

Die Rolle des Ubiquitin-Proteasom-Systems als posttranslationaler Regulationsmechanismus der Glucokinase in den Beta-Zellen des Pankreas

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Kurzzusammenfassung

Das Signal für die glucoseinduzierte Insulinsekretion wird im Stoffwechsel der Beta-Zellen des Pankreas generiert. Hierbei ist die Glucokinase von besonderer Bedeutung, da sie als Glucosensorenzym die extrazelluläre Glucosekonzentration an den intrazellulären Metabolismus koppelt. Die Glucokinaseaktivität wird hauptsächlich durch posttranslationale Mechanismen reguliert. Neben dem endogenen Aktivator, dem bifunktionellen Enzym 6-Phosphofructo-2-kinase/Fruktose-2,6-bisphosphatase, führen auch synthetische Glucokinase-aktivatoren, die als Therapeutika des Typ 2 Diabetes eingesetzt werden sollen, zu einer Steigerung der Enzymaktivität. In der vorliegenden Arbeit konnte ein additiver Effekt des endogenen Aktivators und des chemischen Aktivators LY2121260 auf die glucosestimulierte Insulinsekretion gezeigt werden. Dieser Befund machte deutlich, dass das Wissen um die posttranslationale Glucokinase-regulation wesentlich ist, um neue effiziente Therapieformen für den Typ 2 Diabetes zu entwickeln. Im Rahmen dieser Dissertation konnte das Ubiquitin-Proteasom-System als neuer Regulationsmechanismus der Glucokinase-stabilität und -aktivität identifiziert werden. Eine Hemmung des proteasomalen Abbaus in Beta-Zellen resultierte in einer reduzierten Glucokinaseaktivität und einem vollständigen Verlust der Insulinsekretion, bedingt durch die Akkumulation von fehlgefalteter, inaktiver Glucokinase. Diese führte letztlich zur Anreicherung des Enzyms in Aggresomen. Anhand von fluoreszenzmarkierter Glucokinase konnte ein Mikrotubuli-assoziiierter Transport solcher Aggresome in Beta-Zellen des Pankreas gezeigt werden. Weiterführend konnte nicht nur die direkte Interaktion der Glucokinase mit Ubiquitin, sondern auch mit der E3-Ligase Parkin und mit der Ubiquitin-ähnlichen Domäne des Midnolins nachgewiesen werden. Die Bindung der Ubiquitin-ähnlichen Domäne des Midnolins an die Glucokinase war bei niedriger Glucosekonzentration am stärksten. Die Überexpression dieser Domäne resultierte in einer Inhibierung der Glucokinaseaktivität sowie der glucoseinduzierten Insulinsekretion. Midnolin und Parkin zeigten in Beta-Zellen die höchste Transkriptionsrate bei niedriger Glucosekonzentration. Allerdings zeigte Midnolin im Vergleich zu Parkin ein deutlich höheres Expressionsniveau. Abschließend konnte die Kollokalisierung zwischen Midnolin und der Glucokinase im Zytoplasma von Beta-Zellen des Pankreas gezeigt werden. In Zusammenhang mit der kürzlich ermittelten Reduktion der proteasomalen Aktivität in Beta-Zellen des Pankreas bei Patienten mit Typ 2 Diabetes eröffnen die Ergebnisse dieser Dissertation neue Ansatzpunkte für das Verständnis der Pathogenese der Erkrankung und ihrer Therapie.

Schlagwörter: Diabetes mellitus, Glucokinase, Midnolin, Ubiquitin-Proteasom-System

Abstract

The signal for glucose-induced insulin secretion is generated in the metabolism of the pancreatic beta cells. In this context glucokinase is of special importance, because the enzyme couples the extracellular glucose concentration to the intracellular metabolism. Glucokinase activity is mainly regulated by posttranslational mechanisms. Besides the endogenous activator, the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, synthetic small molecule glucokinase activators, suited as therapeutics for type 2 diabetes, also lead to an increase of glucokinase activity. In the present work an additive effect of the endogenous activator and the chemical activator LY2121260 upon glucose-induced insulin secretion could be shown. This result demonstrates that the knowledge about posttranslational glucokinase regulation is important for the development of efficient type 2 diabetes therapy forms. In this dissertation the ubiquitin proteasome system could be identified as a new regulatory mechanism of glucokinase stability and activity. Inhibition of the proteasomal degradation in beta cells resulted in decreased glucokinase activity and a total loss of glucose-induced insulin secretion due to accumulation of missfolded, inactive glucokinase. Finally, it led to the enrichment of the enzyme in aggresomes. By using fluorescence-tagged glucokinase a microtubule-assisted transport of such aggresomes could be shown in pancreatic beta cells. In further studies not only the direct interaction of glucokinase with ubiquitin, but also with the E3 ligase parkin and the ubiquitin-like domain of midnolin could be demonstrated. Binding of the ubiquitin-like domain of midnolin to glucokinase was strongest at low glucose concentrations. Overexpression of this domain resulted in the inhibition of glucokinase activity as well as of glucose-induced insulin secretion. In beta cells midnolin and parkin showed highest transcription rates at low glucose concentration. However, midnolin revealed a significantly higher expression level than parkin. Finally, the colocalization of midnolin and glucokinase was shown in pancreatic beta cells. Relating to the recently determined reduction of proteasomal activity in pancreatic beta cells of patients with type 2 diabetes, the results of this dissertation pave the way to new approaches for understanding of the pathogenesis of this disease and its therapy.

Keywords: diabetes mellitus, glucokinase, midnolin, ubiquitin proteasome system

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1 Einleitung

1.1 Glucosehomöostase und Diabetes mellitus

Der Diabetes mellitus ist eine der häufigsten Stoffwechselerkrankungen. Zurzeit sind weltweit etwa 346 Millionen Menschen an Diabetes mellitus erkrankt (Sherwin & Jastreboff 2012). Die Zahl der Diabeteserkrankungen nimmt aufgrund der veränderten Lebensumstände, Bewegungsmangel in Kombination mit ungesunder Ernährung, stetig zu und soll bis 2030 auf 552 Millionen ansteigen (Whiting *et al.* 2011). Betroffen sind hiervon nicht nur Erwachsene, sondern auch immer häufiger Kinder und Jugendliche (Sherwin & Jastreboff 2012). Die Grundlage des Diabetes ist eine Hyperglykämie, der eine gestörte Insulinsekretion und/oder Insulinwirkung zu Grunde liegt (Kerner & Brückel 2011). Nach Angaben der WHO definiert sich eine Hyperglykämie über eine Blutzuckerkonzentration von >7 mmol/l (126 mg/dl) im Nüchternzustand, bzw. >11 mmol/l (200 mg/dl) 2 h nach oraler Gabe von 75 g Glucose. Unterteilt werden kann der Diabetes mellitus in zwei Hauptformen. Der Typ 1 Diabetes mellitus ist durch einen absoluten Insulinmangel gekennzeichnet, der durch eine autoimmune Destruktion der Beta-Zellen des Pankreas hervorgerufen wird. Beim Typ 2 Diabetes mellitus liegt hingegen ein relativer Insulinmangel und/oder eine Insulinresistenz der Zielorgane vor. Die Zahl der an Typ 2 Diabetes mellitus erkrankten Patienten macht in den westlichen Industriestaaten 80 - 90% aus, während nur 10 - 20% der Betroffenen einen Typ 1 Diabetes mellitus aufweisen (Waldhäusl & Lenzen 2007). Bei gesunden Menschen wird die Blutglucosekonzentration in einem engen Bereich von 60 – 110 mg/dl konstant gehalten (Waldhäusl & Lenzen 2007). Dies wird durch die hormonelle Regulation der anabolen und katabolen Stoffwechselwege ermöglicht. Das von den Alpha-Zellen des Pankreas sekretierte Hormon Glucagon verstärkt zusammen mit Adrenalin und Cortisol den katabolen Stoffwechsel und sorgt somit für einen Anstieg der Blutglucosekonzentration. Das Peptidhormon Insulin hingegen wird bei einem Anstieg der Blutglucosekonzentration von den Beta-Zellen der Langerhans'schen Inseln des Pankreas sezerniert. In peripheren Geweben steigert Insulin die Glucoseaufnahme, ist verantwortlich für die Speicherung von Nährstoffen, stimuliert anabole und inhibiert katabole Stoffwechselwege. Hauptwirkungsorte sind hierbei Leber, Muskulatur und Fettgewebe. In Leber und Muskulatur erhöht Insulin die Glykogensynthese, im Fettgewebe und in der Leber wird außerdem die Glykolyse und Fettsäuresynthese angeregt. Des Weiteren vermittelt Insulin eine verstärkte Glucoseaufnahme in der Muskulatur und dem

Fettgewebe indem es durch eine rezeptorvermittelte Signalkaskade die Translokation des GLUT4-Transporters an die Plasmamembran erhöht (Leto & Saltiel 2012).

1.2 Glucoseinduzierte Insulinsekretion

Durch seine anabole und antikatabole Wirkung ist Insulin für die Blutglucoseregulation essentiell. Insulin besteht aus 51 Aminosäuren und wird in den Beta-Zellen des Pankreas synthetisiert und von diesen sezerniert. Das reife Insulin besteht aus zwei Peptidketten, die durch Disulfidbrücken miteinander verknüpft sind. Im Rahmen der Insulinbiosynthese wird jedoch zunächst ein einsträngiges Vorläufermolekül, das Präproinsulin, aus der codierenden mRNA am ER translatiert. Infolge der weiteren Prozessierung entsteht nach Abspaltung des Signalpeptids und Ausbildung von Disulfidbrücken das Proinsulin. Dieses wird im Golgi-Apparat in Vesikel verpackt, wo es durch Abspaltung des mittelständigen C-Peptids zum reifen Insulin prozessiert wird.

Der bedeutende Stimulus für die Insulinsekretion ist die Glucose. Ein Anstieg der Blutglucosekonzentration sorgt für eine kontrollierte Freisetzung des Insulins aus den Beta-Zellen der Langerhans'schen Inseln. Diese Kontrolle resultiert aus der Stimulus-Sekretionskopplung, die auf dem Metabolismus der Beta-Zelle beruht (Lenzen & Panten 1988; Lenzen 1990; Lenzen 1992; Lenzen & Tiedge 1994; Matschinsky 2002). Die Aufnahme der Glucose erfolgt sowohl in den Beta-Zellen als auch den Hepatozyten durch erleichterte Diffusion über den niedrigaffinen Glucosetransporter GLUT2 (Orci *et al.* 1989; Bell *et al.* 1990; Kayano *et al.* 1990; Lenzen & Tiedge 1994). Die Glucosekonzentration in der Beta-Zelle reflektiert so die Blutglucosekonzentration, wodurch eine schnelle Reaktion auf Veränderungen möglich ist (Lenzen & Panten 1988). Der erste und auch geschwindigkeitsbestimmende Schritt ist die Phosphorylierung der Glucose zu Glucose-6-phosphat, welcher durch die Glucokinase katalysiert wird (Matschinsky & Ellerman 1968; Iynedjian 1993; Efrat *et al.* 1994; Matschinsky 2002). Der folgende Glucoseabbau über Glykolyse, Citratzyklus und mitochondriale Atmungskette sorgt für einen Anstieg des ATP/ADP-Quotienten. In den Beta-Zellen resultiert dieser Anstieg in der Schließung des ATP-sensitiven Kaliumkanals (K_{ATP}), der sich aus dem Kanalprotein KIR6.2 und dem Sulfonylharnstoffrezeptor SUR1 zusammensetzt (Panten *et al.* 1996; Aguilar-Bryan *et al.* 1998). Die Schließung des K_{ATP} -Kanals führt zu einer Depolarisation der Zellmembran, welche die Öffnung eines spannungsabhängigen Calciumkanals (VDCC) initiiert. Ausgelöst durch den Ca^{2+} Einstrom in die Zelle kommt es zur Exocytose der Insulingranula (Abb. 1.1).

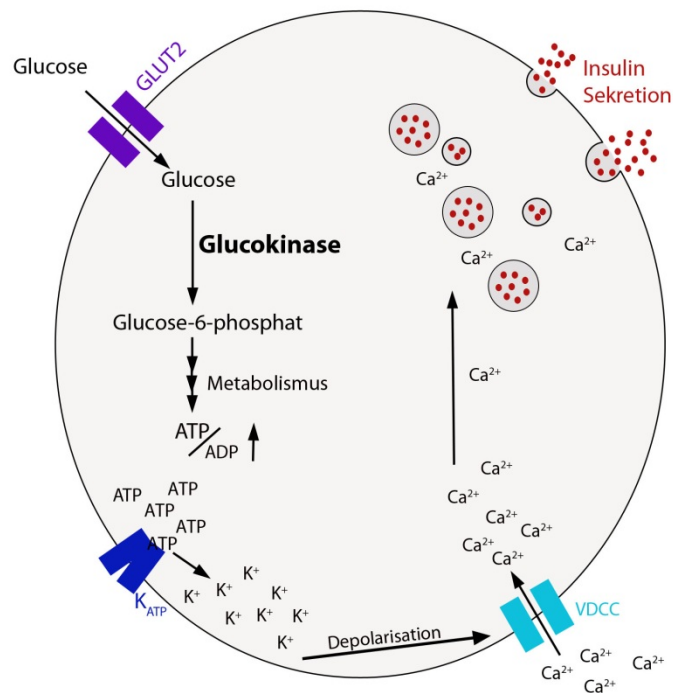


Abb. 1.1 Schematische Darstellung der glucoseinduzierten Insulinsekretion in der Beta-Zelle des Pankreas.

1.3 Das Glucosensorenzym Glucokinase

Das Schlüsselenzym der Stimulus-Sekretionskopplung stellt die Glucokinase dar, welche in den Beta-Zellen des Pankreas die Rolle des Glucosensors übernimmt (Matschinsky *et al.* 1993). Die Glucokinase wird vorwiegend in der Leber und im Pankreas (Lenzen & Panten 1988) exprimiert. Zusätzlich konnte eine, wenn auch geringere, Expression in den neuroendokrinen Zellen des Gehirns und Darms nachgewiesen werden (Jetton *et al.* 1994). Die Glucokinase (Hexokinase IV, EC 2.7.1.2) gehört zur Gruppe der Hexokinasen, unterscheidet sich aber in vielerlei Hinsicht von den Hexokinasen I-III.

Die Hexokinasen, welche als Monomer vorliegen, katalysieren die Übertragung einer Phosphatgruppe von ATP auf die 6-Hydroxylgruppe der Glucose. Die Hexokinasen I-III weisen ein Molekulargewicht von 100 kDa auf und sind durch einen niedrigen $S_{0,5}$ -Wert (20 – 130 $\mu\text{mol/l}$) gekennzeichnet. Durch diese hohe Glucoseaffinität weisen sie bereits bei niedriger Glucosekonzentration maximale Aktivität auf. Zusätzlich zeigen die Hexokinasen I-III eine Feedback-Hemmung durch ihr Produkt Glucose-6-phosphat im physiologischen Bereich (Middleton 1990; Printz *et al.* 1993b).

Die Glucokinase hingegen hat ein Molekulargewicht von etwa 52 kDa, zeigt eine deutlich geringere Glucoseaffinität mit einem $S_{0,5}$ -Wert von 10 mmol/l und wird nicht durch

Glucose-6-phosphat inhibiert (Lenzen 1992; Matschinsky *et al.* 1993; Printz *et al.* 1993a; Printz *et al.* 1993b). Obwohl die Glucokinase als Monomer vorliegt, zeigt das Enzym eine kooperative Reaktionskinetik mit einem Hill-Koeffizienten von 1,5 – 1,7 für das physiologische Substrat D-Glucose (Cardenas *et al.* 1978; Lenzen 1992; Matschinsky *et al.* 1993; Printz *et al.* 1993a; Printz *et al.* 1993b). Der Umkehrpunkt des sigmoidalen Kurvenverlaufs liegt bei 5 mmol/l und somit im Bereich der physiologischen Blutglucosekonzentration (Matschinsky *et al.* 1993). Ein Anstieg der Glucosekonzentration in diesem Bereich resultiert in einer effizienten Zunahme der Glucosephosphorylierung und schließlich in einer schnellen Insulinsekretion. Aufgrund dieser Eigenschaften ist die Glucokinase für die Funktion als Glucosesensor in den pankreatischen Beta-Zellen prädestiniert.

1.3.1 Die Glucokinasegenstruktur

Die Aminosäuresequenz der Glucokinase ist in der Leber und den Beta-Zellen des Pankreas weitgehend identisch, unterscheidet sich jedoch in 11 der 15 N-terminalen Aminosäuren (Andreone *et al.* 1989; Magnuson & Shelton 1989). Die gewebsspezifische Expression der Glucokinase-mRNA erfolgt sowohl in der Leber als auch in den Beta-Zellen des Pankreas ausgehend von einem Glucokinasegen, jedoch mit unterschiedlichen Promotoren (Magnuson *et al.* 1989). Das Gen besteht aus 10 Exons, wobei die Transkription der Exons 2 – 10 in beiden Geweben identisch ist. Die Kontrollregionen liegen mindestens 12 kb auseinander und umfassen sowohl das erste Exon als auch die Promotoren, mit einer Beta-Zell-Kontrollregion stromaufwärts und einer Leberzell-Kontrollregion stromabwärts gelegen. Dieses resultiert in einer Transkriptionseinheit von über 27,5 kb für die Beta-Zell Glucokinase und 15,5 kb für die Leber-Isoform (Magnuson & Shelton 1989; Magnuson 1990). In der Leber wird die Glucokinasetranskription durch Insulin stimuliert und durch Glucagon gehemmt (Iynedjian *et al.* 1988; Iynedjian 2009). In den Beta-Zellen des Pankreas hingegen wird die Glucokinaseexpression durch Glucose stimuliert (Tiedge & Lenzen 1991; Tiedge & Lenzen 1995). Eine Fastenperiode von 48 h reduzierte die Glucokinase-mRNA in Beta-Zellen der Ratte um 53%, während die Expression in der Leber nicht mehr detektierbar war. Dieses deutet darauf hin, dass der Beta-Zell-Promotor des Glucokinasegens relativ insensitive bezüglich kurzfristiger regulatorischer Einflüsse ist (Lenzen & Tiedge 1994).

1.3.2 Die Glucokinaseproteinstruktur

Sowohl die Leber-Isoform als auch die Beta-Zell-Isoform der Glucokinase umfasst 465 Aminosäuren, die sich lediglich in 11 der 15 N-terminalen Aminosäuren unterscheiden (Andreone *et al.* 1989; Magnuson & Shelton 1989). Das Expressionsniveau der Glucokinase beträgt in der Leber 1:1000 und im endokrinen Pankreas nur 1:20000 bezogen auf die Gesamtmasse aller löslichen Proteine (Iynedjian *et al.* 1986). Die Struktur der Glucokinase wurde zunächst auf Basis der Hefe Hexokinase B erstellt, welche eine 33%ige Homologie zur humanen Glucokinase-Isoform besitzt (Printz *et al.* 1993b; St. Charles *et al.* 1994). Die Glucokinase weist auch eine 49,9%ige Homologie zum C-terminalen Teil der Ratten-Hexokinase I und eine 55%ige Homologie zum C-terminalen Teil der Ratten-Hexokinase II auf (Printz *et al.* 1993b). Durch diese Homologien wurden erste Hinweise auf den katalytischen Mechanismus geliefert. Erst im Jahre 2004 gelang es, die Kristallstruktur der humanen Leber-Glucokinase anhand zweier N-deletierter Formen röntgenkristallographisch zu untersuchen (Kamata *et al.* 2004). Mit dieser Analyse konnten erste wichtige Aussagen über die Aktivität und die damit einhergehenden Konformationsänderungen der Glucokinase gemacht werden. Wie bereits für die Hexokinasestruktur beschrieben, unterteilt sich die Glucokinase in zwei Domänen (Anderson *et al.* 1978; St. Charles *et al.* 1994; Kamata *et al.* 2004). Diese beiden Domänen sind durch einen Spalt getrennt, in dem sich das aktive Zentrum befindet (Anderson *et al.* 1978). Die Glucosebindungsstelle wird von Aminosäuren der großen und kleinen Domäne gebildet. Hierbei sind Glu256 und Glu290 der großen Domäne sowie Thr168 und Lys169 der kleinen Domäne wesentlich. Zusätzlich sind Asn204 und Asp205 der Verbindungsregion II an der Ausbildung der Bindungsstelle beteiligt (Kamata *et al.* 2004). Diese Analyse bestätigte die bereits postulierte Beteiligung der Aminosäuren Asn204, Asp205, Glu256 und Glu290 (St. Charles *et al.* 1994). Anhand von Punktmutationen der Aminosäuren Asn204, Glu256 und Glu290 konnte eine Beteiligung dieser Reste an der Bindung der Glucose direkt nachgewiesen werden. Diese Mutationen resultierten in einem Verlust des sigmoidalen Verhaltens der Glucokinase (Xu *et al.* 1994). Etwa 20 Å entfernt von der Glucosebindungsstelle befindet sich das allosterische Zentrum, welches die Aktivator-Bindungsstelle darstellt. Das allosterische Zentrum wird umschlossen von der Verbindungsregion I, dem β 1-Faltblatt und der α 5-Helix der großen Domäne und der α 13-Helix der kleinen Domäne (Kamata *et al.* 2004). Eine schematische Darstellung der Glucokinase nach Bindung von Glucose, ATP und eines Aktivators ist in Abb. 1.2 dargestellt (Kamata *et al.* 2004).

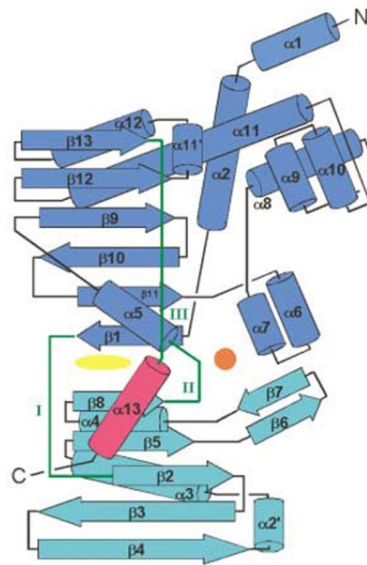


Abb. 1.2 Schematische Darstellung der Sekundärstruktur der Glucokinase mit gebundener Glucose und einem synthetischen allosterischen Aktivator. Die Struktur der Glucokinase wird unterteilt in die große Domäne (dunkelblau) und kleine Domäne (cyan und magenta), die durch die Verbindungsregionen I-III (grün) verbunden sind. Im Interdomänenraum befindet sich die Glucosebindungsstelle (oranger Kreis) sowie 20 Å entfernt das allosterische Bindungszentrum (gelbes Oval) (Kamata *et al.* 2004).

