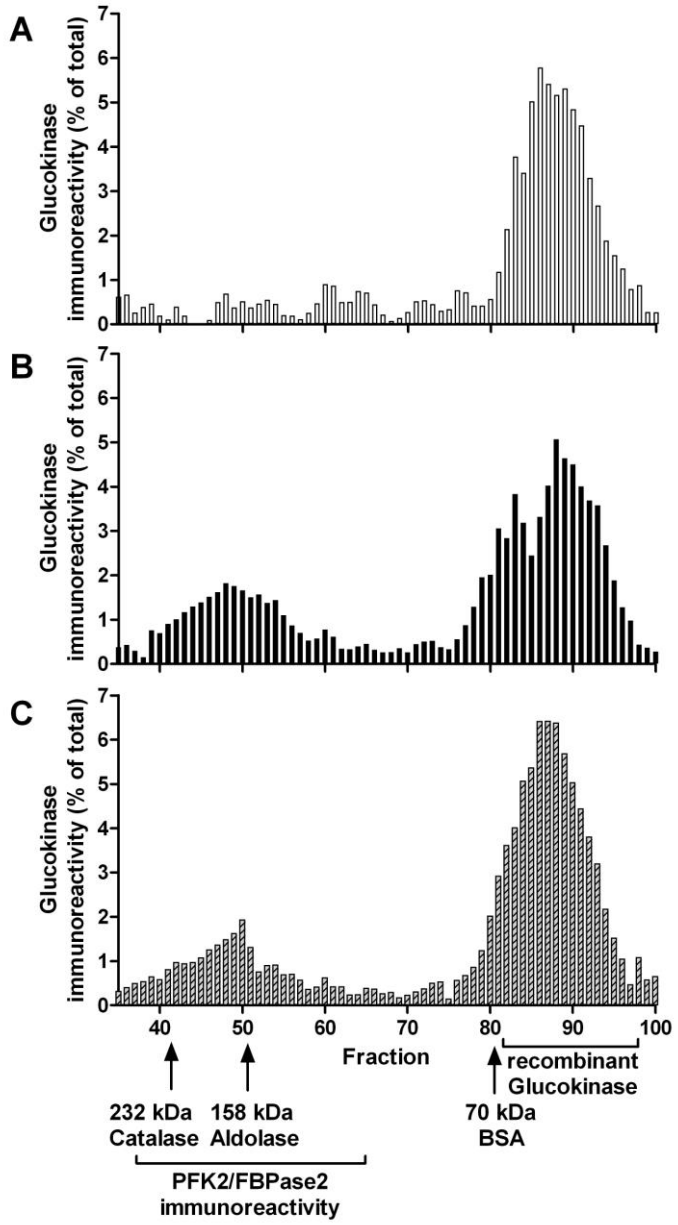

A
Increase in glucokinase activity (mU/mg cellular protein)
55
45
35
25
15
5
1.000
1.560
3.125
6.250
12.500
25.000
50.000
100.000
Glucose (mmol/l)
B
Glucose (mmol/l)
1.000
1.560
3.125
6.250
12.500
25.000
50.000
100.000
Decrease in glucokinase activity (mU/mg cellular protein)
-5
-15
-25
-35
-45

**Fig. 7.**


A
B
C
Glucokinase immunoreactivity (% of total)
0
1
2
3
4
5
6
7
40
50
60
70
80
90
100
Fraction
recombinant Glucokinase
232 kDa Catalase
158 kDa Aldolase
70 kDa BSA
PFK2/FBPase2 immunoreactivity

# 3. Diskussion

Das Glucosesensorenzym Glucokinase, auch Hexokinase Typ IV genannt, hat eine entscheidende Funktion sowohl in der glucose-induzierten Insulinsekretion der β-Zellen des Pankreas als auch im Glucosemetabolismus der Leber. Im Gegensatz zu der transkriptionellen Regulation des Leberisoenzyms durch Insulin wird der Gehalt an Glucokinaseprotein in der β-Zelle kaum durch den Ernährungsstatus beeinflusst (Iynedjian 1989; Tiedge et al. 1999). In beiden Geweben spielen posttranslationale Mechanismen bei der Regulation der Glucokinase Enzymaktivität eine wichtige Rolle. Obwohl das Substrat Glucose der primäre Regulator der Glucokinaseaktivität ist, sind Prozesse wie Protein-Protein-Interaktionen und die Kompartimentierung für die Glucokinase Feineinstellung essentiell und tragen dadurch zur Aufrechterhaltung der Blutglucosehomöostase bei (Baltrusch & Lenzen 2007; Bosco et al. 2000; de la Iglesia et al. 1999; Rizzo et al. 2002; Shiota et al. 1999; Tiedge et al. 1999; Van Schaftingen 1989). Das bifunktionelle Enzym 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase ist ein Bindungspartner der Glucokinase, der das Enzym in β-Zellen posttranslational aktiviert (Baltrusch et al. 2001; Massa et al. 2004). Dabei verstärkt die Interaktion mit der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase die Erhöhung der intrinsischen Glucokinaseaktivität durch Glucose, ohne die für die Glucosesensorfunktion wichtigen Parameter der Glucoseaffinität und der Kooperativität zu beeinflussen (Massa et al. 2004).

## *3.1. Koordinierte Aktivierung des Glucosemetabolismus durch die Überexpression von 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase in Glucokinase überexprimierenden insulinproduzierenden Zellen*

In der vorliegenden Arbeit wurde der Einfluss der Überexpression der β-Zellisoform der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase auf den zellulären Gehalt an ATP und ADP sowie auf die Insulinsekretion in insulinproduzierenden Glucokinase überexprimierenden RINm5F Zellen untersucht. Für die Bestimmung bei verschiedenen Glucosekonzentrationen wurden die Zellen zuvor für eine Stunde in Abwesenheit von Glucose kulti-

viert, um bei einer niedrigen Basalrate den Gehalt an ATP und ADP sowie die Insulinsekretion als direkte Reaktion auf den Glucosestimulus zu messen. Dabei konnte gezeigt werden, dass bei einer Glucosekonzentration von 10 mmol/l die erhöhte Glucokinaseaktivität bei gleichzeitiger Überexpression der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase mit einer effizienten Einschleusung der Glykolyseprodukte in den oxidativen Stoffwechsel einhergeht. In den RINm5F Kontrollzellen ist der Glucosemetabolismus durch eine vergleichsweise hohe Expression der hoch-affinen Hexokinasen gekennzeichnet (Tiedge 2000). Die RINm5F Zellen zeigten keine Veränderungen im Gehalt an ATP und ADP wie auch der Ratio ATP/ADP bei einer Erhöhung der Glucosekonzentration von 3 mmol/l auf 10 mmol/l. Des Weiteren wurde eine relativ hohe basale Insulinsekretion ohne signifikante Erhöhung nach Glucosestimulus in RINm5F Zellen beobachtet. Erst durch die moderate Überexpression der Glucokinase in den RINm5F-GK Zellen erlangten sie die für β-Zellen charakteristische Responsivität auf millimolare Glucosekonzentrationen. Diese zeigte sich in einem Anstieg sowohl der ATP/ADP Ratio als auch der glucose-stimulierten Insulinsekretion. Interessanterweise konnten sowohl die ATP/ADP Ratio als auch die Insulinsekretion durch die gleichzeitige Überexpression der β-Zellisoform der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase im Vergleich zu den RINm5F-GK Zellen glucoseabhängig weiter gesteigert werden, ohne die Zellvitalität zu beeinträchtigen. Damit erfüllt die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase wichtige Voraussetzungen für eine physiologische Aktivierung der Glucokinase.