Wie bereits für die Hexokinasen der Hefe und der Ratte beschrieben (Bennett & Steitz 1978; Aleshin *et al.* 1998a; Aleshin *et al.* 1998b), wurde auch für die Glucokinase postuliert, dass es zwei Konformationen mit unterschiedlicher Glucosebindungsaffinität geben muss (Lin & Neet 1990; Neet *et al.* 1990). Tatsächlich konnte die Glucokinase in zwei verschiedenen Formen kristallographisch dargestellt werden, die, durch eine von der Glucosebindung ausgelöste Konformationsänderung, ineinander übergehen können (Kamata *et al.* 2004). Die Wechselwirkung der Hydroxylgruppen der Glucose mit den Aminosäuren des aktiven Zentrums induziert eine Konformationsänderung, bei der die Glucokinase in die aktive Form (*closed form*) übergeht. Obwohl die aktive Form der Glucokinase nahezu identisch mit der der Hexokinase I ist, konnte anhand der beiden Kristallstrukturen der Glucokinase gezeigt werden, dass die Glucokinase weitaus größere Konformationsänderungen durchläuft, um in die inaktive Form überzugehen (Kamata *et al.* 2004). Diese wurde daher als weit-offene Form (*super-open form*) eingeführt, welche die inaktive Form der Glucokinase beschreibt. Die Unterschiede dieser beiden Grenzkonformationen sind in Abb. 1.3 dargestellt. In der weit-offenen Form entspricht die Struktur der großen Domäne weitgehend der der geschlossenen Form, wohingegen die Anordnung der kleinen Domäne erhebliche Unterschiede aufweist. Zur Verdeutlichung der Konformationsänderung wurde die kleine Domäne in drei Ebenen unterteilt. Die innere Ebene wird durch die $\alpha 13$ -Helix gebildet. Diese formt zusammen mit der $\alpha 5$ -Helix der großen Domäne den Grenzübergang zwischen den beiden Domänen. Die mittlere Ebene umfasst die β -Faltblätter, die äußere Ebene

beinhaltet die drei α -Helices $\alpha 2'$, $\alpha 3'$ und $\alpha 4'$. In der weit-offenen Form sind die mittlere und äußere Ebene als feste Einheit um 99° gedreht. Die innere Ebene rotiert hingegen um 70° in eine andere Richtung. Dadurch ergibt sich, dass die $\alpha 13$ -Helix aus der kleinen Domäne freigegeben wird. Im Laufe der Konformationsänderung kommt es so zum Bruch und zur Neubildung von Wasserstoffbrückenbindungen innerhalb der kleinen Domäne (Kamata *et al.* 2004). Diese Rotationen fallen im Vergleich zur Hexokinase weitaus größer aus, bedingt vor allem durch die Flexibilität der Verbindungsregion I. Die Aminosäureregion Ser64 bis Glu72 stellt eine höchst flexible Einheit dar (Kamata *et al.* 2004). Im Gegensatz dazu ist die entsprechende Sequenz der Hexokinase eine starre Einheit, die zusammen mit den Verbindungsregionen II-III eine Art Gelenk bildet, wodurch die Konformationsänderung der kleinen Domäne nur 12° beträgt (Aleshin *et al.* 1998b; Kamata *et al.* 2004).

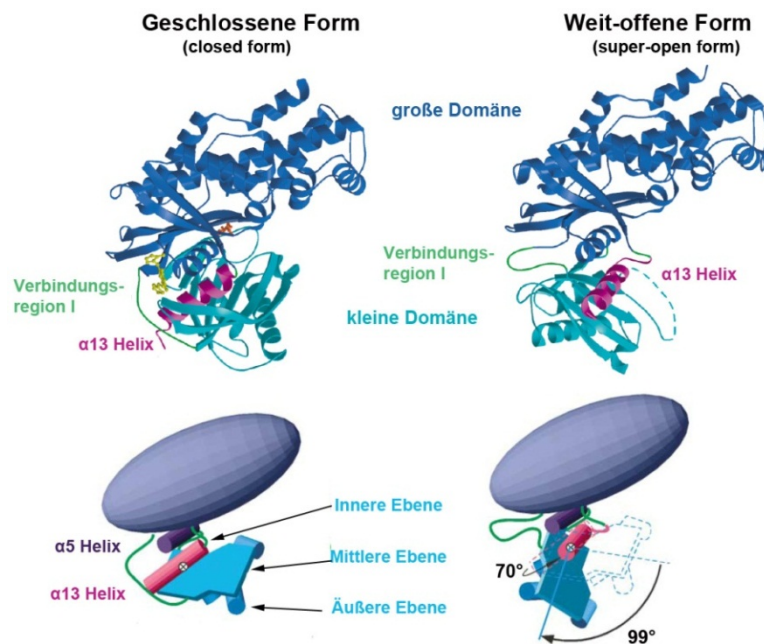


Abb. 1.3 Strukturmodell der Glucokinase in den Grenzkonformationen. Bändermodell und schematische Darstellung der Glucokinase in der geschlossenen (*closed*) (linke Seite) und weit-offenen (*super-open*) (rechte Seite) Form der Glucokinase. Dargestellt ist die Konformationsänderung der kleinen Domäne bei Übergang von der geschlossenen zu der weit-offenen Konformation. Modifiziert nach (Kamata *et al.* 2004).

Anhand der ermittelten Konformationen und den daraus abgeleiteten Strukturdaten entwickelte Kamata *et al.* (2004) ein kinetisches Modell, welches das kooperative Verhalten des monomeren Enzyms grundlegend beschreibt. In diesem Zusammenhang wurde eine weitere Konformation, die offene Form (*open form*), postuliert, welche einer aktiven Zwischenstufe entspricht. In dem Modell wird von zwei Zyklen der Katalyse ausgegangen. Der schnelle Katalysezyklus beschreibt den Übergang zwischen der aktiven geschlossenen

und offenen Form und erlaubt somit eine schnelle Umsetzung der Glucose. Der Übergang zwischen der offenen und inaktiven weit-offenen Form hingegen erfolgt langsamer. Sinkt die Glucosekonzentration, so bleibt die Glucokinase zunächst für einige Zeit in der offenen Form bestehen. Erfolgt keine weitere Glucosebindung, so geht die Glucokinase in die thermodynamisch stabilere weit-offene Form über (Kamata *et al.* 2004). In weiteren Versuchen konnte bestätigt werden, dass sich bei geringen Glucosekonzentrationen ein Gleichgewicht zwischen der offenen und weit-offenen Form einstellt, welches zugunsten der weit-offenen Form verschoben ist (Kim *et al.* 2007; Antoine *et al.* 2009). Im Laufe der Jahre wurde das Modell von Kamata *et al.* (2004) immer mehr erweitert, so dass man aktuell von mehreren intermediären Konformationen zwischen den Grenzkonformationen der weit-offenen und geschlossenen Form ausgeht (Zhang *et al.* 2006; Antoine *et al.* 2009; Larion & Miller 2010; Larion *et al.* 2012).

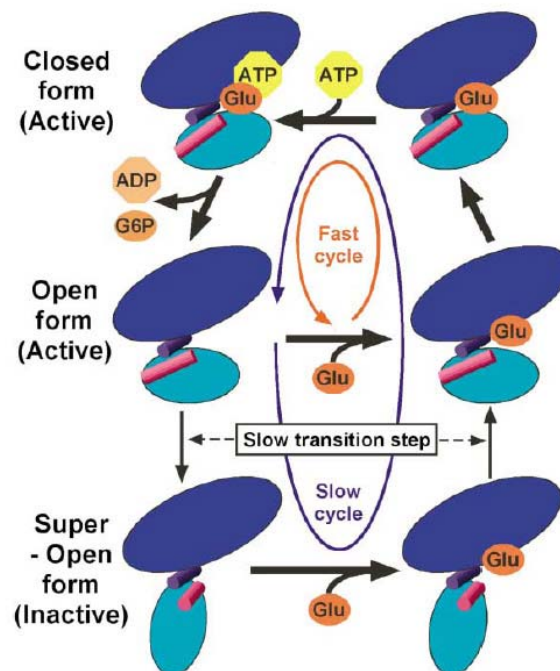


Abb. 1.4 Modelldarstellung der Glucokinasekinetik. In Abwesenheit von Glucose nimmt die Glucokinase die weit-offene (*super-open*) Form ein. Durch Bindung der Glucose geht diese Form beim Durchlaufen des Katalysemechanismus in die offene (*open*) und schließlich in die geschlossene (*closed*) Form über. Glu: Glucose, G6P: Glucose-6-phosphat (Kamata *et al.* 2004).

1.3.3 Exogene Aktivatoren und Inhibitoren der Glucokinase

Wesentlich für die Strukturaufklärung und damit für das kooperative Verhalten der Glucokinase war die Bindung von synthetischen Aktivatoren an die Glucokinase, die zu einer Stabilisierung der aktiven Form (*closed form*) führt (Kamata *et al.* 2004; Antoine *et al.* 2009; Liu *et al.* 2012). Der erste Glucokinaseaktivator wurde 2003 beschrieben (Grimsby *et al.* 2003).

al. 2003). Heute liegt eine von der chemischen Struktur heterogene Substanzklasse vor, die allerdings noch keinen Eingang in die Therapie des Diabetes gefunden hat (Matschinsky 2013). Gemeinsam ist diesen Verbindungen, dass sie an das allosterische Zentrum der Glucokinase binden und zu einer Aktivitätssteigerung führen. Eine Beteiligung der Aminosäuren Val62, Arg63, Met210, Ile221, Tyr214 und Tyr215 konnte für verschiedene Aktivatoren bestätigt werden (Kamata *et al.* 2004; Efanov *et al.* 2005). Zwar sind die synthetischen Glucokinaseaktivatoren in der Lage, die glucosestimulierte Insulinsekretion zu erhöhen und damit die Blutglucosekonzentration zu senken, haben aber auch einen Einfluss auf die Glucoseaffinität. Die Aktivatoren senken den $S_{0,5}$ -Wert der Glucokinase. Aufgrund der erhöhten Glucoseaffinität kommt es zu einem erhöhten Glucoseumsatz bei niedriger Glucosekonzentration mit dem Risiko einer Hypoglykämie (Efanov *et al.* 2005; Liu *et al.* 2012). Aufgrund der strukturellen Veränderungen der Verbindungsregion I sowie der Umlagerung der α 13-Helix können Aktivatoren nicht an die Glucokinase in der weit-offenen Form binden (Kamata *et al.* 2004). Allerdings reichen bereits Glucosekonzentrationen deutlich unterhalb des $S_{0,5}$ -Werts der Glucokinase aus, um die Bindung des Aktivators zu ermöglichen.

Mannoheptulose und der entsprechende Zuckeralkohol Perseitol sind natürliche Bestandteile der Avocadofrucht und können aus dieser isoliert werden (Richtmyer 1970; Kappler-Tanudyaya *et al.* 2007). Beide sind wie die Sedoheptulose C7-Verbindungen. Sedoheptulose wurde erstmals aus *Sedum spectabile* Bor. isoliert (Richtmyer 1970). Es ist in Form von Sedoheptulose-7-phosphat sowohl ein Intermediat des Pentosephosphatweges als auch des Calvin-Zyklus. Mannoheptulose wird von der Glucokinase nicht verstoffwechselt, inhibiert kompetitiv die Enzymaktivität (Tiedge *et al.* 1997a) und reduziert somit die Glucoseoxidation und die Insulinsekretion (Lenzen 1992; Tiedge *et al.* 1997a). Mannoheptulose ist zudem in der Lage, die geschlossene Konformation der Glucokinase zu stabilisieren (Zelent *et al.* 2005; Baltrusch & Lenzen 2007).

1.3.4 Endogene Bindungspartner der Glucokinase

Die Regulation der Glucokinaseaktivität erfolgt hauptsächlich auf posttranslationaler Ebene. Als endogener Aktivator der Glucokinase wurde das bifunktionelle Enzym 6-Phosphofruktose-2-kinase/Fruktose-2,6-bisphosphatase (PFK-2/FBPase-2) mit Hilfe eines *Random Peptide Phage Display Screenings* identifiziert (Baltrusch *et al.* 2001). Die PFK-2/FBPase-2 katalysiert die Synthese und den Abbau von Fructose-2,6-bisphosphat und tritt als Homodimer auf. Für die Dimerisierung ist die Kinasedomäne des Enzyms verantwortlich

(Baltrusch *et al.* 2001; Okar *et al.* 2001). Die Interaktion mit der Glucokinase hingegen wird durch die Bisphosphatasedomäne über das Konsensusmotiv (SL)KVWT vermittelt, wobei aufgrund der entgegengesetzten Lokalisation der Bindungsstellen eine Interaktion mit zwei Glucokinasmolekülen möglich ist (Baltrusch *et al.* 2001). Sowohl in den Hepatozyten als auch in den Beta-Zellen des Pankreas führt diese Interaktion zu einer Steigerung der intrinsischen Aktivität der Glucokinase und zu einer verbesserten glucoseinduzierten Insulinsekretion in den Beta-Zellen. Im Gegensatz zu den synthetischen Aktivatoren wird der $S_{0,5}$ -Wert durch die Interaktion mit der PFK-2/FBPase-2 nicht verändert, wodurch das kooperative Verhalten der Glucokinase erhalten bleibt (Massa *et al.* 2004; Baltrusch *et al.* 2006). Anhand von FRET-Analysen und mit Hilfe eines fluoreszenzbasierten *Mammalian Two Hybrid Systems* konnte gezeigt werden, dass die Bindung der PFK-2/FBPase-2 an die Glucokinase glucoseabhängig erfolgt und eine Interaktion nur bei hohen Glucosekonzentrationen stattfindet (Langer *et al.* 2010).

In den Hepatozyten wird die Glucokinaseaktivität primär von dem nur dort exprimierten Glucokinase Regulatorprotein (GRP) beeinflusst. Das 1989 nachgewiesene Protein fungiert als kompetitiver Inhibitor der Glucokinase und bindet diese in der weit-offenen Konformation (Van Schaftingen 1989; Futamura *et al.* 2006). Dabei sorgt es bei niedrigen Glucosekonzentrationen für eine Translokation der Glucokinase in den Zellkern (Shiota *et al.* 1999). Die Bindung des GRPs an die Glucokinase und damit ihre Hemmung wird durch Fructose-6-phosphat verstärkt. Fructose-1-phosphat sowie Glucose hingegen fördern die Dissoziation des Komplexes (Van Schaftingen 1989; Vandercammen & Van Schaftingen 1990; Agius & Stubbs 2000).

Während aufgrund der Translokation die Glucokinase in Hepatozyten sowohl im Zytoplasma als auch im Nukleus lokalisiert ist, wurde in den Beta-Zellen ausschließlich eine zytoplasmatische Lokalisation festgestellt. In Permeabilisationsexperimenten konnte jedoch eine diffuse Glucokinasefraktion mit hoher Enzymaktivität von einer intrazellulär gebundenen Fraktion mit geringer Aktivität unterschieden werden (Tiedge *et al.* 1999; Stubbs *et al.* 2000). Zum einen wurde eine Bindung der Glucokinase an α -Tubulin beschrieben (Baltrusch & Lenzen 2007), zum anderen wurde bei niedriger Glucosekonzentration die Bindung an sekretorische Insulingranula dargestellt (Toyoda *et al.* 1999; Rizzo *et al.* 2002). Die Bindung an Insulingranula wird durch die NO-Synthase kontrolliert und durch S-Nitrosylierung der Glucokinase aufgehoben (Rizzo & Piston 2003; Markwardt *et al.* 2012). Die Assoziation der Glucokinase an die Mitochondrien wurde in

einem Multiproteinkomplex mit BAD beschrieben (Danial *et al.* 2003), die direkte Interaktion bislang aber nicht nachgewiesen. Weiterhin wurde die Interaktion der Glucokinase mit Ubiquitin bzw. Polyubiquitin postuliert (Bjorkhaug *et al.* 2007). In einem *in vitro* System konnte die Ubiquitinierung der Glucokinase gezeigt und durch Aktivitätsmessungen mit rekombinantem Protein eine Aktivitätssteigerung mit Polyubiquitin und in geringerem Maße Monoubiquitin beschrieben werden (Bjorkhaug *et al.* 2007).

1.4 Das Ubiquitin-Proteasom-System

Der kontrollierte Proteinumsatz spielt in der Zelle eine entscheidende Rolle. Zum einen haben Proteine eine spezifische Lebenszeit, zum anderen müssen solche, die zum Beispiel durch Fehlfaltung oder Mutation funktionslos sind, aus der Zelle entfernt werden, um die Bildung toxischer Aggregate zu verhindern. Zusätzlich werden als eine wichtige Form der zellulären Regulation Schlüsselenzyme aus Signalkaskaden und Stoffwechselwegen schnell abgebaut (Rock & Goldberg 1999). Hierbei spielt das Ubiquitin-Proteasom-System, welches sowohl im Zytosol als auch im Nukleus lokalisiert ist, eine entscheidende Rolle. Im Jahre 2004 haben die Wissenschaftler Aaron Ciechanover, Avram Hershko und Irwin Rose den Nobelpreis für die Entdeckung und Untersuchung des Ubiquitin-gesteuerten Proteinabbaus erhalten. Ubiquitin wurde 1974 zunächst als „*ubiquitous immunopoietic polypeptide*“ isoliert, sollte eine Funktion in der B- und T-Zell-Differenzierung haben und ubiquitär exprimiert sein (Goldstein 1974; Goldstein *et al.* 1975). Der Name wurde später auf Ubiquitin reduziert. Eine rein immunologische Funktion konnte schnell widerlegt werden (Schlesinger & Goldstein 1975; Low & Goldstein 1979). Ubiquitin nimmt eine globuläre, kompakte Form ein, die durch fünf β -Faltblätter, eine α -Helix und einen freien C-Terminus charakterisiert ist (Vijay-Kumar *et al.* 1987; Walters *et al.* 2004; Grabbe & Dikic 2009). Durch einen am C-Terminus befindlichen Glycin-Rest kann Ubiquitin kovalent an Proteine gebunden werden.

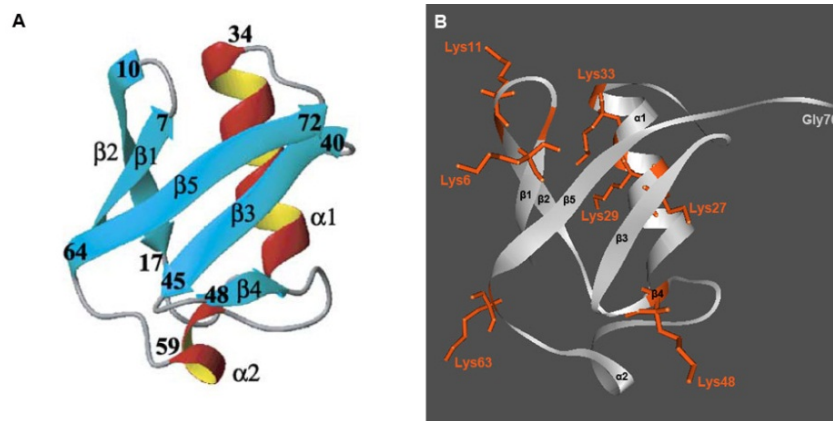


Abb. 1.5 Tertiärstruktur des Ubiquitins. (A) Bändermodell der Ubiquitin-Superfaltung (Walters *et al.* 2004). (B) Ubiquitin enthält sieben Lysinreste (rot), die zur Ausbildung von Polyubiquitinketten befähigt sind. Die Struktur wurde mit dem 3D Molecule Viewer auf Basis des Proteindatenbank-Eintrags pdb.1G6J erstellt.

Die bekannteste Funktion des Ubiquitins ist die Markierung von Proteinen zur proteasomalen Degradation. Dieser Weg umfasst drei enzymatisch katalysierte Schritte (Hershko & Ciechanover 1992; Ciechanover 1994). Zunächst erfolgt die ATP-abhängige Aktivierung des C-terminalen Glycin-Restes durch das Ubiquitin-aktivierende Enzym E1. Hierbei entsteht unter Freisetzung von AMP und Pyrophosphat eine energiereiche Thioesterbindung zwischen dem Glycin-Rest des Ubiquitins und einem Cystein-Rest des E1. Im nächsten Schritt wird das aktivierte Ubiquitin auf einen Cystein-Rest des Ubiquitin-übertragenden Proteins E2 transferiert. Der letzte Schritt wird durch eine Ubiquitin-Ligase E3 katalysiert. Hierbei erfolgt die Bindung des Ubiquitins über seinen Glycin-Rest an die ϵ -Aminogruppe eines Lysin-Restes im Zielprotein (Hershko & Ciechanover 1998). Für den proteasomalen Abbau bedarf es einer Polyubiquitinierung von mindestens vier Ubiquitin-Resten. Durch den wiederholten Ablauf der Ubiquitinierung können weitere Ubiquitinmoleküle auf einen internen Lysin-Rest des bereits gebundenen Ubiquitins übertragen werden. Für eine effiziente Ausbildung von Polyubiquitinketten scheint zusätzlich ein weiterer Faktor (E4) benötigt zu werden, der möglicherweise eine Rolle bei der Auswahl der Ubiquitin-internen Lysin-Reste spielt (Hoppe 2005). Innerhalb des Ubiquitin-Systems gibt es eine hierarchische Struktur (Glickman & Ciechanover 2002). Alle Ubiquitinierungsprozesse werden von einem einzigen E1 eingeleitet, welches das Ubiquitin auf alle E2 Proteine überträgt. Die Spezifität des Systems wird hauptsächlich durch die Vielzahl der E3-Ligasen garantiert (Hershko & Ciechanover 1998). Diese können in zwei Gruppen unterteilt werden, die sich vor allem in der Art der Ubiquitinübertragung unterscheiden. Bei den HECT- (*homologous to E6-AP carboxyl terminus*) Domänen E3s wird das Ubiquitin von E2 zunächst auf einen Cystein-Rest im E3 übertragen, bevor es von dort aus an das Substrat gebunden wird. Die RING- (*really interesting new gene*) Domänen

E3s hingegen vermitteln direkt die Übertragung des Ubiquitins auf das Substrat, ohne vorherige Bindung des Ubiquitins. Der Ablauf der Ubiquitinierung ist in Abb. 1.6 dargestellt.

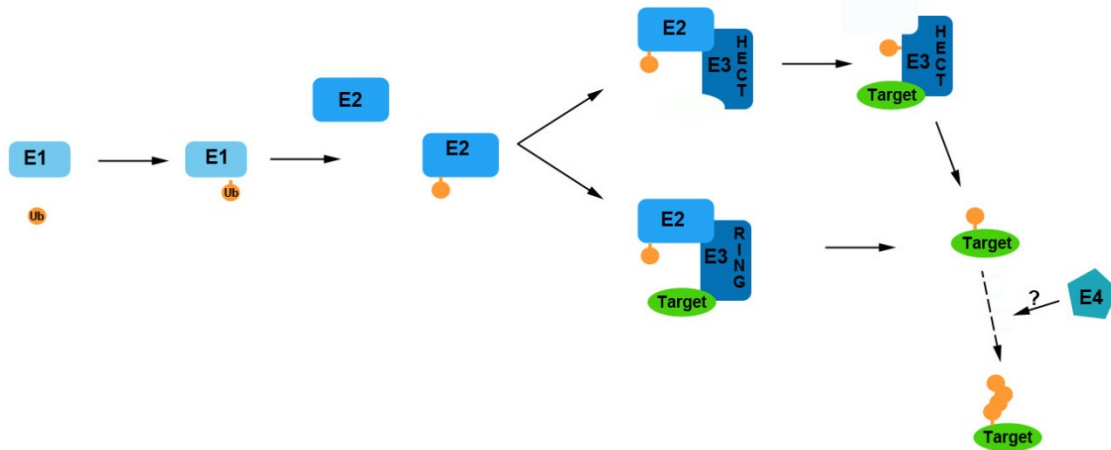


Abb. 1.6 Mechanismus der Ubiquitinierung. Im ersten Schritt erfolgt die ATP-abhängige Aktivierung des Ubiquitins (Ub) durch E1. Anschließend wird das Ubiquitin auf E2 übertragen. Die Ubiquitinierung des Targetproteins erfolgt schließlich mit Hilfe einer Protein-Ligase E3 (HECT- oder RING-Finger E3-Ligase). Für die Polyubiquitinierung wird eventuell ein weiterer Faktor E4 benötigt.