## *3.2. Steigerung der Glucokinase Enzymaktivität* in vitro *durch rekombinant exprimiertes aufgereinigtes 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase Protein und durch einen synthetischen Aktivator*

Das Bindungsmotiv der Glucokinase liegt in der Bisphosphatasedomäne der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase und ist innerhalb der Isoenzyme des bifunktionellen Enzyms hochkonserviert (Baltrusch et al. 2001). Zudem erfolgt die Steigerung der intrinsischen Glucokinase Enzymaktivität in insulinproduzierenden Zellen nicht nur bei Überexpression der β-Zellisoform der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase, sondern gleichermaßen bei Überexpression mit der Leberisoform sowie der kinaseaktiven S32A/H258A mutierten Leberisoform. Das Holoenzym sowie die Bisphosphatasedomäne der Leberisoform der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase der Ratte konnten

für die vorliegende Arbeit rekombinant exprimiert werden. Das aufgereinigte Holoenzym war in der Lage, die Enzymaktivität von aufgereinigter rekombinanter Glucokinase bei einem zehnfachen molaren Überschuss des bifunktionellen Enzyms signifikant zu erhöhen. Dies unterstützt die Annahme einer schwachen Wechselwirkung zwischen Glucokinase und 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase, worauf auch frühere Ergebnisse aus Hefe *Two-Hybrid* Studien hinwiesen (Baltrusch et al. 2001). Der für schwache Wechselwirkungen charakteristische geringe Anteil des Komplexes im Reaktionsgleichgewicht konnte durch die Erhöhung der Konzentration eines Bindungspartners erhöht werden. Gleichermaßen war die Bisphosphatasedomäne der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase in der Lage, bei einem fünffachen molaren Überschuss die Enzymaktivität der Glucokinase signifikant zu erhöhen, ohne die Glucoseaffinität der Glucokinase zu verändern. Ein synthetischer Glucokinase-Aktivator steigerte die Glucokinase Enzymaktivität dagegen unter gleichzeitiger Erhöhung der Glucoseaffinität des Enzyms. Die aktivierenden Effekte der Bisphosphatasedomäne und des Aktivators traten synergistisch auf mit einer maximalen Aktivierung der Glucokinase Enzymaktivität bei gleichzeitiger Erhöhung der Glucoseaffinität. Daher aktivieren die Bisphosphatasedomäne und der synthetische Aktivator die Glucokinase über unterschiedliche Mechanismen. Allgemein gibt es Anzeichen dafür, dass die Interaktion der Enzyme *in vivo* stärker ausfällt als *in vitro* (Baltrusch et al. 2001; Garcia-Herrero 2007; Massa et al. 2004; Payne 2005). Die beobachtete Steigerung der Glucokinase Enzymaktivität durch die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase sowie die separate Bisphosphatasedomäne auch mit aufgereinigten, rekombinanten Proteinen schließt einen amplifizierenden Effekt einer bislang nicht identifizierten intrazellulären Komponente auf die Komplexbildung nicht aus, jedoch ist dies offensichtlich für die Interaktion nicht essentiell.

## 3.3. Glucoseabhängigkeit der Bindung der Glucokinase an die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase wie an das Glucokinase Regulatorprotein

Das Hauptziel der vorliegenden Arbeit war die Charakterisierung der molekularen Mechanismen der Interaktion zwischen Glucokinase und 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase in insulinproduzierenden Zellen im Hinblick auf die Glucoseabhängigkeit. Um Unterschiede im Bindungsverhalten im zellulären Kontext zu untersuchen, wurden Fusionsproteine für die Durchführung von Fluoreszenz-Resonanz-Energietransfer (FRET) und *Mammalian Two-Hybrid* Experimenten generiert. Zur Validierung der FRET Experimente als

auch des *Two-Hybrid* Systems wurde gleichermaßen die Wechselwirkung der Glucokinase mit dem Glucokinase Regulatorprotein untersucht, deren Modulation durch Glucose bereits beschrieben wurde (Baltrusch 2005; Bosco et al. 2000; de la Iglesia et al. 1999; Shiota et al. 1999). Die FRET Effizienz hängt stark von der Entfernung zwischen Donor und Akzeptor des Fluorophorenpaars ab, aber auch von der relativen Orientierung der Dipolmomente von Donor und Akzeptor zueinander. Da FRET typischerweise nur bei einem Abstand der Fluorophore von wenigen Nanometern auftritt, wird die Methode zur Detektion direkter Protein-Protein-Interaktionen eingesetzt. Das Bindungsmotiv für die Glucokinase ist im Monomer der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase einmal vorhanden, und vieles deutet auf eine 1:1 Stöchiometrie innerhalb des Komplexes hin (Baltrusch et al. 2001; Smith 2007). Für die Bindung des Glucokinase Regulatorproteins an die Glucokinase, wodurch die Glucokinase Enzymaktivität kompetitiv inhibiert wird, wurde eine 1:1 Stöchiometrie nachgewiesen (Vandercammen & Van Schaftingen 1990). Als FRET-Donor diente an die Glucokinase fusioniertes ECFP, als FRET-Akzeptor an das Glucokinase Regulatorprotein oder an die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase fusioniertes EYFP. Die durchgeführte FRET Messung basierend auf *sensitized emission* ist eine semi-quantitative Methode, da im Gegensatz zur internen Positivkontrolle, wo EYFP und ECFP in einem Protein exprimiert werden, eine solche Stöchiometrie bei den untersuchten Proteinen nicht vorliegt. Allerdings ist, da die Interaktion unter gleichen zellulären Bedingungen analysiert wurde, ein Vergleich der Wechselwirkung der Glucokinase an das Glucokinase Regulatorprotein mit der Bindung der Glucokinase an die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase möglich. Die stärkere Bindung der Glucokinase an das Glucokinase Regulatorprotein bei niedriger Glucosekonzentration im Vergleich zu einer hohen Glucosekonzentration konnte mittels FRET gezeigt werden. Für die Interaktion der Glucokinase mit der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase konnte FRET nur bei einer Konzentration von 25 mmol/l beobachtet werden. Dies spricht dafür, dass nicht nur die Aktivierung der Glucokinase Enzymaktivität durch die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase, sondern die Bindung der Enzyme selbst glucoseabhängig geschieht. Die FRET Experimente wurden in COS Zellen durchgeführt, die sich durch eine langsamere Aufnahme von Glucose aus dem Kulturmedium sowie eine niedrigere cytosolische Glucosekonzentration im Fließgleichgewicht im Vergleich zu der β-Zelllinie MIN6 auszeichnen (M. Kaminski und S. Baltrusch, unveröffentlichte Daten). Die geringere intrazelluläre Glucosekonzentration könnte ein Grund dafür sein, dass die Glucokinase auch bei einer extrazellulären Glucosekonzentration von 25 mmol/l eine signifikante Bindung an das Glucokinase Regulatorprotein zeigte und dass umgekehrt bei einer extrazellu-

lären Glucosekonzentration von 3 mmol/l kein FRET zwischen der Glucokinase und der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase messbar war. Daneben ist auch eine ungünstige sterische Anordnung der Dipolmomente im Komplex bei niedriger Glucosekonzentration nicht auszuschließen, die dem Auftreten eines FRET Signals entgegenstehen kann.