Wie bereits erwähnt ist für den proteasomalen Abbau eine Polyubiquitinierung mit mindestens vier Ubiquitinmolekülen notwendig, wobei die Verknüpfung der weiteren Ubiquitine über das interne Lys48 erfolgt. Neben Lys48 besitzt Ubiquitin noch sechs weitere Lysin-Reste (Lys6, Lys11, Lys27, Lys29, Lys33 und Lys63), die wahrscheinlich alle zur Ausbildung von Isopeptidbindungen genutzt werden können (Peng *et al.* 2003; Herrmann *et al.* 2007; Husnjak & Dikic 2012). Die Art und Länge der Ubiquitinierung ist entscheidend für das Schicksal der Substrate. So scheint die Ubiquitinierung über Lys63 die Proteinfunktion zu verändern und eine Rolle in der DNA Reparatur sowie zellulären Signal- und Transportwegen zu spielen (Pickart 2000; Li & Ye 2008). Neben diesen Polyubiquitinierungen sind auch Mono- und Multiubiquitinierungen bekannt, deren Funktion zum Teil allerdings noch ungeklärt ist (Weissman 2001; Herrmann *et al.* 2007).

Die Ubiquitinierung ist ein reversibler Prozess. Durch deubiquitinierende Enzyme (DUB) können Ubiquitin-Reste von einem Substrat wieder abgespalten werden und in neuen Ubiquitinierungen recycelt werden. Dieses spielt beispielsweise eine Rolle bei der Prozessierung des Ubiquitins, wobei aus einem zunächst translatierten Polyubiquitin Monomere gebildet werden oder bei dem proteasomalen Abbau, bei dem die Abspaltung des Polyubiquitins für die Funktion des Proteasoms essentiell ist (Wiborg *et al.* 1985; Ozkaynak *et al.* 1987; Amerik & Hochstrasser 2004). Durch das Zusammenspiel von Ubiquitinierung

und Deubiquitinierung entsteht ein dynamischer Ubiquitin-Pool aus wenig freiem und mehrheitlich gebundenem (Poly-) Ubiquitin, welches in Zellen durch die Verwendung eines GFP-Ubiquitin Konjugats sichtbar gemacht werden konnte (Dantuma *et al.* 2006).

1.4.1 Ubiquitin-ähnliche Proteine

Interessanterweise kann nicht nur Ubiquitin selbst die Funktion von Proteinen beeinflussen. Es gibt eine Reihe von Ubiquitin-ähnlichen Proteinen, die mit anderen Proteinen interagieren oder sogar kovalent verknüpft werden können. Die Gruppe der Ubiquitin-ähnlichen Proteine wird in zwei Kategorien unterteilt (Jentsch & Pyrowolakis 2000). Die erste Gruppe bilden die *ubiquitin-like modifier* (UBL, bzw. Typ 1), die ähnlich wie Ubiquitin kovalent über deren C-Terminus verknüpft werden können. Zu dieser Gruppe gehört unter anderem die SUMO-Familie oder Nedd8. Die zweite Gruppe, die *ubiquitin-domain proteins* (UDP, bzw. Typ 2), besitzt wie der Name sagt, eine Ubiquitin-ähnliche Domäne (ULD oder UBQ), kann jedoch nicht kovalent konjugiert werden. Mitglieder dieser heterogenen Gruppe sind beispielsweise RAD23 oder die E3-Ligase Parkin (Jentsch & Pyrowolakis 2000; Walters *et al.* 2004).

Die Ubiquitin-ähnliche Domäne umfasst 45 – 80 Aminosäuren und ähnelt in ihrer Struktur stark dem Ubiquitin (Jentsch & Pyrowolakis 2000; Grabbe & Dikic 2009). Dabei nimmt die Domäne die Ubiquitin-Superfaltung $\beta\beta\alpha\beta\beta\alpha$ ein (Kiel & Serrano 2006). Die ULD ist zumeist Bestandteil eines größeren (Multidomänen) Proteins, wobei die ULD in der Regel am N-Terminus gelegen ist (Buchberger 2002; Walters *et al.* 2002). Die Funktion von Mitgliedern dieser Proteinklasse ist weitreichend. Beispielsweise spielen sie eine Rolle in der Regulation von Enzymaktivitäten, DNA-Reparatur, Apoptose, Proteinfaltung und -abbau (Walters *et al.* 2004; Grabbe & Dikic 2009). Die Zahl der identifizierten ULD-beinhaltenden Proteine nimmt stetig zu. Einige Proteine enthalten ULDs, die eher divergent sind und nicht zwangsläufig mit dem Ubiquitin-Proteasom-System in Verbindung stehen müssen (Grabbe & Dikic 2009). Ein Beispiel für die Vielfalt der Aufgaben von ULD-beinhaltenden Proteinen ist die E3-Ligase Parkin. Parkin gehört der Gruppe der RING-Ligasen an und ist ein Multidomänenprotein, aufgebaut aus einer ULD (N-terminal), einer cysteinreichen RING0 und 2 RING-Domänen (RING1 + 2), die durch eine cysteinreiche IBR (*in between RING*) Domäne getrennt sind (Walden & Martinez-Torres 2012). Die RING2 Domäne ist für die E3-Ligase Aktivität essentiell. Für eine Vielzahl von Substraten wird eine Interaktion mit Parkin und eine Parkin-abhängige Mono- oder Polyubiquitinierung postuliert (Walden & Martinez-Torres 2012). Parkin ist in der Lage, sich selbst zu ubiquitinieren

(Autoubiquitinierung). Normalerweise nimmt Parkin allerdings eine Konformation ein, in der die Autoubiquitinierung verhindert wird. Bei der Regulation der Autoubiquitinierung spielt die ULD eine wichtige Rolle, die an die potentiellen Peptidsequenzen der C-terminalen RING-Sequenz bindet (Chaugule *et al.* 2011). Des Weiteren spielt Parkin eine Rolle in der Autophagie von Mitochondrien (Narendra *et al.* 2008). Hierbei wird Parkin abhängig von Pink1, einer mitochondrialen Kinase, zu defekten Mitochondrien rekrutiert und polyubiquitiniert dort den spannungsabhängigen Anionen-Kanal 1 (VDAC1). Die Polyubiquitinierung scheint weitere Bestandteile des Autophagie-Signalweges zu rekrutieren, die letztlich zur Translokation des defekten Mitochondriums in das Autophagosom führen (Wild & Dikic 2010).

1.4.2 Ubiquitin-Bindungsdomänen

Ubiquitin-Bindungsdomänen (UBD) umfassen 20 - 150 Aminosäuren und sind in der Lage, direkt mit Mono- und/oder Polyubiquitin zu interagieren. Die Affinität ist mit Dissoziationskonstanten zwischen 10 – 500 $\mu\text{mol/l}$ recht gering (Hicke *et al.* 2005). Es gibt eine Vielzahl verschiedener UBDs, wobei die meisten eine α -helikale Struktur ausbilden (Hicke *et al.* 2005; Hurley *et al.* 2006). Eine Gruppe bildet das Ubiquitin-interagierende Motiv (UIM). Dieses Motiv wurde zunächst in einer Untereinheit des Proteasoms identifiziert, wo es an der Erkennung von Polyubiquitin bzw. ubiquitinierten Proteinen beteiligt ist (Deveraux *et al.* 1994; Young *et al.* 1998). Mittlerweile sind mehrere Proteine bekannt, die eine UIM besitzen. Aus Sequenzvergleichen wurde eine 20 Aminosäuren umfassende Konsensussequenz abgeleitet, die die Kernsequenz θ -x-x-Ala-x-x-Ser-x-x-Ac (θ : große, hydrophobe Aminosäure, Ac: saure Aminosäure, x: weniger stark konservierte Aminosäure) beinhaltet (Hofmann & Falquet 2001; Fisher *et al.* 2003). UIMs wurden in Proteinen gefunden, die an der Ubiquitinierung oder dem Ubiquitin Metabolismus beteiligt sind, oder auch mit *ubiquitin-like modifiers* interagieren (Hofmann & Falquet 2001). Zusätzlich konnte gezeigt werden, dass ebenfalls eine Interaktion von Ubiquitin-ähnlichen Domänen mit UIMs möglich ist. So bindet die ULD von hHR23, dem humanen RAD23 Analogon, an die UIM von S5a (Hiyama *et al.* 1999; Fujiwara *et al.* 2004). Im Gegensatz zu der UIM der S5a sind UIMs aus anderen Proteinen auch in der Lage, Monoubiquitin zu binden, wobei die Affinität jedoch gering ist (Fujiwara *et al.* 2004). Daneben fördert die UIM in vielen der UIM-beinhaltenen Proteine die Monoubiquitinierung (Hicke *et al.* 2005; Hurley *et al.* 2006).

1.4.3 Das 26S Proteasom

Die Degradation der polyubiquitinierten Proteine übernimmt das 26S Proteasom, welches die Proteine in Peptide mit einer Länge von 3 – 23 Aminosäuren zerlegt (Glickman & Ciechanover 2002). Das Proteasom besteht aus zwei 19S Untereinheiten (regulatorische Partikel, Regulatorkomplex), die sich an den Enden der zylindrischen 20S Untereinheit (katalytischer Kern) befinden (Abb. 1.7). Die regulatorischen 19S Partikel erkennen ubiquitinierte Proteine, sind verantwortlich für die Deubiquitinierung und Entfaltung der Proteine. Der Regulatorkomplex besteht aus ca. 20 verschiedenen Untereinheiten, die in zwei größere Subkomplexe (Base und Lid) unterteilt werden können (Tanaka *et al.* 2005). Die Erkennung von Polyubiquitinketten erfolgt über die S5a-Untereinheit am Übergang zwischen Lid und Base, welche zwei Ubiquitin-interagierende Motive (UIM) beinhaltet, die mit Polyubiquitinketten, nicht aber mit Monoubiquitin interagieren (Deveraux *et al.* 1994; Young *et al.* 1998). Die notwendige Entfaltung der Proteine erhöht die Spezifität des Proteasoms, da somit native, gefaltete Proteine nicht zufällig abgebaut werden können. Die katalytische 20S Untereinheit ist aus zwei äußeren α -Ringen und zwei inneren β -Ringen aufgebaut, die sich jeweils aus sieben α - bzw. β -Einheiten zusammensetzen ($\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$). Die katalytische Funktion wird dabei von den β -Untereinheiten übernommen und teilt sich auf in Chymotrypsin-, Trypsin- und Peptidylhydrolase-ähnliche Aktivitäten (β_5 -, β_2 - und β_1 -Untereinheit) (Glickman & Ciechanover 2002; Wolf & Hilt 2004; Tanaka *et al.* 2005; Herrmann *et al.* 2007). In Hefen konnte eine Hierarchie der katalytischen Untereinheiten bezüglich der Aktivität und Beteiligung am Abbau zellulärer Proteine: $\beta_5 > \beta_2 \geq \beta_1$ festgelegt werden (Jager *et al.* 1999; Wolf & Hilt 2004). Auch wenn die Mehrheit der Proteine ubiquitiniert werden muss, um proteasomal degradiert zu werden, gibt es einige Ausnahmen. So wird die sehr kurzlebige Ornithindecaboxylase (ODC) ohne Ubiquitinierung vom Proteasom abgebaut, indem der C-Terminus der ODC als Erkennungssignal dient (Hoyt & Coffino 2004).

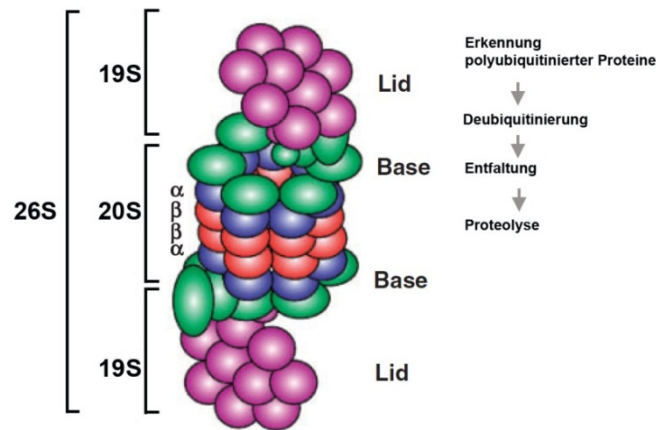


Abb. 1.7 Aufbau des 26S Proteasoms. Das 26S Proteasom besteht aus einem katalytischen Kern (20S) und zwei regulatorischen Einheiten (19S), welche jeweils in Lid und Base unterteilt werden können. Innerhalb der 19S Untereinheit erfolgt die Erkennung der polyubiquitinierten Proteine, die Deubiquitinierung sowie die Entfaltung. Der katalytische Kern ist verantwortlich für die Proteolyse des Proteins. Modifiziert nach (Tanaka *et al.* 2005).

1.4.4 Proteasom-Inhibitoren

Aufgrund der zentralen Rolle des Ubiquitin-Proteasom-Systems sind Proteasom-Inhibitoren bedeutsam, um die zelluläre Protein-Homöostase besser charakterisieren zu können (Wolf & Hilt 2004). Darüber hinaus kommen neuartige, zum Teil aus Naturstoffen abgeleitete Inhibitoren in der Antitumorthherapie zum Einsatz (Rentsch *et al.* 2013). Proteasom-Inhibitoren können entsprechend nach reversibler oder irreversibler Inhibierung unterteilt werden. Nahezu alle Inhibitoren hemmen effektiv die Chymotrypsin-ähnliche Aktivität, welche aufgrund der erhöhten Aktivität der $\beta 5$ -Untereinheit für Analysen des Ubiquitin-Proteasom-Systems von Bedeutung ist. Sie unterscheiden sich jedoch in ihren Effekten auf die anderen Zentren (Kisselev & Goldberg 2001). Zu den reversiblen Inhibitoren zählen die Peptidaldehyde. Das bekannteste Mitglied dieser Gruppe ist das MG132 (z-Leu-Leu-Leu-al). MG132 ist ein potenter Inhibitor vor allem der Chymotrypsin-ähnlichen Aktivität ($K_i = 2 - 4 \text{ nmol/l}$) (Kisselev & Goldberg 2001). Allerdings kann MG132 auch lysosomale Cysteinproteasen und Calpaine hemmen. Hier sind jedoch etwa 10-fach höhere Konzentrationen notwendig (Lee & Goldberg 1998; Kisselev & Goldberg 2001). Spezifischer ist der natürliche, irreversible Inhibitor Lactacystin, welcher neben dem Proteasom nur Cathepsin A hemmt (Lee & Goldberg 1998). Lactacystin selbst kann das Proteasom nicht inhibieren, geht aber bei neutralem pH-Wert spontan in das aktive *clasto*-Lactacystin- β -lacton über. Dieses bindet effizient an Hydroxygruppen der Threonin-Reste in den β -Untereinheiten (Lee & Goldberg 1998; Kisselev & Goldberg 2001). Die selektivste Gruppe der Inhibitoren bilden die Epoxyketone. Epoxomicin inhibiert primär die

Chymotrypsin-ähnliche Aktivität, indem es sowohl mit den Hydroxylgruppen als auch mit den Aminogruppen der katalytischen Threonine interagiert (Kisselev & Goldberg 2001).

1.5 Das Ubiquitin-Proteasom-System in der Beta-Zelle

Das Ubiquitin-Proteasom-System spielt auch in den Beta-Zellen des Pankreas eine wichtige Rolle. Kürzlich konnte in humanen Inseln von Patienten mit Typ 2 Diabetes mellitus gezeigt werden, dass verschiedene Gene des Ubiquitin-Proteasom-Systems herunterreguliert sind. Zeitgleich wurde eine Reduktion der Proteasomaktivität und eine Anreicherung von ubiquitinierten Proteinen gefunden (Bugliani *et al.* 2013). Die Ubiquitin-Carboxyhydrolase 1 (UCHL1) ist beispielsweise in humanen diabetischen Inseln vermindert (Costes *et al.* 2011). Dieses Protein gehört zu der Gruppe der Deubiquitinasen und sorgt somit für die Abspaltung von Ubiquitin bei der proteasomalen Degradation von Proteinen und damit auch für die Regeneration von freiem Ubiquitin. Zudem wurde eine glucoseabhängige Expression von Genen des Ubiquitin-Proteasom-Systems gezeigt. In primären Ratteninseln war die Genexpression von UCHL1, Parkin und UBCH5 (ein Ubiquitin-übertragendes Enzym E2) nach 24 stündiger Inkubation mit 16,7 mmol/l Glucose im Vergleich zu 2,8 mmol/l Glucose signifikant reduziert (Lopez-Avalos *et al.* 2006). Weiterhin gaben Experimente mit Proteasom-Inhibitoren Aufschluss über die Rolle des Proteasoms in den Beta-Zellen. Inhibierung des Proteasoms mit Lactacystin, MG132 oder Epoxomicin resultierte in einer reduzierten glucoseinduzierten Insulinsekretion in Ratten- und Mausinseln sowie in insulinproduzierenden MIN6 Zellen (Kitiphongspattana *et al.* 2005; Kawaguchi *et al.* 2006; Lopez-Avalos *et al.* 2006; Hartley *et al.* 2009). Hierbei scheinen unterschiedliche Mechanismen eine Rolle zu spielen, die jedoch nur teilweise verstanden werden. Zum einen ist die Ubiquitinierung von Untereinheiten des K_{ATP} -Kanals wesentlich. Eine Hemmung des Proteasoms führt zu einer gesteigerten Expression des Kanals an der Zelloberfläche (Yan *et al.* 2005). Die Hemmung des Proteasoms bedingt ebenfalls eine erhöhte Expression des spannungsabhängigen Calciumkanals (VDCC) an der Zelloberfläche (Kawaguchi *et al.* 2006). Hier scheint die Ubiquitinierung der α -Untereinheit bedeutsam. Im Gegensatz zu dem Kaliumkanal konnte bei dem VDCC jedoch keine gesteigerte Aktivität festgestellt werden (Yan *et al.* 2005; Kawaguchi *et al.* 2006).

1.6 Fragestellung

Die Stoffwechselkrankheit Diabetes mellitus ist durch eine chronisch erhöhte Blutglucosekonzentration charakterisiert. Die in den Beta-Zellen des Pankreas exprimierte Glucokinase übernimmt als Glucosesensor eine Schlüsselfunktion zur Aufrechterhaltung der glucoseinduzierten Insulinsekretion. Ein Ausfall des Enzyms ist mit dem Leben nicht vereinbar und Aktivitätsveränderungen führen entsprechend zu einer Hyper- oder Hypoglykämie. Synthetische Glucokinaseaktivatoren haben ein Potential in der Typ 2 Diabetes mellitus Therapie. Endogen spielen vor allem Protein-Protein-Interaktionen in der Regulation der Glucokinaseaktivität eine Rolle in den Beta-Zellen des Pankreas. Das bifunktionelle Enzym 6-Phosphofructo-2-kinase/Fruktose-2,6-bisphosphatase bindet und aktiviert die Glucokinase. Ein inhibierender Interaktionspartner konnte bislang nicht identifiziert werden. Eine Rolle des Ubiquitin-Proteasom-Systems in der Glucokinase-regulation wurde postuliert, ist bislang aber noch wenig verstanden.

Es wurden daher folgende Ziele für die Promotionsarbeit formuliert:

- (1) Der endogene Aktivator 6-Phosphofructo-2-kinase/Fruktose-1,6-bisphosphatase verändert im Gegensatz zu den synthetischen Glucokinaseaktivatoren den $S_{0,5}$ -Wert der Glucokinase nicht. Der Einfluss der 6-Phosphofructo-2-kinase/Fruktose-2,6-bisphosphatase und von chemischen Aktivatoren auf die glucoseinduzierte Insulinsekretion wurde bislang noch nicht vergleichend untersucht. Daher sollte die glucoseinduzierte Insulinsekretion in insulinsezernierenden MIN6 Zellen nach Überexpression der 6-Phosphofructo-2-kinase/Fruktose-2,6-bisphosphatase und Inkubation mit dem Glucokinase-aktivator LY2121260 untersucht werden.
- (2) Die Ubiquitinierung der Glucokinase wurde bislang nur in einem *in vitro* System untersucht. In insulinsezernierenden MIN6 Zellen sollte durch Hemmung des proteasomalen Abbaus der Einfluss des Ubiquitin-Proteasom-Systems auf die Glucokinase *in vivo* untersucht werden. Hierbei sollte geklärt werden, welchen Einfluss die Inhibierung des Proteasoms, mit und ohne gleichzeitiger Hemmung der Proteintranslation, auf die Glucokinase Expression und Enzymaktivität sowie die glucosestimulierte Insulinsekretion hat.
- (3) Um Veränderungen in der zellulären Verteilung der Glucokinase nach Proteasomhemmung darzustellen, sollte die Glucokinase als Fusionskonstrukt

mit dem Fluoreszenzprotein Dendra2 in MIN6 Zellen exprimiert werden. Das Emissionsspektrum im grünen Wellenlängenbereich des Dendra2 kann mittels UV-Licht irreversibel in ein rotes Emissionsspektrum konvertiert werden. Hierdurch ist es möglich, nach einer solchen Photokonversion neu synthetisierte (grüne Fluoreszenz) von bereits zum Startzeitpunkt des Experiments vorhandener Glucokinase (rote Fluoreszenz) zu unterscheiden.

- (4) In der Leber wird die Glucokinase nicht nur durch die 6-Phosphofructo-2-kinase/Fruuctose-2,6-bisphosphatase aktiviert, sondern durch das Glucokinase Regulatorprotein kompetitiv inhibiert. In den Beta-Zellen des Pankreas konnte bislang kein weiterer Interaktionspartner identifiziert werden. Daher wurde ein *Yeast Two-Hybrid Screening* durchgeführt, bei dem ein Proteinfragment mit Homologie zum N-Terminus des Midnolins als potentieller Interaktionspartner der Glucokinase identifiziert werden konnte. Diese Proteinsequenz beinhaltet die Ubiquitin-ähnliche Domäne des Midnolins. Um die Interaktion in Eukaryoten zu bestätigen, sollte mit Hilfe eines fluoreszenzbasierten *Mammalian Two-Hybrid Systems* die Interaktion des Gesamtfragments sowie nur der Ubiquitin-ähnlichen Domäne mit der Glucokinase untersucht werden.
- (5) Weiterhin sollte sowohl das gesamte Proteinfragment als auch die Ubiquitin-ähnliche Domäne in insulinsezernierenden MIN6 Zellen überexprimiert werden. Hierdurch sollte der Einfluss dieser potentiellen Interaktionspartner auf die Expression und Enzymaktivität der Glucokinase sowie die glucoseinduzierte Insulinsekretion charakterisiert werden.