*Two-Hybrid* Systeme wurden im vergangenen Jahrzehnt in der Proteomforschung für die Detektion von Wechselwirkungen zwischen Proteinen etabliert. Sie stellen ein Instrument zur quantitativen Analyse binärer Protein-Protein-Wechselwirkungen dar. In Säugetierzelllinien wie auch in Hefen basieren *Two-Hybrid* Systeme auf der separaten Expression einer Aktivierungsdomäne und einer DNA-Bindungsdomäne, jeweils fusioniert mit einem der potentiellen Bindungspartner. Erst durch die Bindung der zu untersuchenden Proteine aneinander und die resultierende räumliche Nähe von Aktivierungsdomäne und Bindungsdomäne wird ein funktioneller Transkriptionsaktivator rekonstituiert, der die Expression eines Reporterproteins initiiert.

Die Verwendung des Fluoreszenzproteins EYFP als Reporter in den verwendeten modifizierten *Two-Hybrid* Systemen bietet gegenüber den kommerziell erhältlichen Systemen mehrere Vorteile. So ist für die Detektion des Reporterproteins keine Zelllyse erforderlich wie im Fall der Luciferase als Reporterprotein. Bei Verwendung der sezernierten Alkalischen Phosphatase als Reporterprotein ist für die Messung eine Probenentnahme des Zellüberstandes nötig, was den Vergleich von Proben, die zu unterschiedlichen Zeitpunkten entnommen werden, besonders bei schwachen Wechselwirkungen erschwert. Der wohl größte Vorteil des Reporterproteins EYFP in Kombination mit konstitutiv exprimiertem ECFP ist die Möglichkeit der kontinuierlichen Datenerhebung auf Einzelzellebene. Es zeigte sich, dass in den Versuchsansätzen immer auch eine gewisse Anzahl von Zellen eines oder beide der Fluoreszenzproteine unkontrolliert exprimierten mit der Folge einer unphysiologischen Synthese des Fluoreszenzproteins. Durch die Berechnung der EYFP/ECFP Ratio für die quantitative Analyse der Wechselwirkung für Einzelzellen konnten diese Zellen leicht identifiziert und von der weiteren Auswertung ausgeschlossen werden. Bei der Messung der Gesamtheit des gebildeten Reporterproteins, wie in beiden *Mammalian Two-Hybrid* Ursprungssystemen, ist eine solche Differenzierung nicht möglich. Besonders bei der Detektion schwacher Wechselwirkungen wie zwischen der Glucokinase und der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase ist jedoch ein gutes Signal-Rausch-Verhältnis kritisch für die Bewertung der Daten. Die eher niedrige Signalstärke bei schwachen Wechselwirkungen macht die Auswertung einer großen Anzahl von Zellen nötig, um eine Interaktion zu detektieren. Aufgrund der zeitintensiven ma-

nuellen Datenerhebung und Auswertung eröffnete die Etablierung eines halb-automatischen Versuchsansatzes die Möglichkeit, die Fluoreszenzintensitäten sehr vieler Zellen auch über einen größeren Zeitraum hinweg zu analysieren. Die Detektion einer Wechselwirkung erfolgt nach Induktion des Reporters zeitverzögert. Auf diese Weise ist mit den verwendeten *Two-Hybrid* Systemen die Messung von Zeitverläufen möglich. Allerdings ist zu berücksichtigen, dass dies keine Echtzeitaufname ist, da es sich um die Detektion eines Reportergensignals handelt und zudem die Translokation der Proteine in den Zellkern berücksichtigt werden muss.

Mit dem manuellen wie auch mit dem halb-automatischen *Mammalian Two-Hybrid* Versuchsansatz konnte in der MIN6 β-Zelllinie übereinstimmend eine signifikante Wechselwirkung zwischen der Glucokinase und der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase bei einer Glucosekonzentration von 25 mmol/l detektiert werden. Ebenfalls lag mit beiden Systemen bei 3 mmol/l Glucose die EYFP/ECFP Ratio nur schwach über den Werten der Negativkontrolle. Der mit dem semi-automatischen Versuchsansatz zusätzlich erhobene Wert der EYFP/ECFP Ratio bei einer Glucosekonzentration von 10 mmol/l deutet auf einen graduellen Anstieg der Bindungsstärke innerhalb des Komplexes mit steigender Glucosekonzentration hin. Zusammen mit früheren Studien, die einen stärkeren aktivierenden Effekt der Überexpression der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase auf die Glucokinase Enzymaktivität bei steigender Glucosekonzentration zeigten (Baltrusch 2006), stützen die Ergebnisse der FRET und *Two-Hybrid* Analysen die Hypothese, dass die Bindung des bifunktionellen Enzyms eine Stabilisierung der enzymatisch aktiven, geschlossenen Konformation der Glucokinase bewirkt. Die Interaktion mit der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase wäre somit in der Lage, den in dem kinetischen Modell von Kamata *et al.* (Kamata et al. 2004) vorgeschlagenen langsamen Übergang der Glucokinase zu der enzymatisch inaktiven weit offenen Konformation zu verhindern (Abb. 1.4). Kürzlich wurde ein kinetisches Modell entwickelt, das von drei intermediären Konformationen zwischen der weit offenen und der geschlossenen Glucokinase Konformation ausgeht (Antoine 2009). Dabei befinden sich alle fünf Konformationen der Glucokinase im Gleichgewicht, wobei dieses Gleichgewicht durch inhibierende Glucokinase Bindungspartner wie das Glucokinase Regulatorprotein in Richtung der weit offenen Konformation und durch aktivierende Bindungspartner wie synthetische Aktivatoren in Richtung der enzymatisch aktiven geschlossenen Konformation verschoben werden kann. Daraus ergibt sich auch die Hypothese, dass es sich bei der in Zellen beobachteten stärkeren Interaktion zwischen der Glucokinase und der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase im Vergleich zu der

Interaktion zwischen aufgereinigten rekombinanten Enzymen um einen Effekt der Glucose handelt, die den Übergang der Glucokinase von der weit offenen in eine für die Interaktion bevorzugte Konformation begünstigt.

## *3.4. Molekulare Umlagerungen im Homodimer der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase in Abhängigkeit von der cAMP-abhängigen Serin-32 Phosphorylierung und damit vom Ernährungsstatus*

Das bifunktionelle Enzym 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase selbst nimmt wie die Glucokinase eine herausragende Stellung in der Regulation des Kohlenhydratstoffwechsels, insbesondere in der Leber, ein (Okar et al. 2001). Über die Synthese und den Abbau des wichtigen regulatorischen Metaboliten Fructose-2,6-bisphosphat sorgt das Enzym in der Leber für die Umschaltung zwischen Glykolyse und Gluconeogenese. Ein wichtiges Regulationsprinzip der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase stellt dabei die durch Glucagon vermittelte Phosphorylierung des Serin-32 Restes dar, die durch Aktivierung der Bisphosphatase und Inhibierung der Kinase letztendlich zu einer erhöhten Glucosefreisetzung durch die Leber führt (el-Maghrabi 1982; El-Maghrabi et al. 1982; Murray 1984). Molekulare Umlagerungen spielen dabei in der Regulation der Enzymaktivität des bifunktionellen Enzyms eine wichtige Rolle (Kurland 1993; Lin 1994; Zhu 2001). Ob die Dimerisierung der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase, welche über die Kinasedomänen der Monomere vermittelt wird, durch die Serin-32 Phosphorylierung beeinflusst wird, ist bisher unklar, und sollte daher in der vorliegenden Studie untersucht werden. Die Interaktionsstärke zwischen zwei Monomeren des bifunktionellen Enzyms wurde zunächst mittels *Mammalian Two-Hybrid* Analysen gemessen, wobei für die Bewertung der Daten die folgenden Punkte berücksichtigt werden müssen. Erstens wird die Untersuchung einer Homodimerisierung im *Two-Hybrid* System generell zu einer Unterschätzung der Bindungsstärke führen. Bei einer unvermeidbaren Dimerbildung auch von 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase Molekülen, die entweder beide an die Aktivierungsdomäne oder beide an die DNA-Bindungsdomäne fusioniert sind, erfolgt trotz Wechselwirkung keine Induktion des Reporterproteins. Zweitens ist die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase ein für die Glykolyse essentielles Enzym, das in praktisch jeder eukaryotischen Zelle und somit auch in den für die Studie verwendeten COS Zellen exprimiert wird. Kürzlich wurde mittels *Real-time* RT-PCR die Expression der PFKFB1-3 Gene in

der Leber, dem Herzen, dem Gehirn und den β-Zellen von Maus und Ratte analysiert. Dabei konnte gezeigt werden, dass eines der Isoenzyme der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase primär exprimiert wird, jedoch mit zum Teil erheblicher gleichzeitiger Expression der übrigen Isoenzyme (Arden 2008). In der Folge ist mit einer weiteren Reduktion des *Two-Hybrid* Signals durch die Dimerbildung der exogen exprimierten Fusionsproteine mit endogener 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase zu rechnen.

Zunächst überraschend war die Beobachtung, dass die cAMP-abhängige Serin-32 Phosphorylierung, induziert durch die Inkubation mit Forskolin, auf das *Two-Hybrid* Signal für die Dimerisierung des 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase Wildtypenzyms keinen Einfluss hatte, die Signalstärke für das nicht phosphorylierbare S32A/H258A mutierte Enzym jedoch nach Forskolinstimulation anstieg. Dieser Effekt lässt sich im Zusammenspiel mit endogen exprimierter 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase, die cAMP-abhängig phosphoryliert wird, erklären. Wir gehen davon aus, dass es zur Bildung von gemischten Dimeren aus jeweils einem endogenen und einem exogenen Monomer der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase kommt. Dabei stellt sich ein Gleichgewicht ein, da ein unphosphoryliertes endogenes Monomer gleichermaßen ein unphosphoryliertes endogenes oder exogenes Monomer binden kann. Dieser Quenching-Effekt würde erst dann reduziert, wenn es durch die Phosphorylierung zu einer unterschiedlichen Bindungsaffinität innerhalb des Dimers kommt. Nach der Forskolinstimulation würde somit die phosphorylierte endogene 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase von der nicht phosphorylierbaren mutierten exogenen 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase unterscheidbar. Die unterschiedliche Bindungsstärke in phosphoryliertem und unphosphoryliertem Zustand würde nun zur bevorzugten Bildung von gleichartigen Dimeren führen. So würde der Anteil der Dimere aus zwei exogenen Monomeren zunehmen, so dass ein höherer Anteil des S32A/H258A 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase Fusionsproteins zur Induktion des Reporterproteins beitragen würde. Dagegen wären das endogene Enzym und die exogene Wildtyp 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase nach der Forskolinstimulation zu einem identischen Anteil phosphoryliert. Somit ist keine Voraussetzung einer bevorzugten Dimerisierung gegeben. Der Quenching-Effekt des *Two-Hybrid* Signals wäre sowohl vor als auch nach Forskolinstimulation gleichermaßen ausgeprägt, wie es auch in den Experimenten zu beobachten war.

Die Hypothese stützend, dass die Serin-32 Phosphorylierung Ursache des unterschiedlichen Bindungsverhaltens innerhalb des 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase Dimers ist, konnte gezeigt werden, dass Forskolin in COS Zellen den Gehalt an cAMP erhöht,

und zwar unabhängig von der in den *Two-Hybrid* Fusionsproteinen enthaltenen Form der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase. Des Weiteren konnte die Forskolin-induzierte Serin-32 Phosphorylierung der in COS Zellen überexprimierten 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase mit einem phospho-spezifischen Antikörper für das Wildtyp-Enzym nachgewiesen werden. Dagegen konnte für das mutierte Enzym erwartungsgemäß keine Serin-32 Phosphorylierung im *Western Blot* gezeigt werden. Es gibt Hinweise für eine Inhibierung der AMP-abhängigen Proteinkinase durch cAMP (Hurley 2006), weshalb die Phosphorylierung anderer endogen exprimierter Isoenzyme der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase nach Forskolinstimulation nicht ausgeschlossen werden kann. Ein möglicher Einfluss anderer Proteinkinasekaskaden auf die durchgeführten Experimente scheint aufgrund des gezeigten ausgeprägten Effekts auf die Serin-32 Phosphorylierung des Leberisoenzyms jedoch von untergeordneter Rolle zu sein.