2 Publikationen

Titel:

Additive activation of glucokinase by the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and the chemical activator LY2121260

Zeitschrift:

Biochemical Pharmacology 83 (2012) 1300-1306

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Titel:

The ubiquitin proteasome system regulates the stability and activity of the glucose sensor glucokinase in pancreatic beta cells

Zeitschrift:

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Titel:

Identification of the ubiquitin-like domain of midnolin as a new glucokinase interaction partner

Zeitschrift:

The Journal of Biological Chemistry

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Additive activation of glucokinase by the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and the chemical activator LY2121260

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ABSTRACT

The glucose phosphorylating enzyme glucokinase plays a crucial role in stimulus-secretion coupling in pancreatic beta cells and in glucose metabolism in liver. Glucose mediates a shift of the enzyme's conformational equilibrium towards the closed conformation with high glucokinase activity. Further activation of glucokinase is endogenously mediated by interaction with the bisphosphatase domain (FBPase-2) of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) and can be achieved also by a new class of glucokinase activators (GKA), chemical compounds that might be suited for type 2 diabetes therapy. While FBPase-2 increased only the phosphorylating capacity of glucokinase, the GKA LY2121260 augmented in addition the affinity of glucokinase for glucose. PFK-2/FBPase-2 but not LY2121260 antagonized glucokinase inhibition by the competitive glucokinase inhibitor mannoheptulose at increasing glucose concentrations. Interestingly, an additive activation of glucokinase was observed by use of recombinant FBPase-2 together with LY2121260. This new crucial observation could be confirmed with cellular extracts containing the glucokinase and PFK-2/FBPase-2 proteins. Addition of LY2121260 resulted in a further significant increase in glucokinase activity. Because the glucokinase-PFK-2/FBPase-2 complex was conserved under LY2121260 treatment as shown by size exclusion chromatography a concerted action of both activators towards the closed active glucokinase conformation can be anticipated. Thus, as a result of the additive effect of both activators on glucokinase activity, the largest increase of glucose-induced insulin secretion was observed in the combined presence of PFK-2/FBPase-2 and LY2121260.

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1. Introduction

The glucose phosphorylating enzyme glucokinase controls metabolic flux in glycolysis [1,2]. In contrast to the other members of the hexokinase family glucokinase shows a sigmoidal saturation curve and an affinity for glucose in the physiological concentration range between 6 and 8 mmol/l [1–4]. Thus, glucokinase is well suited to play the crucial role of the glucose sensor in the pancreatic beta cell for the control of glucose-induced insulin secretion [1–4]. In liver, with a high expression level, glucokinase is the main regulator of glucose metabolism [5].

Glucose binding induces a global conformational transition of glucokinase [6]. This is the explanation for the positive cooperativity of glucokinase although the enzyme is monomeric [7].

Kinetic models, namely the mnemonical and the slow-transition model have suggested a positive cooperativity of glucokinase [8,9]. A few years ago, two glucokinase conformations were characterized by its crystal structure, a super-opened, catalytically inactive glucokinase conformation and a closed, catalytically active one [10]. In addition there is evidence that further conformational states exist during the transition between the closed and super-opened conformation [10,11]. Three intermediates have been assumed from fluorescence spectroscopy measurements indicating intracellular equilibration of glucokinase conformations depending on the glucose concentration [12].

This glucose-mediated regulation of glucokinase is modulated on the posttranslational level by protein–protein interaction [1,13]. In liver, glucokinase is inhibited by the glucokinase regulatory protein, which binds and shuttles glucokinase at low glucose concentrations to the nucleus [14,15]. The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) is an activating glucokinase binding partner, both in liver and in pancreatic beta cells [2,13,16–19]. The interaction between glucokinase and the bifunctional enzyme is mediated by a motive in the bisphosphatase domain [16]. Activation of glucokinase through PFK-2/FBPase-2

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

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increases the maximal enzyme velocity (V_{max}) value of the enzyme, while the affinity for glucose ($S_{0.5}$) remained unchanged [13,17]. In beta cells glucokinase activation by PFK-2/FBPase-2 occurs at high glucose concentrations and potentiates glucose-induced insulin secretion [13,20].

An increase of insulin secretion to an extent that it induces hypoglycemia was observed in patients carrying activating glucokinase mutations [21]. 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 [21–24]. The region, which accommodates the activating mutations [22,24] is the allosteric binding site of small molecules, which were recently found to activate glucokinase [10,25–29]. Their use as blood glucose lowering agents is an attractive therapeutic concept [30]. The action of the different available compounds varies in their activating potency and by the mode how they modify V_{max} and $S_{0.5}$ of glucokinase [10,25–28]. The recently synthesized glucokinase activator LY2121260 raises the V_{max} and decreases the $S_{0.5}$ value [26]. LY2121260 increased insulin secretion in isolated rat pancreatic islets and stimulated glucose uptake in cultured rat hepatocytes [26]. Furthermore, glucose tolerance was improved in healthy rats [26].

To date the interplay between the endogenous activator PFK-2/FBPase-2 and small molecule glucokinase activators such as LY2121260 in the regulation of glucokinase enzyme activity is unknown. The aim of this study was therefore to analyze and compare the molecular mechanisms of glucokinase activation by PFK-2/FBPase-2 and LY2121260.

2. Materials and methods

2.1. 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) [26] used in this study was kindly provided by Lilly Research Laboratories (Indianapolis, IN). All tissue culture equipment was from Invitrogen (Karlsruhe, Germany) or Greiner-Bio One (Frickenhausen, Germany).

2.2. 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 [17]. 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 [17]. Cells were grown in RPMI 1640 medium (Biochrom AG, Berlin, Germany) supplemented with 10 mmol/l glucose (Sigma–Aldrich), 10% (v/v) fetal calf serum (FCS) (Biowest, Nuaille, France), penicillin and streptomycin (Biochrom AG) in a humidified atmosphere at 37 °C and 5% CO₂. The medium for RINm5F-GK cells was additionally supplemented with 250 µg/ml geneticin (G418) (Invitrogen) and the medium for RINm5F-GK-PFK-2/FBPase-2 cells with 250 µg/ml G418 and 250 µg/ml Zeocin™ (Invitrogen). 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 [17].

2.3. Recombinant glucokinase and FBPase-2 protein

Recombinant beta cell glucokinase was expressed and purified as His₆-tag protein by the pQE30 vector system as described

previously [31]. 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 (GE Healthcare, Freiburg, Germany) as described [19].

2.4. 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 [17]. 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} were estimated by sigmoidal curve fitting. $S_{0.5}$ values for glucose were calculated from Hill plots.

2.5. 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 (GE Healthcare) and eluted with 1 ml/min using ÄKTA® PrimePlus system (GE Healthcare). Resolution of protein separation in the molecular range was calibrated using recombinant glucokinase, bovine serum albumin (70 kDa) (Serva, Heidelberg, Germany), aldolase (158 kDa) and catalase (232 kDa) (Sigma–Aldrich). 15 µl of each fraction was electroblotted to equilibrated polyvinylidene difluoride (PVDF) membranes (Millipore, Schwalbach, Germany) 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 [17].

2.6. Measurement of insulin secretion

MIN6 cells were grown in DMEM medium (Biochrom AG) supplemented with 25 mM glucose, 10% (vol/vol) FCS, penicillin, and streptomycin in a humidified atmosphere at 37 °C and 5% CO₂. Cells were seeded in six-well microplates at a density of 3.5×10^5 cells and grown for three days. Thereafter, cells were transfected with jetPEI (Qbiogene, Montreal, QC, Canada) and 2 µg EYFP, EYFP-PFK-2/FBPase-2 or EYFP-PFK-2/FBPase-2-Mut and grown for further 48 h as described previously [20]. Finally, cells were incubated for 1 h in bicarbonate-buffered Krebs-Ringer solution without glucose supplemented with 0.1% albumin (Sigma–Aldrich) and thereafter stimulated for 1 h with 3 or 10 mmol/l glucose and with or without 10 µM LY2121260. Thereafter 1 ml of the incubation buffer from each well was carefully harvested and gently centrifuged to remove detached cells. In the final supernatants the secreted insulin was measured. Cells were homogenized by sonication in phosphate buffered saline (pH 7.4) and insulin content was measured in soluble fractions. Insulin was measured by ELISA and the protein concentration was quantified by a Bradford protein assay [20].

2.7. Statistical analyses

Data are expressed as means ± SEM. Statistical analyses were performed by ANOVA followed by Bonferroni's test for multiple

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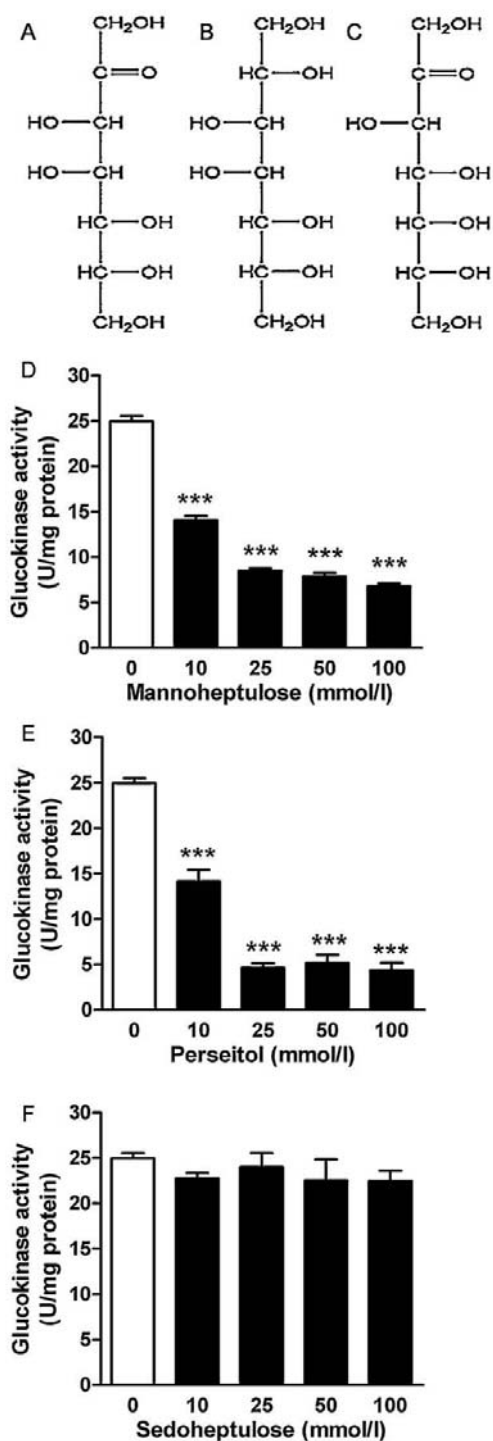


Fig. 1. Inhibition of glucokinase enzyme activities by mannoheptulose, perseitol and sedoheptulose. Chemical structure of mannoheptulose (A), perseitol (B) and sedoheptulose (C). Recombinant beta cell glucokinase was incubated for 5 min with 10, 25, 50 or 100 mmol/l mannoheptulose (D), perseitol (E) or sedoheptulose (F) and

comparisons using the Prism analysis program (Graphpad Inc., San Diego, CA, USA).

3. Results

3.1. Glucokinase inhibition by mannoheptulose and perseitol

Mannoheptulose and sedoheptulose are epimers. The C7 sugars differ only in the configuration of the hydroxyl group at the 4th carbon atom (Fig. 1A–C). Glucokinase was significantly inhibited by mannoheptulose in a concentration-dependent manner (Fig. 1D). This inhibition was sugar conformation selective since sedoheptulose did not affect glucokinase enzyme activity (Fig. 1F). However, the C7 sugar alcohol perseitol with a configuration of the hydroxyl group at the 4th carbon atom corresponding to that of mannoheptulose showed a significant inhibition of glucokinase (Fig. 1E).

3.2. Activation of glucokinase enzyme activity by LY2121260 in the presence of the inhibitors mannoheptulose and perseitol

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 (Fig. 2A and B). Treatment of glucokinase with LY2121260 resulted both in an increase in the maximal enzyme velocity (V_{max} 41.8 ± 0.7 U/mg versus 28.5 ± 1.1 U/mg) and an increase in the affinity to its substrate glucose ($S_{0.5}$ 2.51 ± 0.06 mmol/l versus 7.26 ± 0.46 mmol/l) (Fig. 2A–D). LY2121260 was not able to activate glucokinase in the presence of mannoheptulose (V_{max} 18.0 ± 1.1 U/mg versus 16.4 ± 0.9 U/mg). Actually the affinity of glucokinase for glucose decreased further significantly ($S_{0.5}$ 29.9 ± 2.92 mmol/l versus 17.9 ± 1.57 mmol/l) (Fig. 2A). LY2121260 partially increased glucokinase enzyme activity in the presence of perseitol and evoked a significant increase in both the maximal enzyme velocity (V_{max} 25.3 ± 1.0 U/mg versus 14.8 ± 1.3 U/mg) and the affinity to its substrate glucose ($S_{0.5}$ 3.55 ± 0.43 mmol/l versus 15.1 ± 2.53 mmol/l) (Fig. 2B). Sedoheptulose did not counteract glucokinase activation by LY2121260 (V_{max} 41.5 ± 1.3 U/mg versus 23.5 ± 0.5 U/mg and $S_{0.5}$ 2.57 ± 0.16 mmol/l versus 7.46 ± 0.29 mmol/l) (Fig. 2C).

3.3. Simultaneous glucokinase activation by the chemical compound LY2121260 and 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) [16]. The GK:FBPase-2 complex has a 1:1 stoichiometry [19]. Most likely due to some protein misfolding in the artificial environment a fivefold molar excess of FBPase-2 evoked the highest activation of glucokinase. Incubation of glucokinase with FBPase-2 in a 1:5 molar ratio significantly increased the maximal enzyme velocity (V_{max} 49.8 ± 2.6 U/mg versus 28.7 ± 2.9 U/mg), but did not change the affinity to glucose ($S_{0.5}$ 7.85 ± 0.95 mmol/l versus 7.75 ± 1.85 mmol/l) (Fig. 3A and B). Activation of glucokinase by LY2121260 in the presence of FBPase-2 resulted in a significant further increase of the glucokinase enzyme velocity (V_{max} 62.2 ± 5.6 U/mg versus 40.3 ± 3.1 U/mg) while the $S_{0.5}$ value was comparable ($S_{0.5}$ 1.98 ± 0.26 mmol/l versus 2.21 ± 0.30 mmol/l) (Fig. 3A and B).

after addition of 100 mmol/l glucose enzyme activities were measured spectrophotometrically. Shown are means \pm SEM in U/mg protein from 3 independent experiments. *** $p < 0.001$ compared to control (ANOVA/Bonferroni's test).

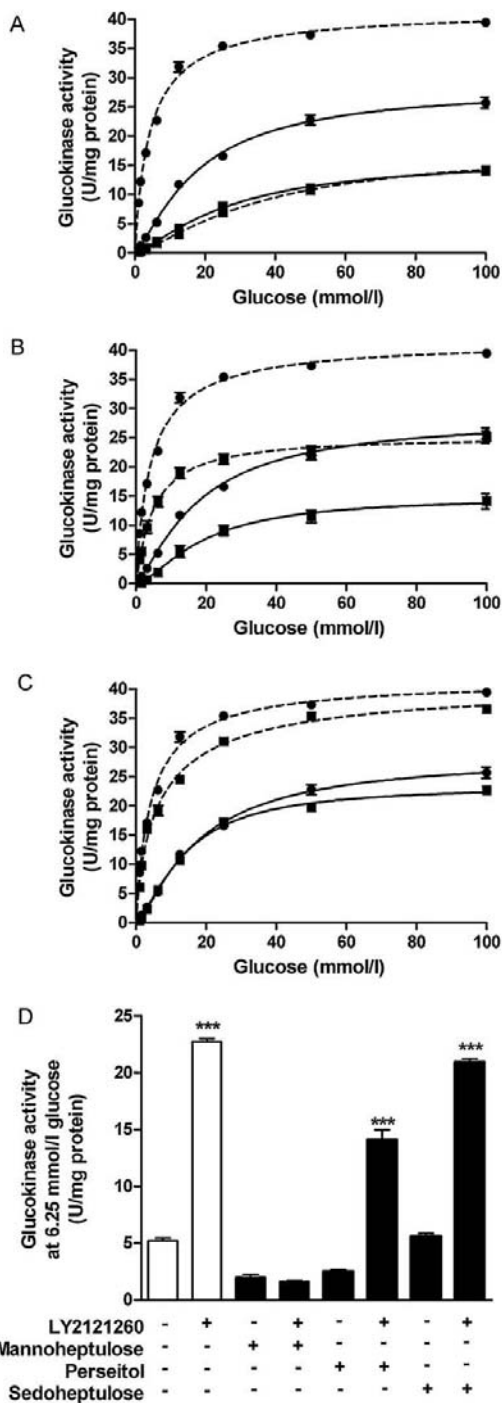


Fig. 2. Effect of the glucokinase activator LY2121260 on glucokinase enzyme activities in the presence of mannoheptulose, perseitol and sedoheptulose. Recombinant beta cell glucokinase was incubated for 5 min without (black circles, solid line), in the presence of 10 μ mol/l LY2121260 (black circles, dashed line), in the presence of 10 μ mol/l mannoheptulose (A), perseitol (B) or sedoheptulose (C) (black square, solid line) or in the presence of both 10 μ mol/l

3.4. Glucokinase activation by the chemical compound LY2121260 and PFK-2/FBPase-2 in the presence of mannoheptulose

The stable insulin-producing cell lines RINm5F-GK and RINm5F-GK-PFK-2/FBPase-2 express the glucokinase protein on the same level [13,17]. The presence of PFK-2/FBPase-2 in cell extracts resulted in a significant increase in glucokinase enzyme activity (31% at 6.25 mmol/l glucose) (Fig. 4A and C). Mannoheptulose inhibited glucokinase enzyme activity in both cell extracts. However, RINm5F-GK-PFK-2/FBPase-2 cell extracts showed in the presence of mannoheptulose a higher glucokinase enzyme activity than RINm5F-GK cell extracts (26% at 6.25 mmol/l glucose) (Fig. 4A and C). Thus, at least in part PFK-2/FBPase-2 was able to counteract glucokinase inhibition by mannoheptulose. Treatment with LY2121260 significantly increased glucokinase enzyme activity (59% at 6.25 mmol/l glucose) (Fig. 4B and C). Both activators together evoked a significant further increase in glucokinase enzyme activity compared to PFK-2/FBPase-2 and LY2121260 alone (47% and 27%, respectively at 6.25 mmol/l glucose) (Fig. 4B and C). LY2121260 was not able to activate glucokinase in the presence of mannoheptulose (Fig. 4B and C).

3.5. Glucokinase-PFK-2/FBPase-2 complex formation in the presence of LY2121260

Glucokinase-PFK-2/FBPase-2 complex formation in RINm5F-GK-PFK-2/FBPase-2 cell extracts was detectable as a higher molecular weight fraction by gel filtration chromatography in comparison to RINm5F-GK cell extracts (Fig. 5A and B). Glucokinase was verifiable by Dot-Blot immunodetection analysis in fractions between 50 and 70 kDa and additionally within a molecular range between 130 and 230 kDa. The first peak (50–70 kDa) corresponded to the elution of separated recombinant glucokinase protein in control experiments. 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. Indeed, the glucokinase-PFK-2/FBPase-2 complex was not abolished by treatment with LY2121260 (Fig. 5C).

3.6. Glucose-induced insulin secretion in dependence upon LY2121260 and PFK-2/FBPase-2

Insulin secretion in PFK-2/FBPase-2 expressing MIN6 cells was comparable to control cells at 3 mmol/l glucose. At 10 mmol/l glucose insulin secretion of PFK-2/FBPase-2 expressing MIN6 cells was significantly higher than in control cells (Fig. 6). Treatment with LY2121260 increased insulin secretion at both, 3 and 10 mmol/l glucose in comparison to control cells (Fig. 6). In PFK-2/FBPase-2 expressing MIN6 cells LY2121260 evoked a significant further increase in insulin secretion at 10 mmol/l glucose (Fig. 6). In contrast, expression of a glucokinase binding deficient mutant PFK-2/FBPase-2 protein reduced insulin secretion in MIN6 cells at 10 mmol/l glucose (Fig. 6). Only at 3 mmol/l glucose, but not at 10 mmol/l glucose LY2121260 increased insulin secretion in mutant PFK-2/FBPase-2 expressing MIN6 cells (Fig. 6).

LY2121260 and 10 mmol/l mannoheptulose (A), perseitol (B) or sedoheptulose (C) (black square, dashed line). After addition of the indicated glucose concentration enzyme activities were measured spectrophotometrically. (D) Shown are means \pm SEM in U/mg protein from 4 independent experiments. Comparison of glucokinase enzyme activities in the presence of LY2121260 (white bars) and mannoheptulose, perseitol or sedoheptulose (black bars) measured at 6.25 mmol/l glucose. *** p < 0.001 compared to mannoheptulose, perseitol or sedoheptulose without LY2121260, respectively (ANOVA/Bonferroni's test).

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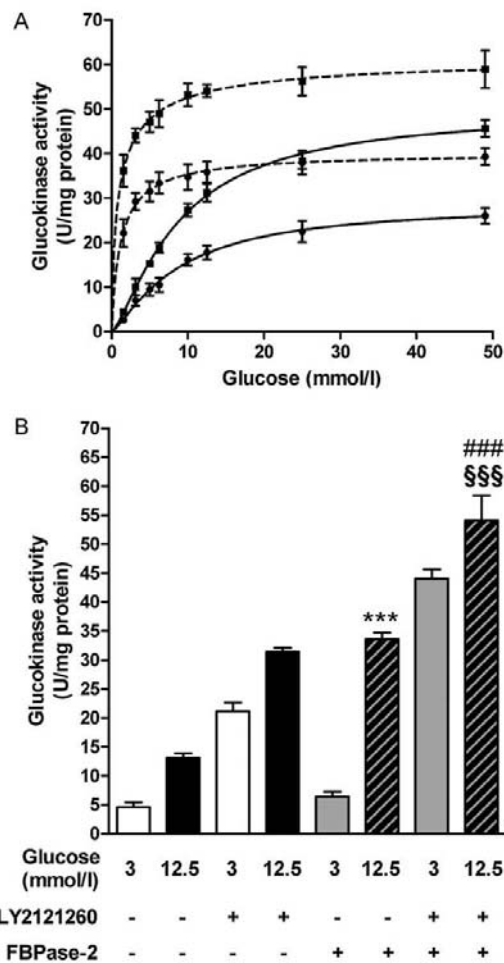


Fig. 3. Effect of the glucokinase activator LY2121260 on glucokinase enzyme activities in the presence of FBPase-2. Recombinant beta cell glucokinase was incubated for 5 min without (black circles, solid line) or in the presence of 10 $\mu\text{mol/l}$ LY2121260 (black circles, dashed line). Recombinant beta cell glucokinase was incubated for 20 min with FBPase-2 in a molar ratio of 1:5 without (black square, solid line) or in the presence of 10 $\mu\text{mol/l}$ LY2121260 (black square, dashed line). After addition of the indicated glucose concentration enzyme activities were measured spectrophotometrically. Shown are means \pm SEM in U/mg protein from 3 independent experiments. (B) Comparison of glucokinase enzyme activities measured at 3 (white and gray bars) or 12.5 (black and gray striped bars) mmol/l glucose with or without FBPase-2 and 10 μM LY2121260 as indicated. *** $p < 0.001$ compared to control at 12.5 mmol/l glucose; \$\$\$ $p < 0.001$ compared to LY2121260 alone at 12.5 mmol/l glucose; **** $p < 0.001$; compared to FBPase-2 alone at 12.5 mmol/l glucose (ANOVA/Bonferroni's test).