Nachdem die *Two-Hybrid* Analysen Hinweise auf eine unterschiedliche Bindungsstärke innerhalb des 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase Dimers in Abhängigkeit des Phosphorylierungsstatus lieferten, sollte mittels FRET geklärt werden, ob die phosphorylierten oder die unphosphorylierten Monomere stärker interagieren. Dafür wurden Fusionsproteine der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase in COS Zellen exprimiert, in denen das photoaktivierbare Fluoreszenzprotein Dendra2 N-terminal an die Kinasedomäne des bifunktionellen Enzyms fusioniert wurde. Die grüne Form des Dendra2 diente in den Experimenten als FRET Donor, die rote Form als FRET Akzeptor. Die partielle Umwandlung des grünen Fluorophors in das rote erfolgte durch die kontrollierte Exposition von aufgereinigtem rekombinanten Fusionsprotein mit UV-Licht. Die räumliche Nähe, die Voraussetzung für FRET ist, lag nur in dem frisch isolierten und daher partiell phosphorylierten Wildtyp-Enzym vor. Der Verlust des Signals nach Hydrolyse des Serin-Phosphatrestes deutet auf eine schwächere Bindung zwischen den Monomeren mit einer größeren Entfernung zwischen den Kinasedomänen der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase hin.

Zusammenfassend kann festgestellt werden, dass das postulierte Modell einer Koordination des Kohlenhydratstoffwechsels auf molekularer Ebene durch die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase und die Glucokinase sowohl in der Leber als auch in den β-Zellen des Pankreas durch die Ergebnisse dieser Arbeit bestätigt werden konnte. Frühere Studien zeigten, dass in insulinproduzierenden Zellen die Aktivierung der Glucokinase durch das 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase Leberisoenzym durch Forskolin verhindert wird, nicht jedoch für das S32A/H258A mutierte Enzym (Massa et al. 2004). Des Weiteren führt in Hepatocyten die Überexpression der 6-Phosphofructo-2-kinase/Fructose-

2,6-bisphosphatase für den Wildtyp und das mutierte Enzym in Abwesenheit von Glucagon zu einer erhöhten cytoplasmatischen Lokalisation der Glucokinase (Payne et al. 2005). Jedoch konnte nur die S32A/H258A mutierte 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase die Reduktion des zellulären Gehalts an Fructose-2,6-bisphosphat, die Inhibierung der Glykolyse und die Reduktion der frei beweglichen Glucokinase-Fraktion durch Glucagon verhindern (Payne et al. 2005). Auch sind in dem Glucokinase:6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase Komplex die Enzymaktivitäten der Glucokinase und der 6-Phosphofructo-2-kinase erhöht, während die Aktivität der Fructose-2,6-bisphosphatase unverändert ist (Smith et al. 2007). Somit ist die koordinierte Aktivierung des Glucosestoffwechsels durch einen Komplex aus enzymatisch aktiver Glucokinase und unphosphorylierter 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase, moduliert durch die Glucosekonzentration, ein wahrscheinliches Szenario. Die Interaktion ist daher ein interessanter Ansatzpunkt für die Entwicklung neuer anti-hyperglykämischer Therapieformen.

# 4. Zusammenfassung

Die Aufrechterhaltung der Blutglucosehomöostase wird durch ein komplexes Zusammenspiel anaboler und kataboler Stoffwechselwege gewährleistet. Eine herausragende Rolle kommt dabei dem Enzym Glucokinase in der Leber und den β-Zellen des Pankreas zu. Die Glucokinase ist in der Lage, den glykolytischen Flux an die Blutglucosekonzentration zu koppeln, und hat somit die Funktion eines Glucosesensors inne. In den β–Zellen des Pankreas entsteht das Signal für die glucose-induzierte Insulinsekretion im Glucosemetabolismus, wobei die Phosphorylierung der Glucose durch die Glucokinase den geschwindigkeitsbestimmenden Schritt in diesem Prozess darstellt. In der Leber reguliert die Glucokinase im Zusammenspiel mit weiteren Enzymen den Glucosemetabolismus. Bei der Adaption der Glucokinase Enzymaktivität an kurzfristige Schwankungen der Blutglucosekonzentration spielen posttranslationale Regulationsmechanismen wie Protein-Protein-Interaktionen und die intrazelluläre Kompartimentierung eine wichtige Rolle. Nachdem das leberspezifische Glucokinase Regulatorprotein als inhibierender Bindungspartner der Glucokinase schon seit zwei Jahrzehnten bekannt ist, konnte vor einigen Jahren die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase als ein aktivierendes Glucokinase-bindendes Protein identifiziert werden. Das als Homodimer vorliegende bifunktionelle Enzym wird in unterschiedlichen Isoformen in allen Geweben exprimiert. Es ist durch die Synthese und den Abbau von Fructose-2,6-bisphosphat, dem wichtigsten allosterischen Aktivator der Phosphofructokinase 1, an der Regulation der Glykolyse beteiligt. In der vorliegenden Arbeit konnte gezeigt werden, dass die Steigerung der intrinsischen Glucokinase Enzymaktivität durch gleichzeitige Überexpression sowohl der Leberisoform als auch der β-Zellisoform der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase in insulinproduzierenden Zellen zu einer Erhöhung der zellulären ATP/ADP Ratio und in der Folge zu einer gesteigerten Insulinsekretion führt. Die Aktivierung der Glucokinase Enzymaktivität durch die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase konnte zudem mit aufgereinigten, rekombinanten Proteinen gezeigt werden. Die Beteiligung bislang unbekannter intrazellulärer Komponenten an der Komplexbildung kann dadurch zwar nicht völlig ausgeschlossen werden, ist jedoch nicht essentiell. Für die weitere Untersuchung der Interaktion zwischen Glucokinase und 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase wurden in dieser Arbeit unterschiedliche fluoreszenz-

basierte *Mammalian Two-Hybrid* Systeme in insulinproduzierenden Zellen etabliert und modifiziert. Beiden Systemen ist gemeinsam, dass eine Herpes Simplex Virus VP16 Aktivierungsdomäne und die GAL4 DNA-Bindungsdomäne der Hefe jeweils als Fusionsprotein mit einem der Wechselwirkungspartner exprimiert werden. Nur eine direkte Bindung der untersuchten Proteine aneinander führt zur Rekonstitution des funktionellen Transkriptionsaktivators und damit zur Induktion des Reporterproteins EYFP. Um eine quantitative Analyse durchführen zu können, wurde zusätzlich zu den *Two-Hybrid* Expressionsvektoren das ECFP Gen unter Kontrolle eines konstitutiv aktiven Promotors in den Zellen exprimiert. Die Messung der Fluoreszenzintensitäten erfolgte in lebenden Zellen und erlaubte die Auswertung auf Einzelzellebene. Die Population der untersuchten Zellen konnte durch Verwendung eines automatisierten Fluoreszenzmikroskopsystems erhöht werden. Mit den Systemen konnte die Interaktion zwischen Glucokinase und 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase in der lebenden Zelle unter Modifikation der extrazellulären Bedingungen untersucht werden. Eine entscheidende Rolle bei der Modulation der Bindung der Glucokinase an die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase konnte für das Substrat Glucose nachgewiesen werden. Mit steigender Glucosekonzentration stieg die Bindungsstärke innerhalb des Komplexes an. Parallel wurde die Bindung der Glucokinase an die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase mit fluoreszierenden Fusionsproteinen mittels FRET untersucht. Mit diesem semi-quantitativen Versuchsansatz konnte eine Wechselwirkung der Proteine ebenfalls nur bei hoher Glucosekonzentration gezeigt werden. Die Experimente stützen somit die Hypothese, dass die bisher nicht identifizierte Bindungsstelle im Glucokinase Protein erst mit der Bindung eines Glucosemoleküls durch die Glucokinase und der damit einhergehenden Konformationsänderung zugänglich wird.

Ein weiteres Ziel der vorliegenden Arbeit war die Untersuchung des Einflusses der Serin-32 Phosphorylierung der Leberisoform der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase auf die Dimerisierung des Enzyms. Hierzu wurden die Zellen mit Forskolin inkubiert, das wie Glucagon in der Lage ist, den intrazellulären cAMP Gehalt zu erhöhen. Im Gegensatz zu der β-Zellisoform der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase wird die Leberisoform bei Nahrungskarenz durch die cAMP-abhängige Proteinkinase A phosphoryliert. Die Modulation der Dimerisierung, welche über die Kinasedomänen des bifunktionellen Enzyms vermittelt wird, wurde mittels *Two-Hybrid* und FRET Messungen analysiert. Es konnte gezeigt werden, dass die 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase cAMP-abhängig an Serin-32 phosphoryliert wird und dies mit einer Erhöhung der Bindungsstärke innerhalb des Dimers einhergeht. In vorangegangenen Experimenten konnte gezeigt

werden, dass die Glucokinase bevorzugt an die dephosphorylierte 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase bindet. Damit ergibt sich ein direktes Zusammenspiel von Glucokinase und 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase in der Leber nach Nahrungsaufnahme, welches eine effiziente Einschleusung der Glucose in die Glykolyse gewährleistet.

Die durchgeführten Studien unterstreichen die Bedeutung der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase in der physiologischen Aktivierung der Glucokinase. Eine genaue Charakterisierung dieser Aktivierung, bei der die Glucosesensorfunktion der Glucokinase erhalten bleibt, kann perspektivisch neue Möglichkeiten in der antihyperglykämischen Therapie im Typ 2 Diabetes mellitus eröffnen.

# 5. Literaturverzeichnis

**Agius L. & Stubbs M. (2000).** Investigation of the mechanism by which glucose analogues cause translocation of glucokinase in hepatocytes: evidence for two glucose binding sites. *Biochem J* 346 Pt 2:413-21.

**Aguilar-Bryan L., Clement J.P.t. & Nelson D.A. (1998).** Sulfonylurea receptors and ATP-sensitive potassium ion channels. *Methods Enzymol* 292:732-44.

**Andreone T.L., Printz R.L., Pilkis S.J., Magnuson M.A. & Granner D.K. (1989).** The amino acid sequence of rat liver glucokinase deduced from cloned cDNA. *J Biol Chem* 264:363-9.

**Antoine M., Boutin J.A. & Ferry G. (2009).** Binding kinetics of glucose and allosteric activators to human glucokinase reveal multiple conformational states. *Biochemistry* 48:5466-82.

**Arden C., Hampson L.J., Huang G.C., Shaw J.A., Aldibbiat A., Holliman G., Manas D., Khan S., Lange A.J. & Agius L. (2008).** A role for PFK-2/FBPase-2, as distinct from fructose 2,6-bisphosphate, in regulation of insulin secretion in pancreatic beta-cells. *Biochem J* 411:41-51.

**Baltrusch S. (2004).** Interaction of GK with the Bifunctional Enzyme 6-Phosphofructo-2-Kinase/Fructose-2,6-Bisphosphatase (6PF2K/F26$P_2$ase). (ed.) *In Matschinsky, F.M., Magnuson, M.A. (eds) Glucokinase and Glycemic Disease: From Basics to Novel Therapeutics. Front Diabetes. Karger, Basel*. 262-74

**Baltrusch S., Francini F., Lenzen S. & Tiedge M. (2005).** Interaction of glucokinase with the liver regulatory protein is conferred by leucine-asparagine motifs of the enzyme. *Diabetes* 54:2829-37.

**Baltrusch S., Langer S., Massa L., Tiedge M. & Lenzen S. (2006).** Improved metabolic stimulus for glucose-induced insulin secretion through GK and PFK-2/FBPase-2 coexpression in insulin-producing RINm5F cells. *Endocrinology* 147:5768-76.

**Baltrusch S. & Lenzen S. (2007).** Novel insights into the regulation of the bound and diffusible glucokinase in MIN6 beta-cells. *Diabetes* 56:1305-15.

**Baltrusch S., Lenzen S., Okar D.A., Lange A.J. & Tiedge M. (2001).** Characterization of glucokinase-binding protein epitopes by a phage-displayed peptide library. Identification of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase as a novel interaction partner. *J Biol Chem* 276:43915-23.

**Baltrusch S. & Tiedge M. (2006).** Glucokinase Regulatory Network in Pancreatic {beta}-Cells and Liver. *Diabetes* 55 Suppl 2:S55-64.

**Bell G.I., Kayano T., Buse J.B., Burant C.F., Takeda J., Lin D., Fukumoto H. & Seino S. (1990).** Molecular biology of mammalian glucose transporters. *Diabetes Care* 13:198-208.

**Bosco D., Meda P. & Iynedjian P.B. (2000).** Glucokinase and glucokinase regulatory protein: mutual dependence for nuclear localization. *Biochem J* 348 Pt 1:215-22.

**Brocklehurst K.J., Payne V.A., Davies R.A., Carroll D., Vertigan H.L., Wightman H.J., Aiston S., Waddell I.D., Leighton B., Coghlan M.P. & Agius L. (2004).** Stimulation of hepatocyte glucose metabolism by novel small molecule glucokinase activators. *Diabetes* 53:535-41.

**Davidson H.W., Rhodes C.J. & Hutton J.C. (1988).** Intraorganellar calcium and pH control proinsulin cleavage in the pancreatic beta cell via two distinct site-specific endopeptidases. *Nature* 333:93-6.

**de la Iglesia N., Veiga-da-Cunha M., Van Schaftingen E., Guinovart J.J. & Ferrer J.C. (1999).** Glucokinase regulatory protein is essential for the proper subcellular localisation of liver glucokinase. *FEBS Lett* 456:332-8.

**Efanov A.M., Barrett D.G., Brenner M.B., Briggs S.L., Delaunois A., Durbin J.D., Giese U., Guo H., Radloff M., Gil G.S., Sewing S., Wang Y., Weichert A., Zaliani A. & Gromada J. (2005).** A novel glucokinase activator modulates pancreatic islet and hepatocyte function. *Endocrinology* 146:3696-701.

**Efrat S., Tal M. & Lodish H.F. (1994).** The pancreatic beta-cell glucose sensor. *Trends Biochem Sci* 19:535-8.

**el-Maghrabi M.R., Claus T.H., Pilkis J. & Pilkis S.J. (1982).** Regulation of 6-phosphofructo-2-kinase activity by cyclic AMP-dependent phosphorylation. *Proc Natl Acad Sci U S A* 79:315-9.