4. Discussion

Glucokinase is the glucose sensor in pancreatic beta cells and the key regulator of glucose metabolism in liver [1–4]. Therapeutically activation of glucokinase is a promising concept to counteract hyperglycemia in individuals with type 2 diabetes. For this purpose several chemical glucokinase activators have been developed recently [10,25–29]. The activators bind to an allosteric site of the glucokinase protein. Thereby they prevent relaxation to the super-opened inactive conformation and increase enzyme activity [10–12,32,33]. However, little is known on how these activators interfere with the endogenous posttranslational gluco-

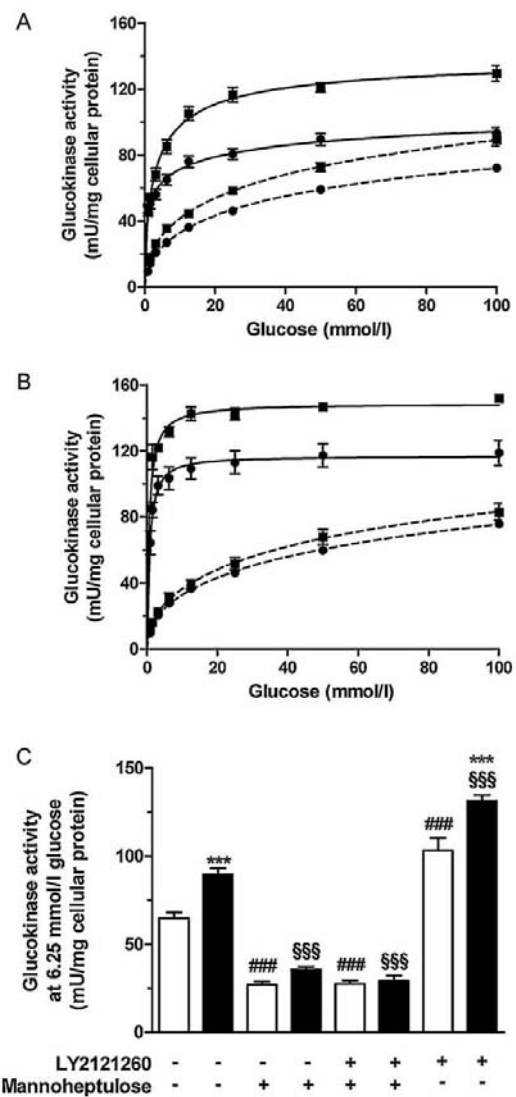


Fig. 4. Effects of the glucokinase activator LY2121260 and mannoheptulose on glucokinase enzyme activities in cell extracts of RINm5F-GK and RINm5F-GK-PFK-2/FBPase-2 cells. Glucokinase enzyme activities were measured spectrophotometrically after sonication of RINm5F-GK cells (circles) and RINm5F-GK-PFK-2/FBPase-2 cells (squares) and 5 min incubation without (A, solid line), in the presence of 10 mmol/l mannoheptulose (A, dashed line), in the presence of 10 $\mu\text{mol/l}$ LY2121260 (B, solid line), or in the presence of both 10 $\mu\text{mol/l}$ LY2121260 and 10 mmol/l mannoheptulose (B, dashed line). Shown are means \pm SEM in mU/mg cellular protein from 4 independent experiments. (C) Comparison of glucokinase enzyme activities in cell extracts of RINm5F-GK cells (white bars) and RINm5F-GK-PFK-2/FBPase-2 cells (black bars) in the presence of LY2121260 and mannoheptulose measured at 6.25 mmol/l glucose. *** $p < 0.001$ compared to untreated RINm5F-GK cells; \$\$\$ $p < 0.001$ compared to untreated RINm5F-GK-PFK-2/FBPase-2 cells; **** $p < 0.001$ RINm5F-GK-PFK-2/FBPase-2 cells compared with likewise treated RINm5F-GK cells (ANOVA/Bonferroni's test).

kinase regulation in beta cells and liver. Glucokinase inhibition through the glucokinase regulatory protein in liver is counteracted by glucokinase activators [32,33]. In this study glucokinase activation by the endogenous interaction partner PFK-2/FBPase-2 and the chemical compound LY2121260 was studied.

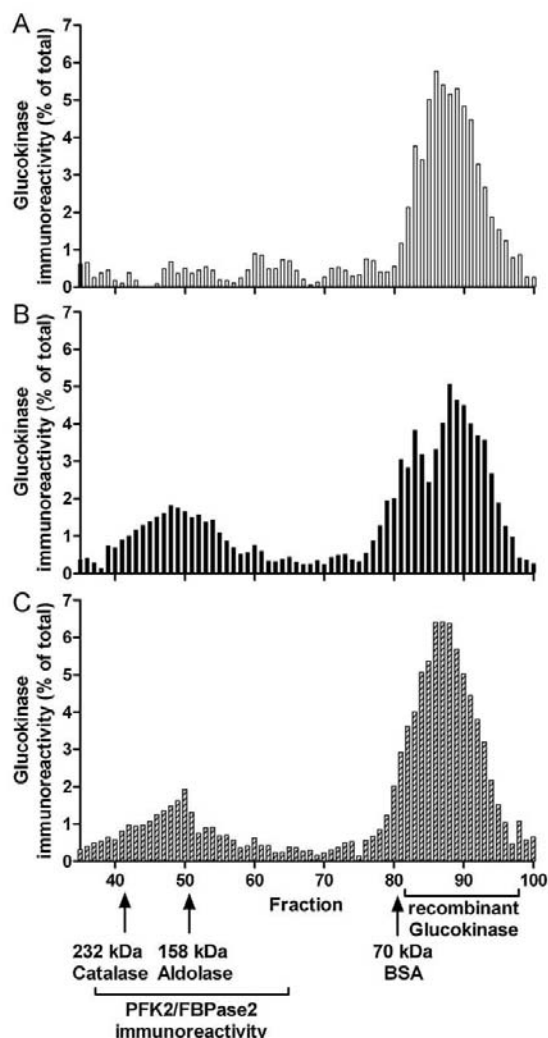


Fig. 5. Glucokinase-PFK-2/FBPase-2 complex formation in RINm5F cells depending on LY2121260. Protein extracts of RINm5F-GK cells (A) and RINm5F-GK-PFK-2/FBPase-2 cells (B and C) were incubated for 1 h with 25 mmol/l glucose alone (A and B) or together with 10 μM LY2121260 (C) and separated by gel filtration 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.

In agreement with previous studies [16,19,20] both the bifunctional enzyme PFK-2/FBPase-2 and LY2121260 increased the maximal velocity of glucokinase. While the low affinity for glucose was maintained during activation through PFK-2/FBPase-2, LY2121260 increased the affinity of glucokinase for glucose. The results of the present study demonstrate for the first time, that the glucokinase:PFK-2/FBPase-2 complex was not diminished by the glucokinase activator LY2121260. Using different approaches we could show that LY2121260 was able to further activate glucokinase in the presence of PFK-2/FBPase-2. This combined effect of the physiological and the pharmacological activator significantly increased insulin secretion at stimulatory glucose concentrations. The importance of PFK-2/FBPase-2 expression for

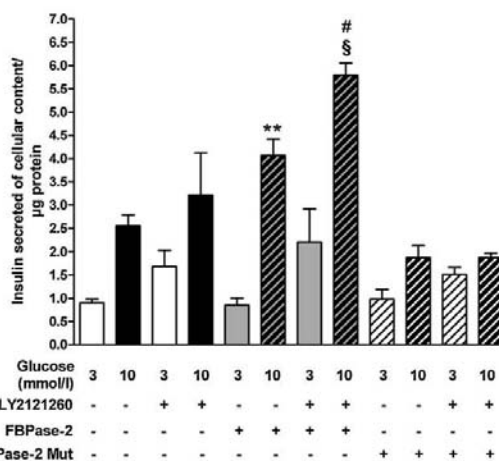


Fig. 6. Effect of the glucokinase activator LY2121260 on glucose-induced insulin secretion in the presence of PFK-2/FBPase-2. Insulin-secreting MING cells were transfected with EYFP (white and black bars), EYFP-PFK-2/FBPase-2 (gray and gray striped bars), or EYFP-PFK-2/FBPase-2-Mut (white striped and black striped bars) and cultured for 48 h. Cells were starved for 1 h and thereafter stimulated for 1 h with 3 (white, gray and white striped bars) or 10 (black, gray striped and black striped bars) mmol/l glucose with or without 10 μM LY2121260 as indicated. Insulin secretion is shown as insulin secreted per cellular insulin content and protein. Data are expressed as means ± SEM of 3 individual experiments. ***p* < 0.01 compared to control at 10 mmol/l glucose; #*p* < 0.05 compared to LY2121260 alone at 10 mmol/l glucose; \$*p* < 0.05 compared to FBPase-2 alone at 10 mmol/l glucose (ANOVA/Bonferroni's test).

the insulin secretory action of the glucokinase activator LY2121260 could be confirmed with a binding deficient mutant PFK-2/FBPase-2 protein. In the presence of this mutant PFK-2/FBPase-2, insulin secretion was not increased by LY2121260.

The 7-carbon sugar mannoheptulose is a competitive inhibitor of glucokinase [31,34] and like glucose stabilizes the closed glucokinase conformation [35,36]. It has been shown that glucokinase activators not only increase the affinity of glucokinase for its substrate glucose but also for its competitive inhibitor mannoheptulose [27,29]. Thus, in the presence of mannoheptulose glucokinase activators did not increase glucokinase activity and insulin secretion [27,29]. Likewise, LY2121260 did also not activate glucokinase in the presence of mannoheptulose. In contrast, PFK-2/FBPase-2 was able to activate glucokinase with increasing glucose concentrations in the presence of mannoheptulose. This can be explained by the fact, that glucose displaced the competitive inhibitor mannoheptulose from the catalytic site with increasing glucose concentrations. For this process a free conformational equilibration of glucokinase is required, which is supported by PFK-2/FBPase-2, but hindered by LY2121260.

The heptose mannoheptulose has a high affinity to the catalytic center of glucokinase [31]. To prove that the configuration of the hydroxyl group at the 4th carbon atom is crucial for the binding to glucokinase, the effect of sedoheptulose, the epimer of mannoheptulose, was determined. And in fact sedoheptulose did not inhibit glucokinase. Furthermore the action of perseitol, the sugar alcohol of mannoheptulose [37], with the same configuration of the hydroxyl group at the 4th carbon atom on glucokinase was analyzed. Interestingly, perseitol could be identified in the present study as a new inhibitor of glucokinase. LY2121260 was at least in part able to counteract inhibition by perseitol. It can be hypothesized therefore that the alcohol perseitol stabilizes rather an intermediate glucokinase conformation than the closed one. Thus, LY2121260, by promoting the closed conformation, can

induce periseitol release and make the binding site accessible for glucose.

Unfortunately, an analysis of glucokinase inhibition by periseitol in intact cells was not possible, since neither insulin-secreting MIN6 and RINm5F cells nor COS cells took up this sugar alcohol. Thus, it can be assumed that neither the GLUT 1 glucose transporter, mainly expressed in COS cells [38], nor the GLUT 2 glucose transporter, mainly expressed in beta cells [39], can channel periseitol in contrast to mannoheptulose. The fructose transporter GLUT 5 which has been proposed to mediate intestinal uptake of sugar alcohols [38] is not expressed in pancreatic beta cells [40].

In conclusion, this study proves that PFK-2/FBPase-2 facilitates the glucose effect on the glucokinase conformational equilibrium thereby activating the enzyme. In addition, it could be demonstrated that the bifunctional enzyme PFK-2/FBPase-2 and the glucokinase activator LY2121260 have an additive activating effect on glucokinase enzyme activity thereby maximizing the insulin secretory capacity of the beta-cells.

Acknowledgements

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**The ubiquitin proteasome system regulates the stability and activity of the
glucose sensor glucokinase in pancreatic beta cells**

Short title: Ubiquitination of glucokinase

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Synopsis:

The ubiquitin proteasome system is important to maintain pancreatic beta cell function. Inhibition of the proteasome significantly reduced glucose-induced insulin secretion. Key regulators of the stimulus-secretion cascade seem to be affected by protein misfolding, if the proteasome is down-regulated as recently reported in humans with type 2 diabetes. It remains unknown, however, whether the glucose sensor enzyme glucokinase is involved in this process. A direct interaction between glucokinase and ubiquitin could be shown in vivo by fluorescence resonance energy transfer suggesting regulation of glucokinase by the proteasome. After proteasome inhibition glucokinase activity was significantly reduced in MIN6 cells, whereas the protein content was increased indicating protein misfolding. Enhancing the availability of chaperones by cycloheximide could induce refolding and restored glucokinase activity. Glucokinase aggregation due to proteasome blocking with MG132, bortezomib, epoxomicin or lactacystin could be detected in MIN6 cells, primary beta cells and hepatocytes using fluorescence-based assays. Glucokinase aggresome formation proceeded microtubule assisted and was avoided by cycloheximide. Thus, our results provide support for glucokinase misfolding and aggregation in case of a diminished capacity of the ubiquitin proteasome system in pancreatic beta cells. In the type 2 diabetic situation this could contribute to reduced glucose-induced insulin secretion.

Key words:

glucokinase, ubiquitin proteasome system, pancreatic beta cells, aggresomes, protein misfolding, glucose-induced insulin secretion

INTRODUCTION

The cellular protein abundance and turnover rate is controlled by the translation rate and the ubiquitin proteasome system. Under physiological conditions the proteasome degrades ubiquitinated proteins, once their specific lifetime has been reached [1]. In case of oxidative and endoplasmic reticulum stress, the ubiquitin proteasome system eliminates misfolded proteins [1, 2]. In pancreatic beta cells the balance between protein synthesis, folding and degradation is important to maintain proper glucose-induced insulin secretion [2]. Incubation of human, mouse and rat pancreatic islets as well as MIN6 beta cells with a proteasome inhibitor resulted in a significantly reduced glucose-induced insulin secretion, whereas the insulin content remained unchanged [3-5]. Thus, in addition to insulin biosynthesis [3], regulation of proteins involved in the pathway of insulin secretion such as the ATP-dependent potassium and voltage-dependent calcium channels by the ubiquitin proteasome system has been discussed [2, 5, 6]. However, the impact of the ubiquitin proteasome system on other key regulators of the insulin secretion machinery is far less understood, but of growing interest [2].

Recently, it has been reported that in pancreatic islets of humans with type 2 diabetes the activity of the ubiquitin proteasome system is reduced compared to healthy individuals [7, 8]. Using quantitative RT-PCR it was demonstrated that genes of the 26S proteasome were down-regulated [7]. This is in agreement with another recently published study, reporting accumulation of ubiquitinated proteins in beta cells of humans with type 2 diabetes [8]. Furthermore low expression and activity of the ubiquitin carboxyl-terminal hydrolase L1 has been shown in this study indicating reduced access of ubiquitinated proteins to the proteasome in humans with type 2 diabetes [8]. Thus, both studies underline that alterations in the ubiquitin proteasome system can contribute to the pathogenesis of type 2 diabetes.

The glucose phosphorylating enzyme glucokinase catalyses not only the first step of glycolysis in beta cells, but has the important role of the glucose sensor [9-13]. Knockdown of glucokinase resulted in a loss of glucose-induced insulin secretion [14]. In pancreatic beta cells glucokinase is mainly regulated on the posttranslational level [15-22]. The enzyme is activated by glucose and the bifunctional enzyme phosphofructo-2-kinase/fructose-2,6-bisphosphatase [16, 18, 19, 22]. In vitro it has been described that recombinant glucokinase is ubiquitinated, suggesting quality control of glucokinase by the proteasome [23]. Using a multiplexing strategy the stability of ~ 8000 human proteins has been investigated. This cell-based approach revealed for the long-living protein GAPDH (gene ID 2597) a lifetime index of 6.24. For glucokinase (gene ID 2645) a significantly lower lifetime index of 4.34 has been observed, indicating medium half-life and regulation by the proteasome [24]. Thus, the aim of this study was to investigate the impact of the ubiquitin proteasome system on glucokinase activity in pancreatic beta cells.

EXPERIMENTAL

Materials

Z-Leu-Leu-Leu-al (MG132) and the ProteoStat® Aggresome detection Kit were from ENZO Life Sciences (Lörrach, Germany), cycloheximide (CHX), camptothecin and nocodazole from Sigma-Aldrich (Steinheim, Germany), epoxomicin from Merck (Darmstadt, Germany) and bortezomib from NEB (Ipswich, MA, USA). All primers, including random hexamer primers, and chemicals for TaqMan assays were obtained from Life Technologies (Karlsruhe, Germany). The RevertAid™ H Minus M-MuLV reverse transcriptase was from Fermentas (St. Leon-Rot, Germany). The GoTaq® Taq polymerase was purchased from Promega (Mannheim, Germany), and dNTPs from Genecraft (Münster, Germany).

Plasmids

The cDNA of human beta cell glucokinase was subcloned in frame (ApaI and BamHI restriction sites) in the pDendra2-C vector (Evrogen, Moscow, Russia). Generation of pECFP-glucokinase has been described previously [25]. ECFP was replaced by mCherry (Clontech, Mountain View, CA) using AgeI and BspEI to generate mCherry-glucokinase. Tubulin from the pEYFP-Tub vector (Clontech) was subcloned into the Dendra2-C vector using XhoI and BamHI to generate Dendra2- α -tubulin. pEGFP-C1-Ub (addgene plasmid 11928) and pEGFP-C1-Ub^{K0,G76V} (addgene plasmid 11932) were generated and deposited by Dantuma et al. [26]. The enhanced green fluorescence protein (EGFP) was replaced by the enhanced yellow fluorescence protein (EYFP) using AgeI and BsrGI restriction sites to produce EYFP-ubiquitin and EYFP-ubiquitin^{K0,G76V}. The pcDNA3.3-d2eGFP vector (addgene plasmid 26821) was deposited by Rossi et al. [27, 28].

Cell culture, isolation of primary cells and treatment

MIN6 and COS cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Biochrom, Berlin, Germany) supplemented with 25 mmol/l glucose, 10% (vol/vol) fetal calf serum, 10 U/ml penicillin, 10 μ g/ml streptomycin, and 2 mmol/l glutamine in a humidified atmosphere at 37°C and 5% CO₂. MIN6 cells were transfected with the vector DNA by the use of jetPEI (Qbiogene, Montreal, Canada). Stable clones were selected through resistance against G418 (1,200 μ g/ml) and characterized further for Dendra2-glucokinase by fluorescence microscopy. Cells were seeded and grown 48 h prior to experiments. Pancreatic islets were isolated from 11 weeks old female NMRI mice by collagenase digestion in bicarbonate-buffered Krebs-Ringer solution. Beta cells were obtained by dispersion in calcium-free Krebs-Ringer solution and kept in RPMI-1640 medium supplemented with 5 mmol/l glucose for 24 h. Primary hepatocytes were isolated from C57/BL6 mice [25]. Isolated cells were suspended in Williams' medium E supplemented with 10 mmol/l glucose, 5% (vol/vol) fetal calf serum, 1 x 10⁻⁴ mmol/l dexamethasone, and 1 x 10⁻⁵ mmol/l insulin. Hepatocytes were seeded at a density of 4 x 10⁴ cells on glass cover slips and incubated for 24 h in a humidified atmosphere at 37°C and 5% CO₂. MG132 (10 μ mol/l), epoxomicin (1 μ mol/l), CHX (10 μ g/ml), and bortezomib (10, 50, 100 nmol/l) were added to the culture medium for 3 or 12 h, and camptothecin (2.5, 5, 10 μ mol/l) for 36 h.

Glucokinase enzyme activity

MIN6 cells were seeded in 10 cm dishes at a density of 3 x 10⁶ cells and treated as indicated. Glucokinase enzyme activity was measured in an enzyme-coupled photometric assay [22]. Cells were homogenized in phosphate buffered saline (pH 7.4) and the protein concentration was quantified by a Bio-Rad protein assay. Enzyme activity measured spectrophotometrically at 1 mmol/l glucose was subtracted from the value obtained at 10 mmol/l glucose to compensate for cellular hexokinase activity.

Measurement of insulin secretion

MIN6 cells were seeded in six-well plates at a density of 3 x 10⁵ cells and treated as indicated. Finally, cells were incubated for 1 h in bicarbonate-buffered Krebs-Ringer solution without glucose supplemented with 0.1% albumin and thereafter stimulated for 1 h with 3 or 25 mmol/l glucose. Thereafter, 1 ml of the incubation buffer from each well was carefully harvested and gently centrifuged to remove detached cells. In these supernatants the secreted insulin was determined by RIA against a rat insulin standard. The protein concentration was quantified by Bradford protein assay.

MTT cell viability and caspase-3 activity assays

MIN6 cells were seeded in 96-well plates at a density of 20,000 cells and treated as indicated. Cell viability was then determined by a microplate-based MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [29]. MIN6 cells were seeded at a density of 50,000 cells per well and caspase-3 positive cells were detected using the NucView™ 488 Caspase-3 assay kit (Biotium, Hayward, CA, USA). The total number of cells was determined by nuclear staining with 1 µmol/l Hoechst33342 for 15 min and corresponding fluorescence images were taken automatically using a HC 387/11-433(50)-517(40)-613(60) filter set (AHF Analysentechnik, Tübingen, Germany) and quantified with a scanR/ IX81 microscope system (Olympus, Hamburg, Germany) as described previously [30].

Western Blot analyses

MIN6 cells and hepatocytes were seeded in 6 cm dishes at a density of 2.5×10^5 cells and treated as indicated. Finally, cells were homogenized by sonication in lysis buffer and insoluble material was pelleted by centrifugation. Total cellular protein was fractionated by reducing 10% SDS-PAGE and electroblotted to polyvinylidene difluoride membranes. Nonspecific binding sites of the membranes were blocked with Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, NE, USA) for 30 min at room temperature. Blots were incubated with glucokinase antibody (sc-7908, diluted 1:200, or sc-1980, diluted 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for beta cell and liver glucokinase, respectively and GAPDH (sc-137179, diluted 1:2000, Santa Cruz Biotechnology) at 4 °C overnight, followed by incubation with the appropriate IRDye secondary antibodies (Li-Cor Biosciences) for 30 min at room temperature. Specific protein bands were visualized in the Li-Cor Infrared Imaging System (Li-Cor Biosciences). Quantification of specific protein bands was performed using Odyssey application software (Li-Cor Biosciences).

qPCR-analysis

Total RNA was isolated from MIN6 or MIN6 Dendra-GK cells using the Qiagen RNeasy Kit (Qiagen, Hilden, Germany) and glucokinase gene expression was measured with TaqMan assays (human GK: Hs01564555_m1, mouse GK: Mm00439129_m1). The reactions were performed using the ViiA 7 real-time PCR system (Life Technologies) [31, 32]. The housekeeping genes Actb, G6pdx, Gapdh, Ppia were used for normalization. Data analysis was performed with qBasePLUS (Biogazelle, Zulte, Belgium) [31, 32].

Immunocytochemistry

MIN6 cells and hepatocytes at a density of 80,000 cells, and primary beta cells from 20 islets were seeded on glass cover slips and treated as indicated. Thereafter cells were washed twice with PBS and fixed overnight with 4% paraformaldehyde. Cells were permeabilized for 30 min with 0.5% Triton, 3 mM EDTA in ProteoStat® aggresome assay buffer (pH 8) on ice. Nonspecific binding sites were blocked with 1% BSA, 1% Triton-X 100 in PBS for 20 min at room temperature. Cells were immunostained as described previously [30] with goat anti glucokinase antibody (sc1979, diluted 1:100, Santa Cruz Biotechnology) and the appropriate Cy2 secondary antibody (diluted 1:200, Dianova, Hamburg, Germany) for 1 h at room temperature. Thereafter cells were stained with the aggresome detection reagent containing nuclear staining (Hoechst 33342) and Proteostat® red dye. Cells were mounted with Mowiol/DABCO anti-photobleaching mounting media (Sigma, St. Louis, MO). Finally, samples were analyzed with an Olympus Fluoview1000 confocal microscope system (Olympus). For sequential scanning the multi-line argon laser (488 nm) to excite Cy2, the diode laser (405 nm) to excite Hoechst 33342, and the DPSS-laser (559 nm) to excite the Proteostat® dye and an UPLSAPO 60 x 1.35 NA oil-immersion objective were used. Hoechst33342 was detected at a 425 to 475 nm wavelength range together with Cy2 at 500 to

545 nm. Light emitted by ProteoStat[®] dye was detected at 575 to 675 nm. Image processing was done using FV10-ASW software (Olympus).

Live-cell imaging

Analyses of MIN6 Dendra2-GK cells and fluorescence resonance energy transfer (FRET) experiments were performed with a cellR/Olympus IX81 inverted microscope system (Olympus) equipped with a Cellcubator (Olympus) using 60% humidity, 37°C and 5% CO₂. Glass bottom dishes were fixed on the microscope stage and images were taken with an UPLSAPO 60 x 1.35 NA oil-immersion objective (Olympus). ET 470/40 and 556/20 filter sets (AHF) were used to excite green and red Dendra2, respectively. Dendra2 green and red emission were detected using a 510/30-630/100 dualband filter (AHF). A S360/40 filter (AHF) was used for photoconversion of Dendra2. Image processing was done using xcellence software (Olympus). The FRET setup has been described previously [19]. Sensitized emission-based FRET efficiency (FRET_N) was calculated from the enhanced cyan fluorescence protein (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. [33].

Data analyses

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

RESULTS

Effect of MG132 and CHX on glucose-induced insulin secretion and cell viability

MIN6 beta cells were responsive to a glucose stimulus, whereas treatment with the reversible proteasome inhibitor MG132, the translation inhibitor CHX, or a combination of both for 12 h led to a loss of insulin secretion at basal (Fig. 1A) and stimulating (Fig. 1B) glucose concentrations. Treatment with bortezomib, a proteasome inhibitor specific for the β5 proteasome subunit, significantly reduced both basal and glucose-induced insulin secretion in a concentration-dependent manner. Cell viability determined by MTT test was reduced only by 10 and 30% for bortezomib (50 nmol/l) and MG132 and/or CHX, respectively (Fig. 1C) and the number of caspase-3 positive MIN6 cells was only 2-fold increased (Fig. 1D). In contrast, camptothecin with a comparable effect on insulin secretion (Fig. 1A and B) decreased cell viability by 50% (Fig. 1C) and resulted in a 7-fold higher number of caspase-3 positive cells (Fig. 1D). Therefore loss of cell viability could not be the major reason for the loss of insulin secretion observed by proteasome inhibition.

Effect of MG132, epoxomicin, lactacystin, and CHX on glucokinase enzyme activity and protein content

Treatment of MIN6 cells with MG132 significantly decreased the activity of the glucose sensor enzyme glucokinase by around 50% (Fig. 2A). Although CHX had no effect on glucokinase activity compared to control, co-treatment with MG132 could counteract the reduction caused by MG132. In agreement with previous studies [17], glucokinase overexpressing MIN6 Dendra2-GK cells showed a higher glucokinase activity compared to MIN6 cells alone (data not shown). Treatment with MG132 and/or CHX resulted in a significantly reduced activity compared to control (Fig. 2B). To test whether the reduction in glucokinase activity was due to a reduced protein content, western blot analyses were performed. Quantification of the typical line at ~ 53 kDa showed that MG132 treatment of

MIN6 cells resulted in a significant increase in the glucokinase protein content (Fig. 2C). 12 h treatment of MIN6 cells with more specific proteasome inhibitors, namely epoxomicin (1 $\mu\text{mol/l}$) or lactacystin (10 $\mu\text{mol/l}$) resulted also in a higher glucokinase protein content (data not shown). Glucokinase protein expression was reduced after 12 h treatment with the translation inhibitor CHX (Fig. 2C). Simultaneous inhibition of the proteasome and translation resulted in a protein expression comparable to the control situation. To test if also long-living proteins are influenced by treatment with MG132 and/or CHX, the immunoreactivity of GAPDH was quantified (Fig. 2D). In contrast to the effect on glucokinase, 12 h treatment with the inhibitors alone or in combination had no significant effect on GAPDH expression in MIN6 cells. qPCR analyses revealed a downregulation of endogenous glucokinase gene expression after treatment with MG132 and/or CHX after 3 and 12 h by $\sim 40\%$ and 60% , respectively (Suppl. Fig. 1A). MIN6 Dendra2-GK cells showed a comparable response to MG132 and CHX with respect to Gck expression (Suppl. Fig. 1B), whereas human GK mRNA under control of the CMV promoter was solely significantly influenced by 12 h treatment with CHX (Suppl. Fig. 1C) indicating that inhibition of the translation has only an effect on transgene expression.

Aggregation of endogenous glucokinase after proteasome inhibition

Glucokinase aggregation after inhibition of the proteasome was further analyzed by fluorescence microscopy. MIN6 cells were treated with MG132 and/or CHX for 12 h and thereafter stained for glucokinase and the ProteoStat dye specific for aggresomes [34]. In MIN6 cells, glucokinase was homogeneously distributed in the cytoplasm (Fig. 3A). Inhibition of degradation by MG132 resulted in small aggregates which colocalize with the aggresome detection marker (Fig. 3B). The aggregates were distributed in the cytoplasm with a somewhat higher density in the perinuclear region. Treatment with CHX had nearly no effect on glucokinase distribution (Fig. 3C). Simultaneous inhibition of translation and degradation reduced cytoplasmic glucokinase aggregation (Fig. 3D). Likewise treatment with the $\beta 5$ proteasome subunit specific inhibitor bortezomib evoked formation of glucokinase aggregates in the cell cytoplasm which colocalizes with the aggresome detection marker. For quantification (Fig. 3H), regions of interest were determined in the cytoplasm. Proteasome inhibition increased the range of pixel intensity significantly representing aggregation of GK, whereas co-treatment with CHX could diminish this aggregation significantly. Aggregation of endogenous glucokinase could be confirmed in primary mouse beta cells (Fig. 3F). 12 h treatment with MG132 resulted in glucokinase aggregates in the cytoplasm, which colocalize with the aggresome detection marker (Fig. 3G).

Impact of MG132 and CHX on glucokinase in hepatocytes

Glucokinase expression in hepatocytes is significantly higher compared to pancreatic beta cells [35]. After incubation at high glucose glucokinase showed a homogenous distribution in mouse hepatocytes (Fig. 4A). Inhibition of the proteasome evoked a strong aggregation of glucokinase in the cytoplasm with significant colocalization with the ProteoStat dye (Fig. 4B). Simultaneous inhibition of the translation could counteract this aggregation (Fig. 4C). Proteasome inhibition resulted in an increase of glucokinase in relation to GAPDH expression in hepatocytes (Fig. 4D), which could be partly diminished by co-treatment with CHX.

Interaction of glucokinase with ubiquitin

In vitro ubiquitination of glucokinase has been reported previously using a rabbit reticulocyte lysate system [23]. In addition to wild type conjugation-efficient EYFP-ubiquitin, a mutant conjugation-deficient EYFP-ubiquitin^{K0,G76V} lacking all internal lysine residues and the C-terminal glycine residue [26] was used to investigate ubiquitination of glucokinase in vivo. EYFP-ubiquitin (Fig. 5A) and EYFP-ubiquitin^{K0,G76V} (Fig. 5B) were visible in the cytoplasm and the nucleus in MIN6. EYFP-ubiquitin fluorescence was highest in the nucleus. In the

cytoplasm a punctuate pattern was observed, partly co-localized with glucokinase. In contrast, the conjugation-deficient mutant showed a homogeneous expression pattern throughout the cytoplasm and the nucleus. For FRET analyses, ECFP-GK and EYFP as control, EYFP-ubiquitin WT or mutant were co-overexpressed in COS cells. In ECFP-GK and EYFP-ubiquitin co-overexpressing cells FRET was 4.5 times higher than in the negative control (Fig. 5C). In contrast, no interaction was detected with the EYFP-ubiquitin^{K0,G76V} mutant (Fig. 5C).

Effect of proteasome inhibition on mCherry-glucokinase in comparison to d2EGFP in MIN6 cells

The enhanced green fluorescence protein (eGFP) has a long half-life and high protein stability and showed homogenous distribution in mammalian cells after transfection [27, 28]. Peptide sequences which are rich in proline, glutamic acid, serine, and threonine, shortly named PEST-sequences are known to serve as a signal for protein degradation. Subcloning of a PEST-sequence to the C-terminus of the eGFP resulted in d2eGFP, an eGFP variant with short half-life [27, 28]. MIN6 cells were transiently transfected with mCherry-glucokinase, a red fluorescence glucokinase fusion protein and d2eGFP and analysed over 9 h (Fig. 6A). The horizontal intensity profile demonstrated distribution of both fluorescent proteins at 0, 3, 6 and 9 h (Fig. 6B). MG132 induced aggregation of mCherry-glucokinase (Fig. 6C) as observed for endogenous glucokinase in MIN6 cells (Fig. 3). Aggregated mCherry-glucokinase near the cell nucleus gained over time, reflected by the strong increase in fluorescence at the aggregation point in the profile (Fig. 6D). In contrast, despite the short half-life d2eGFP showed a homogenous distribution without aggregation (Fig. 6C). The intensity profile is comparable at 0, 3, 6 and 9 h (Fig. 6D). Treatment of MIN6 cells with epoxomicin (Suppl. Fig. 2A and B) and bortezomib (Suppl. Fig. 2 C and D) resulted in the same effect.

Aggregation of Dendra2-GK in MIN6 cells

MIN6 cells, transiently transfected (Fig. 7A-C) or stably overexpressing a Dendra2 fusion protein with glucokinase (Fig. 7D-L) were incubated with MG132 alone or in combination with CHX. No differences were detected in the distribution of Dendra2-glucokinase compared to endogenous glucokinase (Fig. 3), mCherry-glucokinase (Fig. 6), and between transiently transfected or stably overexpressing cells (Fig. 7A and D). The proteasome inhibitors MG132 (Fig. 7B and E), lactacystin (Fig. 7G), epoxomicin (Fig. 7H-L), and bortezomib (Suppl. Fig. 3B and C) evoked aggregation of Dendra2-glucokinase in the cytoplasm with a higher localization around the nucleus, whereas co-treatment with CHX could counteract this aggregation (Fig. 7C and F). In the three-dimensional slice view the compactness of Dendra2-glucokinase aggregation is clearly visible (Fig. 7I). Smaller aggregates arranged around a dense core, resulting in a growing globular aggregate. The homogeneous distribution of Dendra2-glucokinase (Fig. 7J) changed to small (Fig. 7K) and globular (Fig. 7L) aggregates 6 and 8 h after treatment with epoxomicin, respectively. The fluorescent protein Dendra2 was homogeneously distributed in MIN6 cells (Suppl. Fig. 3D) and did not aggregate after 12 h treatment with MG132 (Suppl. Fig. 3E) or bortezomib (Suppl. Fig. 3F). Susceptibility of the tubulin network to proteasome inhibitors, because of hyperacetylation of α -tubulin has been reported previously [36]. Dendra2- α -tubulin showed a comparable filamentous structure in MIN6 cells (Suppl. Fig. 3G) as the endogenous protein [17]. After 12 h treatment with MG132 or bortezomib tubulin bundles and focal aggregates were detectable (Suppl. Fig. 3H and I).

Live-cell imaging of MIN6 Dendra-GK cells after treatment with MG132

Using the special feature of Dendra2, irreversible photoconversion [37], the effect of the proteasome inhibitor MG132 on folded (red Dendra2) and newly synthesized Dendra2-GK (green Dendra2) in living cells can be analysed over time. MIN6 Dendra2-GK cells were treated with MG132 and fluorescence images of living cells were taken every hour after initial irreversible photoconversion of green to red Dendra2 (Fig. 8A). After 5 h of treatment small aggregates were visible in the cytoplasm with a somewhat higher concentration near the nucleus. After 9 h the small aggregates accumulated in the perinuclear region forming a dense globular aggregate. After 24 h a high amount of aggregated Dendra2-GK was detectable around the nucleus. Nocodazole, an inhibitor of microtubule polymerisation prevented accumulation of the aggregates near the nucleus and after 12 h small aggregates were distributed in the whole cytoplasm (Fig. 8B).

DISCUSSION

In pancreatic beta cells the rate of transcription and translation of the glucose sensor glucokinase appears to be very stable [38, 39]. Regulation of glucokinase enzyme activity occurs mainly on the posttranslational level [21] by interaction with the bifunctional enzyme phosphofructo-2-kinase/fructose-2,6-bisphosphatase [16, 18, 19, 22], cytoplasmic compartmentation via binding to insulin granules mediated through S-nitrosylation [15, 20], to mitochondria [12], and association with tubulin filaments [17]. However, another aspect of posttranslational regulation, namely glucokinase protein stability remains *in vivo* an unexploited field. Unstructured regions and amino acids which are correlated with protein instability were mostly postulated from *in vitro* experiments. However, a global protein stability profiling, which has been recently performed in mammalian cells indicates the need for cellular approaches to investigate protein half-life regulation [24]. In the present study we could demonstrate ubiquitination of glucokinase *in vivo* by FRET analyses, as previously demonstrated *in vitro* [23].

Thermal instability, structural changes and self-association of glucokinase mutations causing maturity onset diabetes of the young have been shown [40-45]. We investigated aggregation of the wild-type glucokinase protein using the computer algorithm TANGO [46]. This program predicted between ¹⁸¹valine and ¹⁸⁵leucine, and between ³⁰²valine and ³¹¹aspartic acid regions with high beta sheet aggregation propensities. Taken additionally into account that the glucokinase protein has a high flexibility and can be present in at least five different conformations by undergoing extensive conformational changes [10, 47, 48] it becomes obvious that glucokinase has high susceptibility to protein misfolding and, thus, requires a sufficient control by the ubiquitin proteasome system.

Two decades ago the half-life of glucokinase in rat hepatocytes has been estimated to be 12.7 h [49]. In the same study the half-life of lactate dehydrogenase was 17 h, and the authors concluded that nonautophagic mechanisms are involved in cytoplasmic enzyme turnover [49]. Interestingly, the recently performed cellular proteome-scale protein-turnover analyses using the proteasome inhibitor MG132 suggested that both proteins are regulated by the proteasome and provided in agreement with the results obtained by Kopitz et al. [49] a higher lifetime index for lactate dehydrogenase (gene ID 3945) than for glucokinase (4.80 versus 4.34) [24]. Treatment of MIN6 cells with the proteasome inhibitor MG132 for 3 h resulted in an increase of the glucokinase protein by 50% [40]. Extending the incubation time to 12 h, which corresponds to the calculated half-life of the enzyme, we observed a comparable increase in glucokinase protein. As control GAPDH immunoreactivity was taken. The content of this long-living protein (lifetime index 6.24) remained unchanged. Glucokinase gene expression decreased, most likely as an adaptive process, after incubation with MG132, but notably did

not increase and, thus, cannot account for causing the higher glucokinase protein content. However, despite the high protein content we observed a 50% decrease in glucokinase activity, indicating formation of inactive misfolded glucokinase in the absence of proteasome activity. In fact, incubation of MIN6 cells with MG132 and in addition with CHX, a compound inhibiting translation and increasing the availability of chaperones, restored glucokinase activity to a certain extent.

On the single cell level we observed direct evidence for regulation of glucokinase by the ubiquitin proteasome system. Using a novel fluorescence-based assay [34] it could be demonstrated in MIN6 cells, primary mouse beta cells and hepatocytes that glucokinase is within aggresomes after inhibition of the proteasome and that this aggregation could be at least in part avoided by CHX through chaperone-mediated refolding. The same effect could be shown using different classes of proteasome inhibitors, namely the peptide aldehyde MG132, a reversible proteasome inhibitor, the peptide epoxyketone epoxomicin and the naturally occurring lactacystin, both irreversible proteasome inhibitors interacting with β subunits of the proteasome, and bortezomib, which has with the $\beta 5$ proteasome subunit a specific target structure [50]. Fluorescent fusion proteins of glucokinase were used in this study to investigate the process of protein aggregation in living MIN6 cells over time. We are aware that the glucokinase protein content in MIN6 cells after overexpression was somewhat higher than in primary beta cells and in the range of that of hepatocytes. However, aggregation due to proteasome inhibition resulted from transiently and stably overexpressed glucokinase in MIN6 cells in a manner comparable to that of the endogenous protein.

The use of a fusion protein between glucokinase and the photoconvertible protein Dendra2 [37] in time series analyses, which allows discrimination between older and newly synthesized protein argue in favour of microtubule assisted aggresome formation in beta cells. At the beginning of proteasome inhibition, small glucokinase containing aggresomes were distributed in the cytoplasm, whereas later on they formed bigger aggregates and were transported to a so-called microtubule organizing centre (MTOC) [51, 52], forming a “ribbon like” huge aggresome near the nucleus [53] (Fig. 8C). Consistently, blocking both microtubule-polymerisation and proteasomal degradation prevented formation at the MTOC. Because unfolded polypeptides can attach to such MTOC located aggresomes this is a dangerous knock-on process for the cell, which has been associated so far with neurodegenerative diseases, cancer and metabolic syndromes [53]. Impaired clearance of misfolded and ubiquitinated proteins due to reduced proteasome activity has been recently reported in patients with type 2 diabetes [7, 8]. In agreement with previous studies [3, 4] we demonstrated that insulin secretion is significantly reduced after inhibition of the proteasome. Despite its protective effect against MG132 induced glucokinase aggregation CHX could not restore insulin secretion, because it interrupts translation, and thus, biosynthesis of insulin and other key proteins of beta cells. An effect of MG132 on cell viability was detectable and has been described previously [3, 4], but in the used concentration of 10 $\mu\text{mol/l}$ the number of apoptotic cells was negligible compared to that of the cytotoxic quinoline alkaloid camptothecin, which had in our study a comparable effect on insulin secretion. In addition, bortezomib, at a concentration of 10 nmol/l , caused a significant aggregation of glucokinase and a reduction of glucose-induced insulin secretion by 80 %, but had virtually no effect on cell viability. This clearly indicates that beyond autophagy [2, 54], the ubiquitin proteasome system is essential for regulating key factors of the stimulus-secretion cascade in pancreatic beta cells.

In conclusion, we demonstrated that glucokinase is regulated by the ubiquitin proteasome system to avoid misfolding and reduced activity of the enzyme. In addition, our fluorescence-based single cell analyses suggest a microtubule assisted aggresome formation of misfolded proteins in beta cells. This helps to understand the pathogenesis of type 2 diabetes, where a

reduced proteasome activity has been reported [7, 8]. Inclusion of glucokinase in aggresomes in beta cells of patients with type 2 diabetes could impair glucose recognition and, thus, contribute at least in part to a reduced glucose-induced insulin secretion.

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FIGURES LEGENDS

Fig.1. Treatment with MG132, bortezomib and/or CHX results in loss of insulin secretion in MIN6 cells. MIN6 cells (white bars) were treated with MG132 (10 $\mu\text{mol/l}$, black bars), CHX (10 $\mu\text{g/ml}$, grey bars), MG132 + CHX (grey striped bars), bortezomib (10, 50, 100 nmol/l , white cross-striped bars) for 12 h, or camptothecin (2.5, 5, 10 $\mu\text{mol/l}$, white lengthwise striped bars) for 36 h. For insulin secretion cells were starved for 1 h (A, B) and thereafter stimulated with 3 mmol/l glucose (A) or 25 mmol/l glucose (B). Insulin secretion is expressed per insulin content per protein content. Cell viability was measured with the MTT assay (C) and caspase-3 positive cells were quantified using an automated fluorescence microscopy approach (D). Data are means \pm SEM of 3 - 10 experiments. $**p<0.01$, $***p<0.001$ compared to control, $^{\circ\circ}p<0.001$ compared to MG132 (ANOVA/Bonferroni's multiple comparison test).

Fig. 2. Treatment with MG132 reduces glucokinase enzyme activity but increases glucokinase protein content in MIN6 cells. MIN6 cells (white bars) were treated with MG132 (10 $\mu\text{mol/l}$, black bars), CHX (10 $\mu\text{g/ml}$, grey bars) or MG132 + CHX (grey striped bars) for 12 h. Cell extracts were prepared from MIN6 cells (A, C, D) or MIN6 Dendra2-GK cells (B) stably overexpressing glucokinase. Glucokinase enzyme activity (A, B) measured at 1 mmol/l glucose was subtracted from the value obtained at 10 mmol/l glucose to exclude the cellular hexokinase activity. Cell extracts (C, D) were analysed by SDS-PAGE and immunoblotted using antibodies against glucokinase and GAPDH. The glucokinase line (~ 53 kDa) (C) and GAPDH line (~ 30 kDa) (D) were quantified. Shown are means \pm SEM of 3 - 10 individual experiments. $*p<0.05$, $**p<0.01$, and $***p<0.001$ (ANOVA/Bonferroni's multiple comparison test).

Fig. 3. Proteasome inhibition evokes cytoplasmic aggregation of glucokinase in beta cells, partly avoidable by co-treatment with CHX. MIN6 cells (A, H white bar) were treated with MG132 (10 $\mu\text{mol/l}$, B, H black bar), CHX (10 $\mu\text{g/ml}$, C, H grey bar), MG132 + CHX (D, H grey striped bar) or bortezomib (50 nmol/l , E, H white cross-striped bar) for 12 h. Finally, cells were fixed and stained for glucokinase (green), for aggresomes (red) and for DNA (blue). Representative images are shown (A-E). Scale bar, 10 μm . For quantification (H) regions of interest were determined and the range of pixel intensity was measured as a marker of cellular aggregation. Data are expressed as means \pm SEM of 3 - 4 individual experiments. $***p<0.001$ compared to control, $\#p<0.05$, and $###p<0.001$ compared to MG132 treatment (ANOVA/Bonferroni's multiple comparison test). Primary mouse beta cells (F) were treated with MG132 (G) for 12 h. Finally, cells were fixed and stained for glucokinase (green), aggresomes (red) and DNA (blue). Images shown were obtained from z-stacks after deconvolution and are representative of 3 individual experiments. Scale bar, 5 μm .