**El-Maghrabi M.R., Fox E., Pilkis J. & Pilkis S.J. (1982).** Cyclic AMP-dependent phosphorylation of rat liver 6-phosphofructo 2-kinase, fructose 2,6-bisphosphatase. *Biochem Biophys Res Commun* 106:794-802.

**Garcia-Herrero C.M., Galan M., Vincent O., Flandez B., Gargallo M., Delgado-Alvarez E., Blazquez E. & Navas M.A. (2007).** Functional analysis of human glucokinase gene mutations causing MODY2: exploring the regulatory mechanisms of glucokinase activity. *Diabetologia* 50:325-33.

**Gidh-Jain M., Takeda J., Xu L.Z., Lange A.J., Vionnet N., Stoffel M., Froguel P., Velho G., Sun F., Cohen D. & et al. (1993).** Glucokinase mutations associated with non-insulin-dependent (type 2) diabetes mellitus have decreased enzymatic activity: implications for structure/function relationships. *Proc Natl Acad Sci U S A* 90:1932-6.

**Grimsby J., Sarabu R., Corbett W.L., Haynes N.E., Bizzarro F.T., Coffey J.W., Guertin K.R., Hilliard D.W., Kester R.F., Mahaney P.E., Marcus L., Qi L., Spence C.L., Tengi J., Magnuson M.A., Chu C.A., Dvorozniak M.T., Matschinsky F.M. & Grippo J.F. (2003).** Allosteric activators of glucokinase: potential role in diabetes therapy. *Science* 301:370-3.

**Guertin K.R. & Grimsby J. (2006).** Small molecule glucokinase activators as glucose lowering agents: a new paradigm for diabetes therapy. *Curr Med Chem* 13:1839-43.

**Hasemann C.A., Istvan E.S., Uyeda K. & Deisenhofer J. (1996).** The crystal structure of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase reveals distinct domain homologies. *Structure* 4:1017-29.

**Hurley R.L., Barre L.K., Wood S.D., Anderson K.A., Kemp B.E., Means A.R. & Witters L.A. (2006).** Regulation of AMP-activated protein kinase by multisite phosphorylation in response to agents that elevate cellular cAMP. *J Biol Chem* 281:36662-72.

**Iynedjian P.B. (1993).** Mammalian glucokinase and its gene. *Biochem J* 293 ( Pt 1):1-13.

**Iynedjian P.B., Gjinovci A. & Renold A.E. (1988).** Stimulation by insulin of glucokinase gene transcription in liver of diabetic rats. *J Biol Chem* 263:740-4.

**Iynedjian P.B., Mobius G., Seitz H.J., Wollheim C.B. & Renold A.E. (1986).** Tissue-specific expression of glucokinase: identification of the gene product in liver and pancreatic islets. *Proc Natl Acad Sci U S A* 83:1998-2001.

**Iynedjian P.B., Pilot P.R., Nouspikel T., Milburn J.L., Quaade C., Hughes S., Ucla C. & Newgard C.B. (1989).** Differential expression and regulation of the glucokinase gene in liver and islets of Langerhans. *Proc Natl Acad Sci U S A* 86:7838-42.

**Jetton T.L., Liang Y., Pettepher C.C., Zimmerman E.C., Cox F.G., Horvath K., Matschinsky F.M. & Magnuson M.A. (1994).** Analysis of upstream glucokinase promoter

activity in transgenic mice and identification of glucokinase in rare neuroendocrine cells in the brain and gut. *J Biol Chem* 269:3641-54.

**Kamata K., Mitsuya M., Nishimura T., Eiki J. & Nagata Y. (2004).** Structural basis for allosteric regulation of the monomeric allosteric enzyme human glucokinase. *Structure (Camb)* 12:429-38.

**Kamata K., Mitsuya M., Nishimura T., Eiki J. & Nagata Y. (2004).** Structural basis for allosteric regulation of the monomeric allosteric enzyme human glucokinase. *Structure* 12:429-38.

**Kayano T., Burant C.F., Fukumoto H., Gould G.W., Fan Y.S., Eddy R.L., Byers M.G., Shows T.B., Seino S. & Bell G.I. (1990).** Human facilitative glucose transporters. Isolation, functional characterization, and gene localization of cDNAs encoding an isoform (GLUT5) expressed in small intestine, kidney, muscle, and adipose tissue and an unusual glucose transporter pseudogene-like sequence (GLUT6). *J Biol Chem* 265:13276-82.

**Kurland I.J., Li L., Lange A.J., Correia J.J., el-Maghrabi M.R. & Pilkis S.J. (1993).** Regulation of rat 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Role of the NH2-terminal region. *J Biol Chem* 268:14056-64.

**Lange A.J., Espinet C., Hall R., el-Maghrabi M.R., Vargas A.M., Miksicek R.J., Granner D.K. & Pilkis S.J. (1992).** Regulation of gene expression of rat skeletal muscle/liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Isolation and characterization of a glucocorticoid response element in the first intron of the gene. *J Biol Chem* 267:15673-80.

**Leighton B., Atkinson A. & Coghlan M.P. (2005).** Small molecule glucokinase activators as novel anti-diabetic agents. *Biochem Soc Trans* 33:371-4.

**Lenzen S. (1990).** Hexose recognition mechanisms in pancreatic B-cells. *Biochem Soc Trans* 18:105-7.

**Lenzen S. (1992).** Glucokinase: signal recognition enzyme for glucose-induced insulin secretion. (ed.) *In Flatt, P.R. (ed.) Nutrient regulation of insulin secretion. Portland Press, London and Chapel Hill*. 101-25

**Lenzen S. & Panten U. (1988).** Signal recognition by pancreatic B-cells. *Biochem Pharmacol* 37:371-8.

**Lenzen S. & Tiedge M. (1994).** Regulation of pancreatic B-cell glucokinase and GLUT2 glucose transporter gene expression. *Biochem Soc Trans* 22:1-6.

**Lin K., Kurland I.J., Li L., Lee Y.H., Okar D., Marecek J.F. & Pilkis S.J. (1994).** Evidence for NH2- and COOH-terminal interactions in rat 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *J Biol Chem* 269:16953-60.

**Magnuson M.A. (1990).** Glucokinase gene structure. Functional implications of molecular genetic studies. *Diabetes* 39:523-7.

**Magnuson M.A., Andreone T.L., Printz R.L., Koch S. & Granner D.K. (1989).** Rat glucokinase gene: structure and regulation by insulin. *Proc Natl Acad Sci U S A* 86:4838-42.

**Magnuson M.A. & Shelton K.D. (1989).** An alternate promoter in the glucokinase gene is active in the pancreatic beta cell. *J Biol Chem* 264:15936-42.

**Massa L., Baltrusch S., Okar D.A., Lange A.J., Lenzen S. & Tiedge M. (2004).** Interaction of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) with glucokinase activates glucose phosphorylation and glucose metabolism in insulin-producing cells. *Diabetes* 53:1020-9.