Fig. 4. MG132 causes not only an increase in glucokinase protein but also cytoplasmic aggregation of glucokinase in hepatocytes. Primary hepatocytes (A) were treated with MG132 (10 $\mu\text{mol/l}$, B, D black bar), CHX (10 $\mu\text{g/ml}$, D grey bar) or MG132 + CHX (C, D grey striped bar) for 12 h. Finally, cells were fixed and stained for glucokinase (green), aggresomes (red) and DNA (blue). Representative images of 2 individual experiments are shown (A-C). Scale bar, 10 μm . Cell extracts were analysed by SDS-PAGE and immunoblotted using antibodies against glucokinase and GAPDH. Glucokinase expression is shown in relation to GAPDH expression (D). Data expressed as means \pm SEM of 3 individual experiments.

Fig. 5. In vivo detection of colocalization and interaction between glucokinase and ubiquitin. MIN6 cells were transiently transfected with ECFP-glucokinase and EYFP-ubiquitin WT (A) or the conjugation-deficient mutant EYFP-ubiquitin^{K0,G76V} (B).

Fluorescence images were taken in living cells 2 days after transfection. In the merged images, ECFP is depicted in green, EYFP in red, and co-localization is indicated in yellow. Sensitized emission-based FRET efficiency was calculated in COS cells (C) transfected with ECFP-GK and EYFP (white bar), ECFP-glucokinase and EYFP-ubiquitin WT (black bar) or EYFP-ubiquitin^{K0,G76V} (white striped bar). Shown are means \pm SEM of 3 individual experiments, *** $p < 0.001$ (ANOVA/Bonferroni's multiple comparison test).

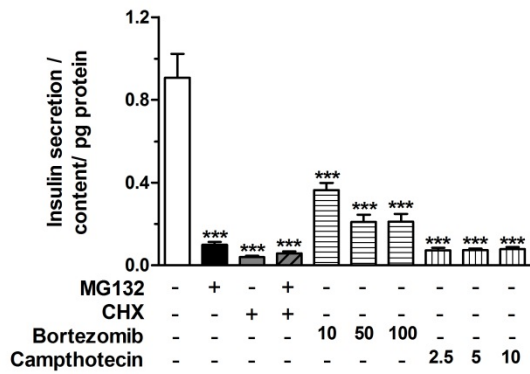
Fig. 6. MG132 evokes aggregation of mCherry-glucokinase but not of the short half-life protein d2eGFP in MIN6 cells. MIN6 cells were transiently transfected with mCherry-glucokinase and d2eGFP. 24 h after transfection medium without MG132 (A) or with MG132 (10 $\mu\text{mol/l}$, C) was added and fluorescence pictures were taken directly, and after 3, 6 and 9 h. The horizontal fluorescence intensity profiles (B, D) of mCherry-glucokinase (red) and d2eGFP (green) without (B) or with MG132 (D) illustrate the distribution at the red line in the corresponding images. Scale bar, 10 μm . Fluorescence images shown are representative of 3 individual experiments.

Fig. 7. Aggregation pattern of Dendra2-glucokinase after treatment with MG132, lactacystin or epoxomicin in MIN6 cells. MIN6 cells transiently transfected with Dendra2-glucokinase (A-C) or MIN6 Dendra2-GK cells (D-H) were incubated with MG132 (10 $\mu\text{mol/l}$, B, E), MG132 + CHX (C, F), lactacystin (10 $\mu\text{mol/l}$, G) or epoxomicin (1 $\mu\text{mol/l}$, H-L) for 12 h. Three-dimensional distribution pattern of cytoplasmic aggregated Dendra2-glucokinase after treatment with epoxomicin (I). MIN6 Dendra2-GK cells were treated with epoxomicin and fluorescence images of the same cell were taken directly (J), and 6 (K), and 8 h (L) thereafter. Fluorescence images shown are representative of 2 - 4 individual experiments.

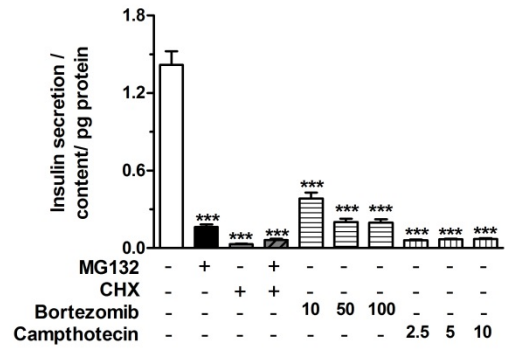
Fig. 8. Time-resolved description of Dendra2-glucokinase aggresome formation after treatment with MG132. (A) MIN6 Dendra2-GK cells were treated with MG132 (10 $\mu\text{mol/l}$). Directly after addition of the proteasome inhibitor MG132, part of the green Dendra2-glucokinase was photoconverted to the stable red form. Representative fluorescence images are shown 5, 7, 9, 12 and 24 h after photoconversion. (B) MIN6 Dendra2-GK cells were treated with MG132 (10 $\mu\text{mol/l}$) and nocodazole (10 $\mu\text{g/ml}$) for 12 h. Images shown are representative of two individual experiments. (C) The aggregation process of glucokinase starting in the cell periphery and resulting in aggresomes close to the nucleus is depicted in the scheme.

Figure 1

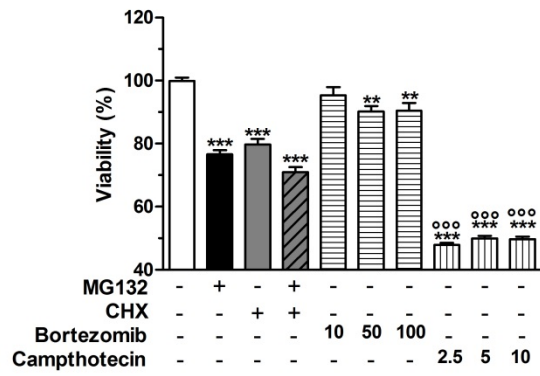
A



B



C



D

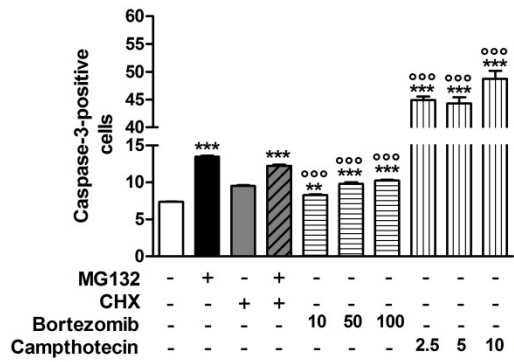


Figure 2

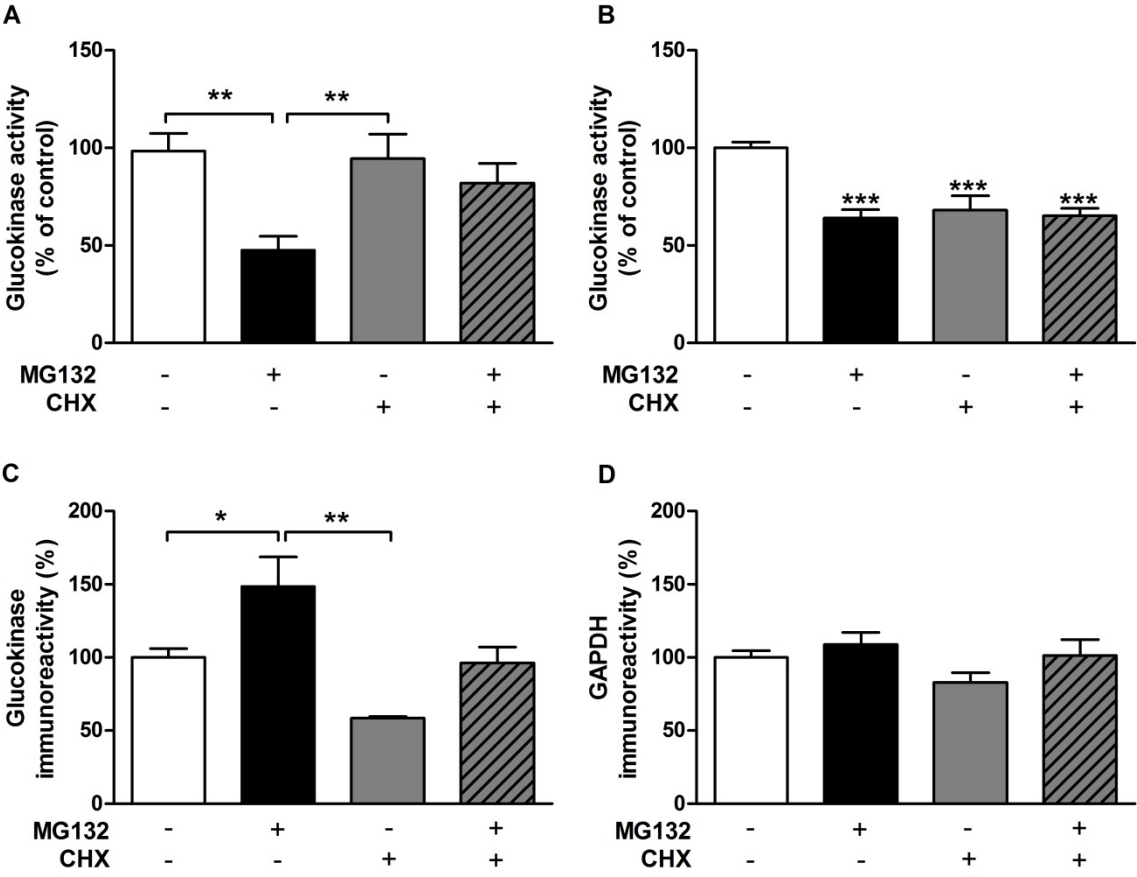


Figure 3

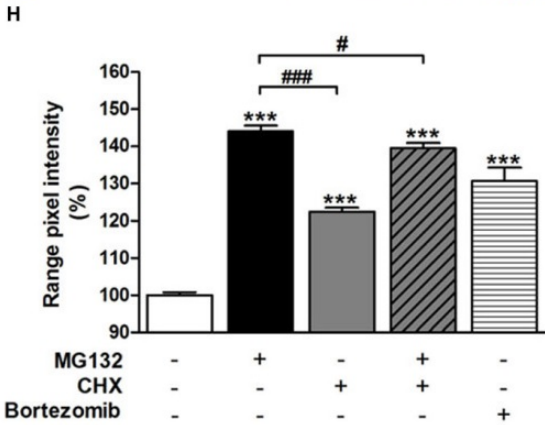
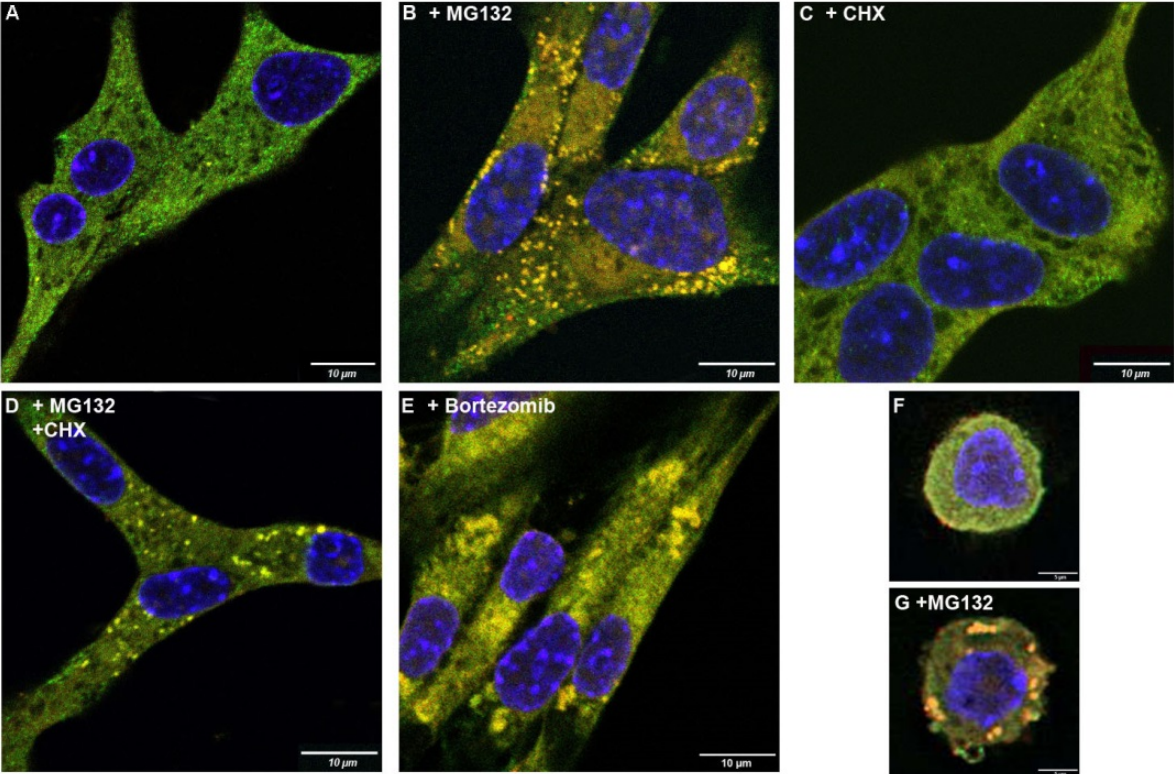


Figure 4

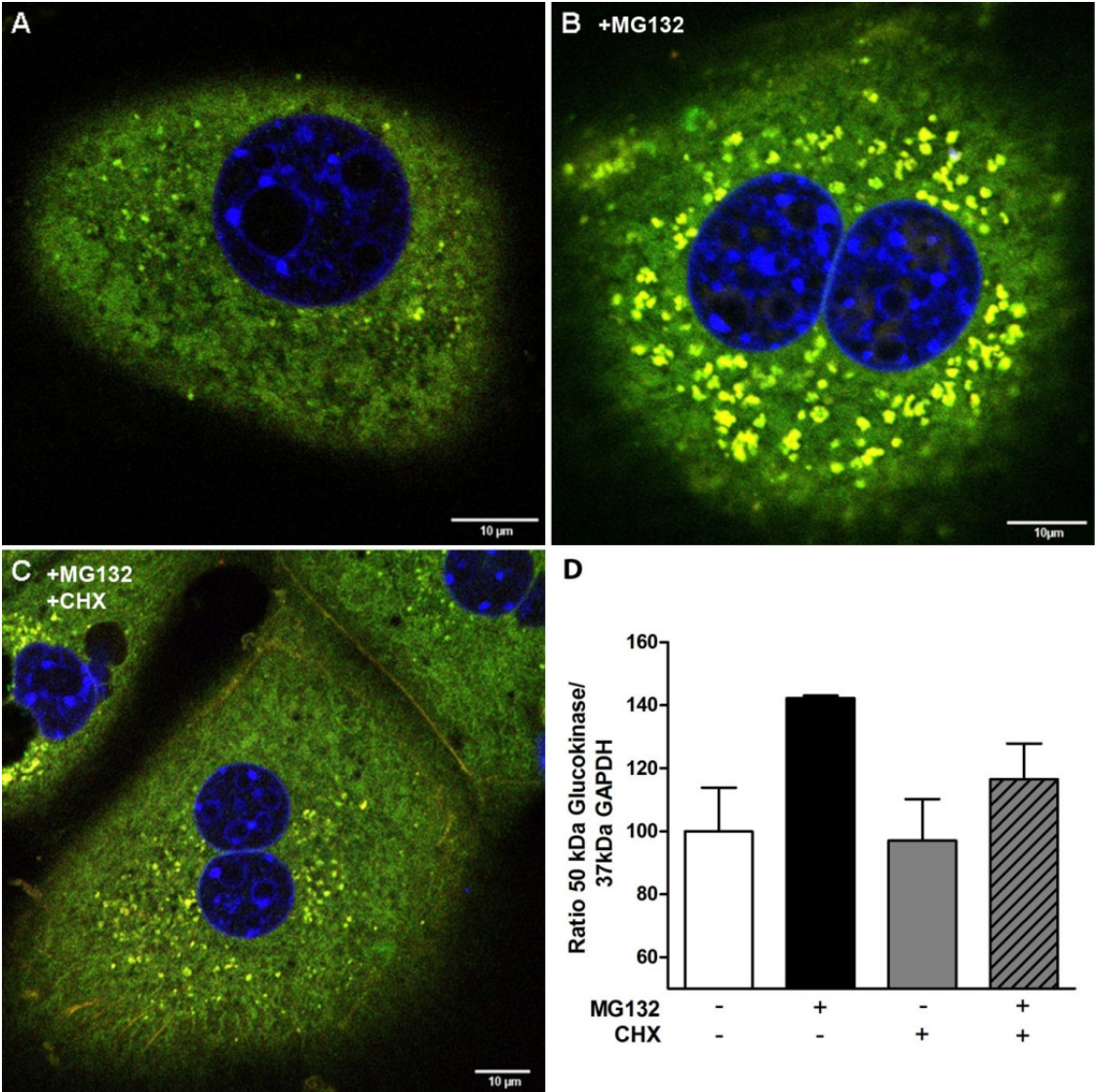


Figure 5

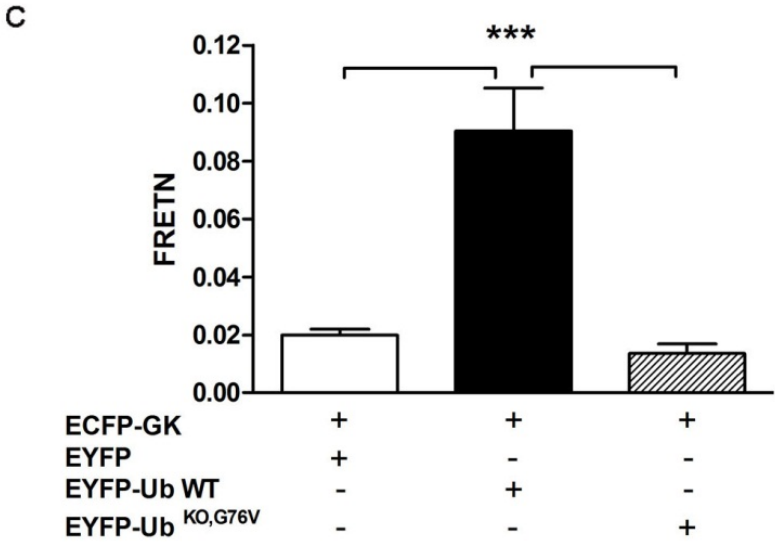
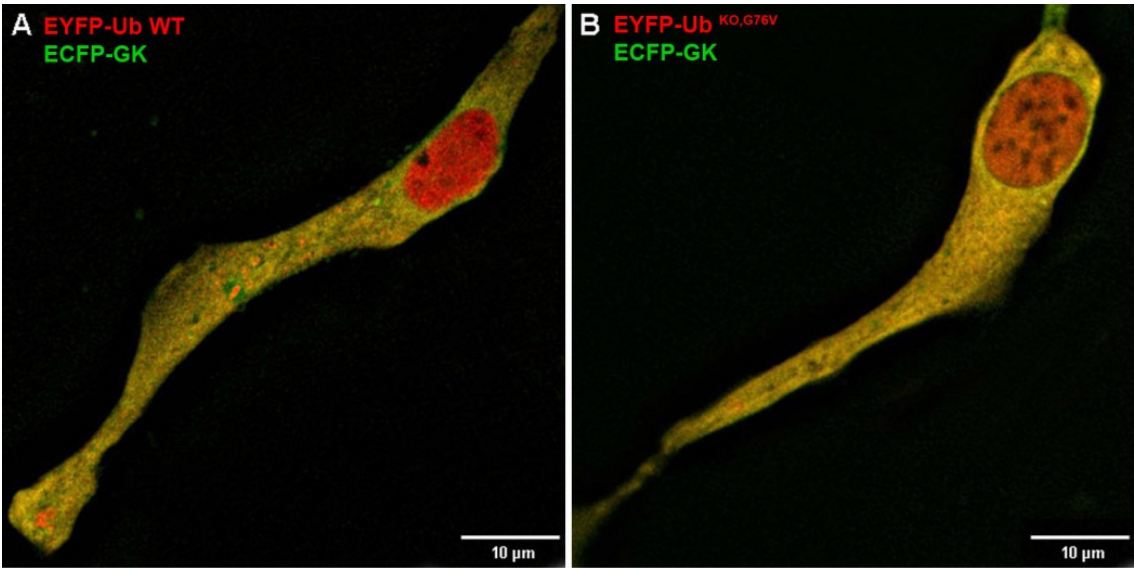