**Matschinsky F.M. (1990).** Glucokinase as glucose sensor and metabolic signal generator in pancreatic beta-cells and hepatocytes. *Diabetes* 39:647-52.

**Matschinsky F.M. (2002).** Regulation of pancreatic beta-cell glucokinase: from basics to therapeutics. *Diabetes* 51 Suppl 3:S394-404.

**Matschinsky F.M. (2009).** Assessing the potential of glucokinase activators in diabetes therapy. *Nat Rev Drug Discov* 8:399-416.

**Middleton R.J. (1990).** Hexokinases and glucokinases. *Biochem Soc Trans* 18:180-3.
**Murray K.J., El-Maghrabi M.R., Kountz P.D., Lukas T.J., Soderling T.R. & Pilkis S.J. (1984).** Amino acid sequence of the phosphorylation site of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *J Biol Chem* 259:7673-81.
**Okar D.A. & Lange A.J. (1999).** Fructose-2,6-bisphosphate and control of carbohydrate metabolism in eukaryotes. *Biofactors* 10:1-14.
**Okar D.A., Manzano A., Navarro-Sabate A., Riera L., Bartrons R. & Lange A.J. (2001).** PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose-2,6-bisphosphate. *Trends Biochem Sci* 26:30-5.
**Panten U., Schwanstecher M. & Schwanstecher C. (1996).** Sulfonylurea receptors and mechanism of sulfonylurea action. *Exp Clin Endocrinol Diabetes* 104:1-9.
**Payne V.A., Arden C., Wu C., Lange A.J. & Agius L. (2005).** Dual role of phosphofructokinase-2/fructose bisphosphatase-2 in regulating the compartmentation and expression of glucokinase in hepatocytes. *Diabetes* 54:1949-57.
**Pierreux C.E., Urso B., De Meyts P., Rousseau G.G. & Lemaigre F.P. (1998).** Inhibition by insulin of glucocorticoid-induced gene transcription: involvement of the ligand-binding domain of the glucocorticoid receptor and independence from the phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways. *Mol Endocrinol* 12:1343-54.
**Pilkis S.J., Claus T.H., Kurland I.J. & Lange A.J. (1995).** 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase: a metabolic signaling enzyme. *Annu Rev Biochem* 64:799-835.
**Pilkis S.J., Weber I.T., Harrison R.W. & Bell G.I. (1994).** Glucokinase: structural analysis of a protein involved in susceptibility to diabetes. *J Biol Chem* 269:21925-8.
**Printz R.L. & Granner D.K. (2005).** Tweaking the glucose sensor: adjusting glucokinase activity with activator compounds. *Endocrinology* 146:3693-5.
**Printz R.L., Koch S., Potter L.R., O'Doherty R.M., Tiesinga J.J., Moritz S. & Granner D.K. (1993).** Hexokinase II mRNA and gene structure, regulation by insulin, and evolution. *J Biol Chem* 268:5209-19.
**Rizzo M.A., Magnuson M.A., Drain P.F. & Piston D.W. (2002).** A functional link between glucokinase binding to insulin granules and conformational alterations in response to glucose and insulin. *J Biol Chem* 277:34168-75.
**Shiota C., Coffey J., Grimsby J., Grippo J.F. & Magnuson M.A. (1999).** Nuclear import of hepatic glucokinase depends upon glucokinase regulatory protein, whereas export is due to a nuclear export signal sequence in glucokinase. *J Biol Chem* 274:37125-30.
**Smith W.E., Langer S., Wu C., Baltrusch S. & Okar D.A. (2007).** Molecular coordination of hepatic glucose metabolism by the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase:glucokinase complex. *Mol Endocrinol* 21:1478-87.
**St Charles R., Harrison R.W., Bell G.I., Pilkis S.J. & Weber I.T. (1994).** Molecular model of human beta-cell glucokinase built by analogy to the crystal structure of yeast hexokinase B. *Diabetes* 43:784-91.
**Takeda J., Gidh-Jain M., Xu L.Z., Froguel P., Velho G., Vaxillaire M., Cohen D., Shimada F., Makino H., Nishi S. & et al. (1993).** Structure/function studies of human beta-cell glucokinase. Enzymatic properties of a sequence polymorphism, mutations associated with diabetes, and other site-directed mutants. *J Biol Chem* 268:15200-4.
**Tiedge M., Elsner M., McClenaghan N.H., Hedrich H.J., Grube D., Klempnauer J. & Lenzen S. (2000).** Engineering of a glucose-responsive surrogate cell for insulin replacement therapy of experimental insulin-dependent diabetes. *Hum Gene Ther* 11:403-14.
**Tiedge M., Krug U. & Lenzen S. (1997).** Modulation of human glucokinase intrinsic activity by SH reagents mirrors post-translational regulation of enzyme activity. *Biochim Biophys Acta* 1337:175-90.

**Tiedge M., Steffeck H., Elsner M. & Lenzen S. (1999).** Metabolic regulation, activity state, and intracellular binding of glucokinase in insulin-secreting cells. *Diabetes* 48:514-23.
**Van Schaftingen E. (1989).** A protein from rat liver confers to glucokinase the property of being antagonistically regulated by fructose 6-phosphate and fructose 1-phosphate. *Eur J Biochem* 179:179-84.
**Vandercammen A. & Van Schaftingen E. (1990).** The mechanism by which rat liver glucokinase is inhibited by the regulatory protein. *Eur J Biochem* 191:483-9.
**Watanabe F., Sakai A., Furuya E. & Uyeda K. (1994).** Molecular cloning and tissue specific expression of fructose 6-phosphate,2-kinase:fructose 2,6-bisphosphatase of rat brain. *Biochem Biophys Res Commun* 198:335-40.
**Wu C., Okar D.A., Stoeckman A.K., Peng L.J., Herrera A.H., Herrera J.E., Towle H.C. & Lange A.J. (2004).** A potential role for fructose-2,6-bisphosphate in the stimulation of hepatic glucokinase gene expression. *Endocrinology* 145:650-8.
**Xu L.Z., Harrison R.W., Weber I.T. & Pilkis S.J. (1995).** Human beta-cell glucokinase. Dual role of Ser-151 in catalysis and hexose affinity. *J Biol Chem* 270:9939-46.
**Xu L.Z., Weber I.T., Harrison R.W., Gidh-Jain M. & Pilkis S.J. (1995).** Sugar specificity of human beta-cell glucokinase: correlation of molecular models with kinetic measurements. *Biochemistry* 34:6083-92.
**Xu L.Z., Zhang W., Weber I.T., Harrison R.W. & Pilkis S.J. (1994).** Site-directed mutagenesis studies on the determinants of sugar specificity and cooperative behavior of human beta-cell glucokinase. *J Biol Chem* 269:27458-65.
**Zhu Z., Ling S., Yang Q.H. & Li L. (2001).** Involvement of the chicken liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase sequence His444-Arg-Glu-Arg in modulation of the bisphosphatase activity by its kinase domain. *Biochem J* 357:513-20.

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