Figure 6

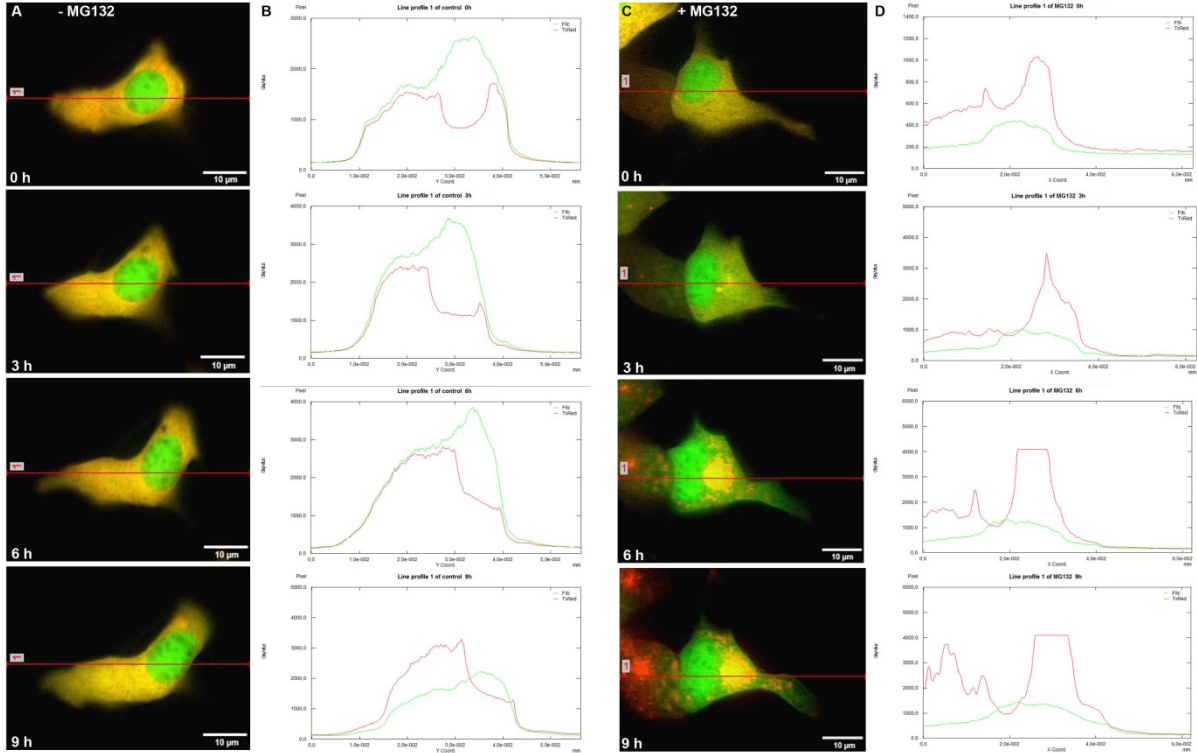


Figure 7

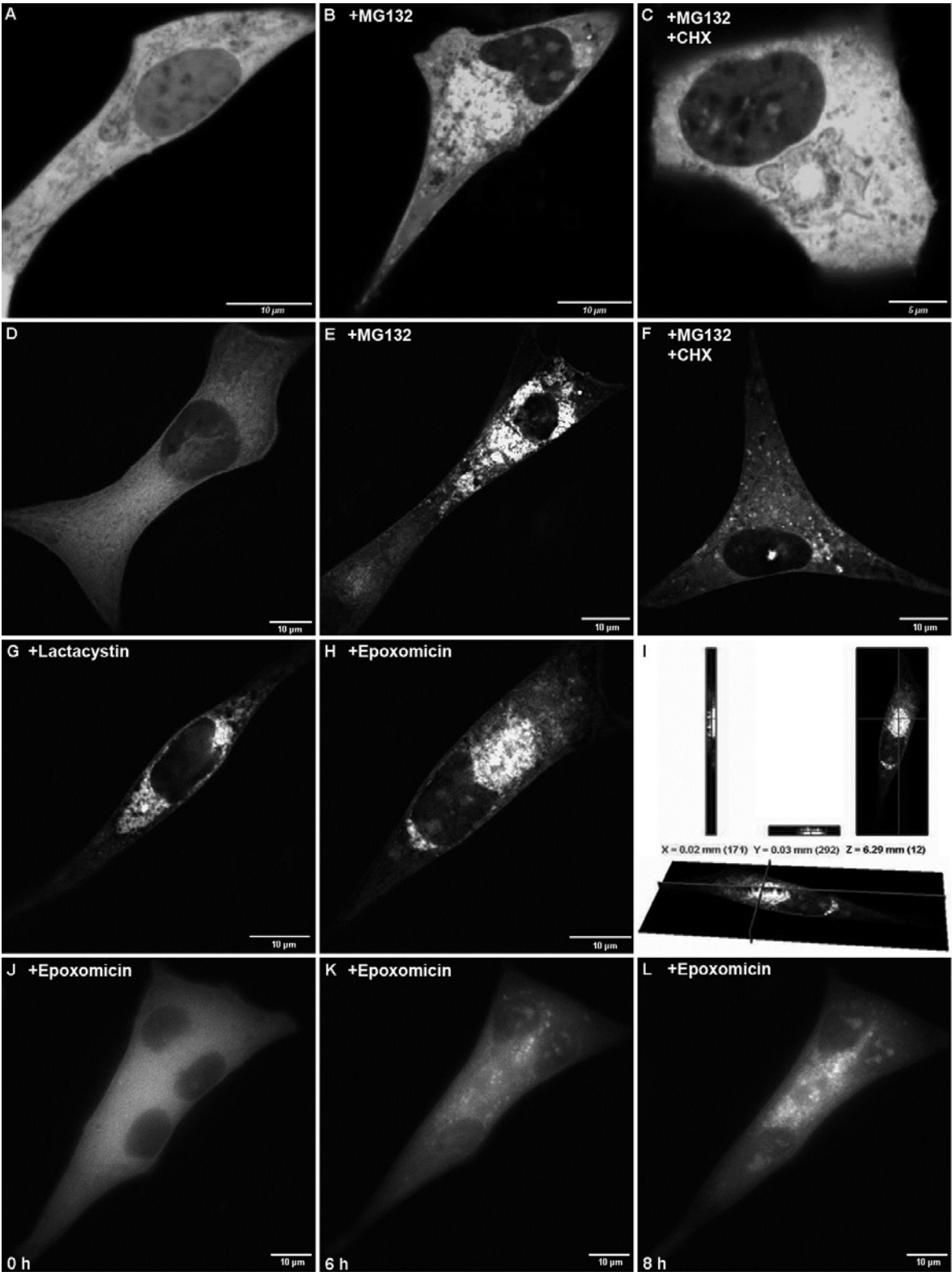
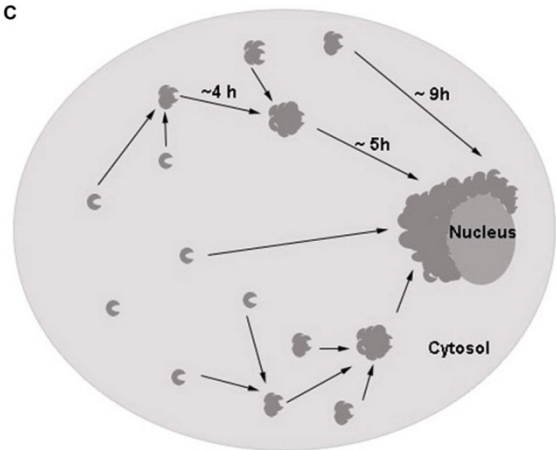
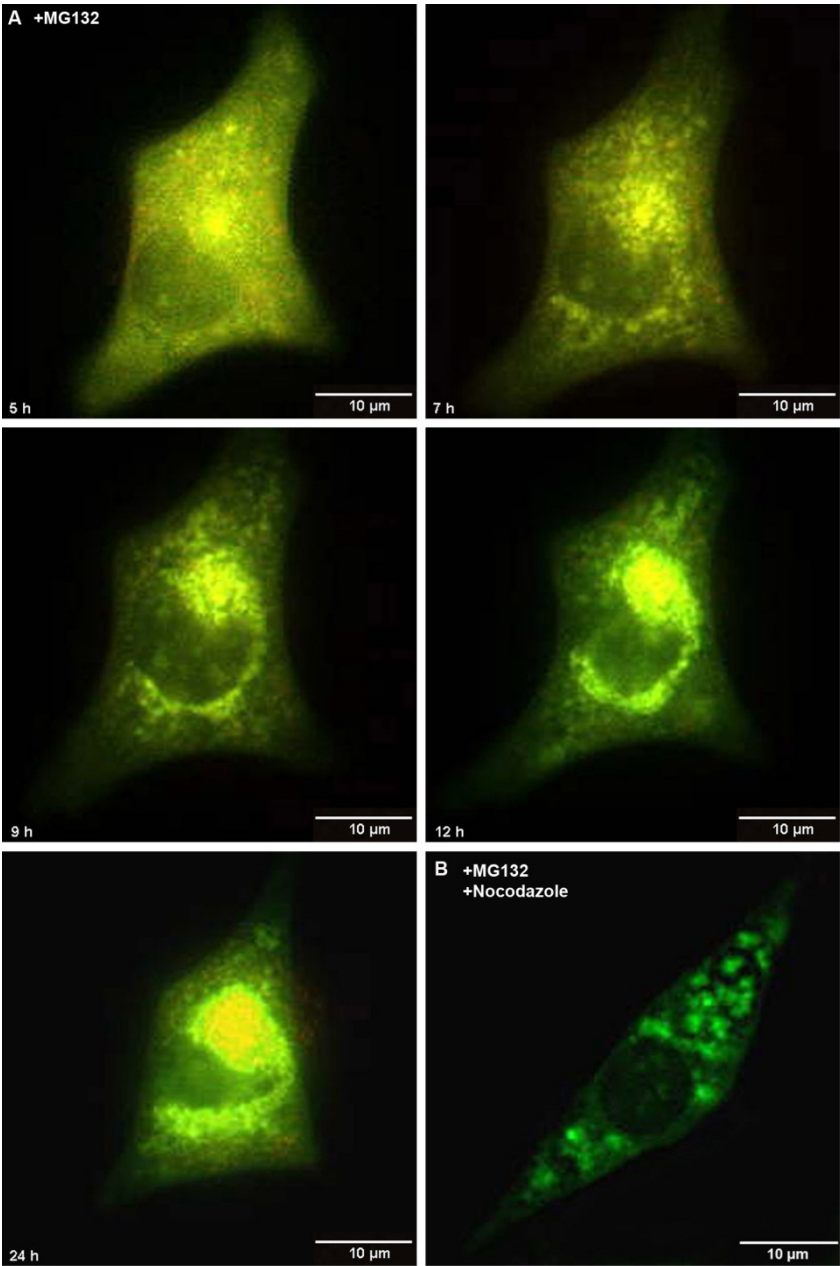


Figure 8



Supplement

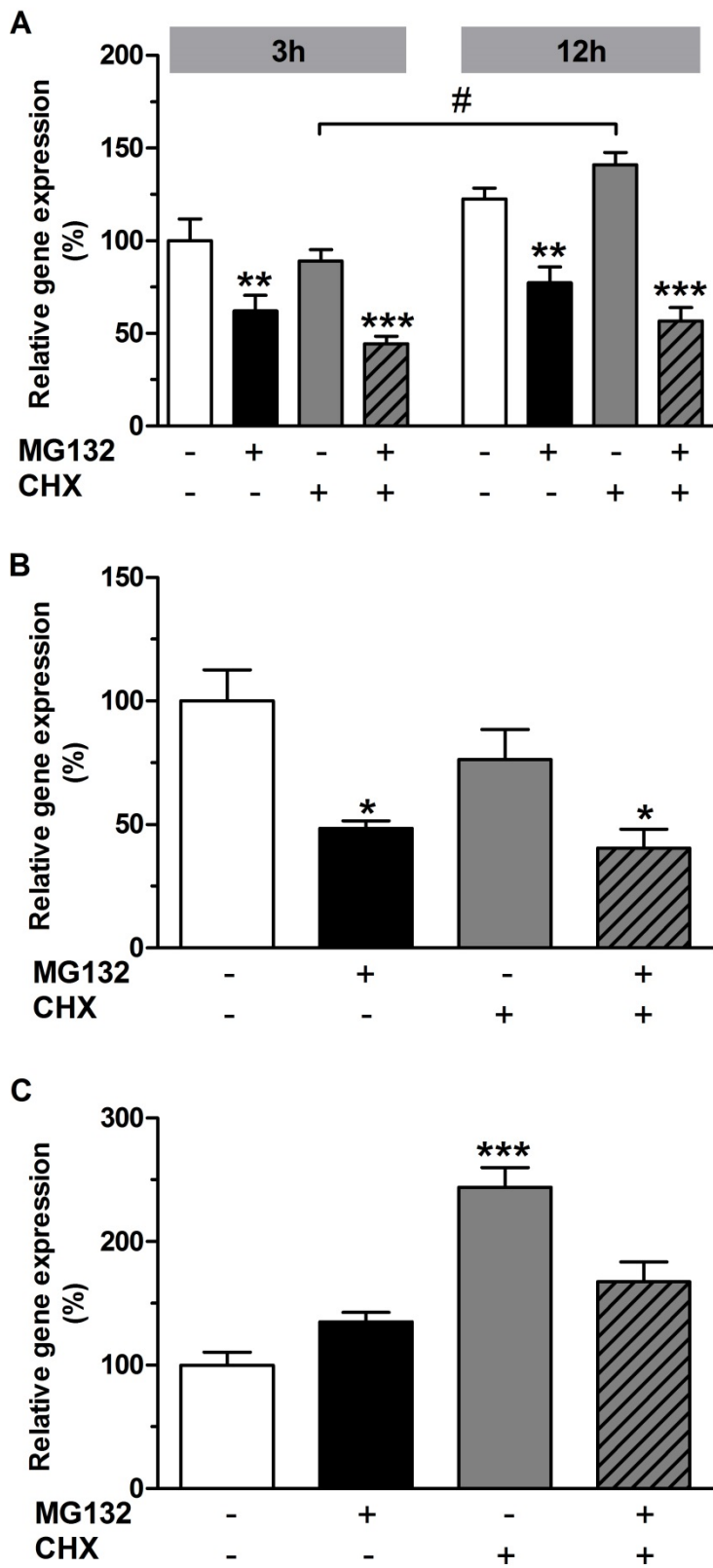
FIGURES LEGENDS

Suppl. Fig. 1. Downregulation of endogenous glucokinase gene expression after incubation with MG132 in MIN6 cells. (A) MIN6 cells (white bars) were treated with MG132 (10 $\mu\text{mol/l}$, black bars), CHX (10 $\mu\text{g/ml}$, grey bars) or MG132 + CHX (grey striped bars) for 3 or 12 h. Finally, total RNA was isolated and expression of mouse Gck gene (TaqMan assay Mm00439129_m1) was analysed. (B, C) MIN6 Dendra2-GK cells (white bars) were treated with MG132 (black bars), CHX (grey bars) or MG132 + CHX (grey striped bars) for 12 h. Finally, total RNA was isolated and the expression of the mouse Gck gene (B) and the overexpressed human GCK gene (C) was analysed. Relative expression levels normalized to housekeeping genes ActB, G6pdx, Gapdh, Ppia are shown. Data expressed as means \pm SEM of 3 - 6 experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to control, # $p < 0.05$ compared to 3 h (ANOVA/Bonferroni's multiple comparison test).

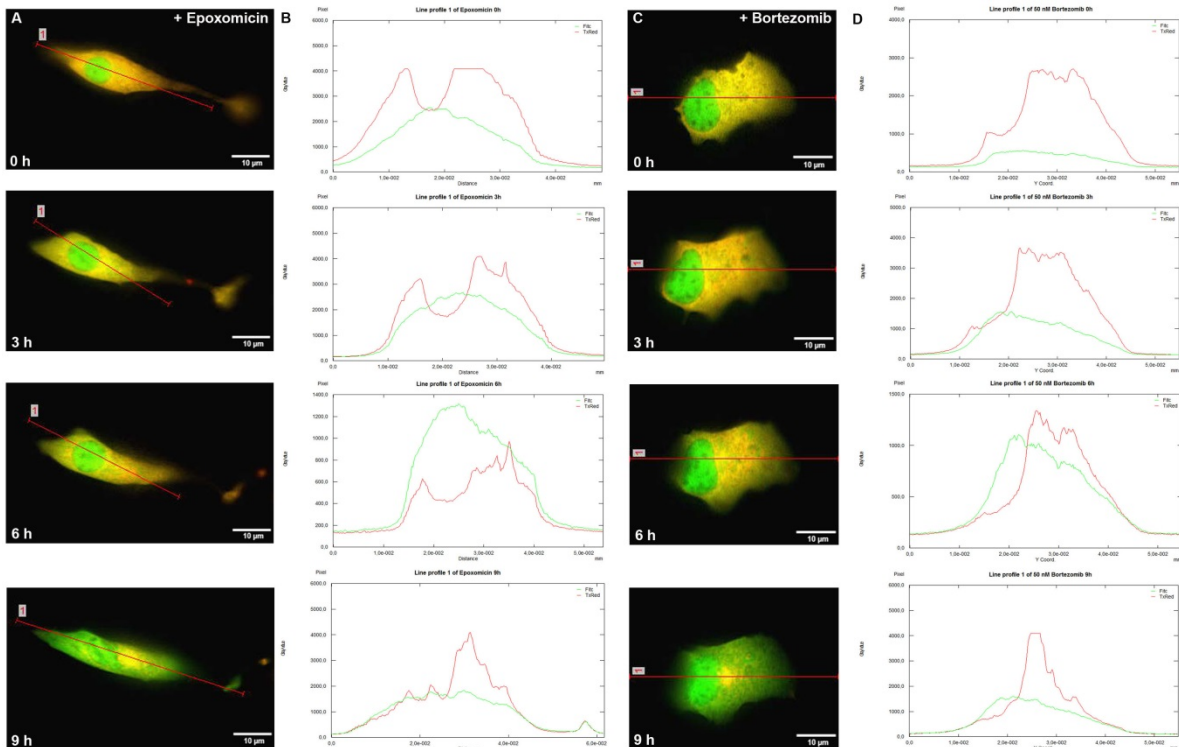
Suppl. Fig. 2. Epoxomicin and bortezomib evoke aggregation of mCherry-glucokinase but not of the short half-life protein d2eGFP in MIN6 cells. MIN6 cells were transiently transfected with mCherry-glucokinase and d2eGFP. 24 h after transfection medium with epoxomicin (1 $\mu\text{mol/l}$, A) or with bortezomib (50 nmol/l , C) was added and fluorescence pictures were taken directly, and after 3, 6 and 9 h. The horizontal fluorescence intensity profiles (B, D) of mCherry-glucokinase (red) and d2eGFP (green) with epoxomicin (B) or bortezomib (D) illustrate the distribution at the red line in the corresponding images. Scale bar, 10 μm . Fluorescence images shown are representative of 2 individual experiments.

Suppl. Fig. 3. MG132 and bortezomib evoke aggregation of Dendra2-glucokinase or Dendra2-tubulin but not of Dendra2 in MIN6 cells. MIN6 Dendra2-GK cells (A - C) and MIN6 cells transiently transfected with Dendra2 (D - F), Dendra2-tubulin (G - I) were incubated with bortezomib (10 nmol/l , B) or bortezomib (50 nmol/l , C, F, I) or MG132 (10 $\mu\text{mol/l}$, E, H), for 12 h. Scale bar, 10 μm . Fluorescence images shown are representative of 2 individual experiments.

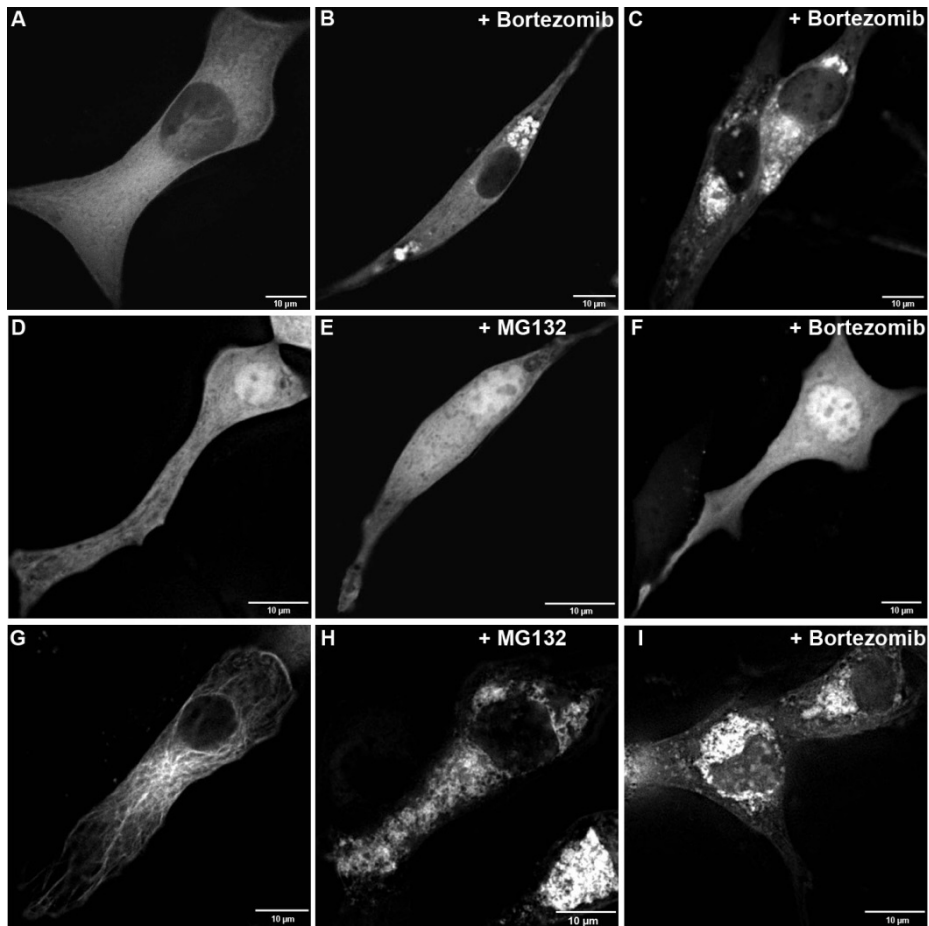
Suppl. Figure 1



Suppl. Figure 2



Suppl. Figure 3



Identification of the ubiquitin-like domain of midnolin as a new glucokinase interaction partner*

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*Running title: *Glucokinase interaction with the ULD of midnolin*

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Key words: Glucokinase; insulin secretion; pancreatic islets; midnolin; parkin; ubiquitin; ubiquitin-like domain; two-hybrid system; fluorescence resonance energy transfer;

Background: Glucokinase activity is regulated on the posttranslational level in pancreatic beta-cells.

Results: Searching a pancreatic islet yeast two-hybrid library the ubiquitin-like domain of midnolin was identified to bind and inhibit glucokinase at low glucose thereby reducing insulin-secretion.

Conclusion: Midnolin is a new glucokinase interaction partner.

Significance: This is the first report demonstrating midnolin protein expression in pancreatic beta cells.

SUMMARY

Glucokinase acts as a glucose sensor in pancreatic beta cells. Its posttranslational regulation is important, but not yet fully understood. Therefore, a pancreatic islet yeast two-hybrid library was produced and searched for glucokinase binding proteins. A

protein sequence containing a full-length ubiquitin-like domain was identified to interact with glucokinase. Mammalian two-hybrid and fluorescence resonance energy transfer analyses confirmed the interaction between glucokinase and the ubiquitin-like domain in insulin-secreting MIN6 cells and revealed highest binding affinity at low glucose. Overexpression of parkin, an ubiquitin E3 ligase exhibiting an ubiquitin-like domain with high homology to the identified, diminished insulin secretion in MIN6 cells but had only some effect on glucokinase activity. Overexpression of the elucidated ubiquitin-like domain or midnolin, containing exactly this ubiquitin-like domain, significantly reduced both intrinsic glucokinase activity and glucose-induced insulin secretion. Midnolin has been to date classified as a nucleolar protein regulating mouse development. However, we could not

confirm localization of midnolin in nucleoli. Fluorescence microscopy analyses revealed localization of midnolin in nucleus and cytoplasm and co-localization with glucokinase in pancreatic beta cells. In addition we could show that midnolin gene expression in pancreatic islets is up-regulated at low glucose and that the midnolin protein is highly expressed in pancreatic beta cells and also in liver, muscle, and brain of the adult mouse and cell lines of human and rat origin. Thus, the results of our study suggest that midnolin plays a role in cellular signaling of adult tissues and regulates glucokinase enzyme activity in pancreatic beta cells.

Expression of the glucose phosphorylating enzyme glucokinase in pancreatic beta cells, liver, and neuroendocrine cells is tissue-specifically regulated by two distinct promoters in a single gene [1-3]. In pancreatic beta cells glucokinase catalyzes the rate-limiting step for glucose-induced insulin secretion and in liver glucokinase regulates glucose uptake [3-10]. Complete knockdown of glucokinase in mice is lethal and animals heterozygous for the gene knockout develop diabetes [11]. This emphasizes the pivotal role of glucokinase as a glucose sensor maintaining blood glucose homeostasis [3-10].

Hormones stimulate glucokinase regulation on the posttranslational level [12-14]. However, in pancreatic beta cells in particular glucose regulates glucokinase protein content and activity [15, 16]. Glucose induces large-scale disorder-order transitions shifting the glucokinase protein to a closed conformation with high phosphorylating activity [17-20]. In

addition ubiquitination of glucokinase and the resulting degradation by the proteasome are relevant quality control steps ensuring cellular glucokinase protein content with high phosphorylating capacity [21-24].

Finally specific protein interactions modulate the phosphorylating activity of glucokinase in a tissue-specific manner. In liver the glucokinase regulatory protein inhibits glucokinase at low glucose and in addition shuttles the protein to the nucleus [25-28]. Both in liver and pancreatic beta cells the bifunctional enzyme phosphofructo-2-kinase/fructose-2,6-bisphosphatase activates glucokinase at high glucose [4, 29-32]. Thus, these soluble regulators attenuate or potentiate glucokinase activity, respectively, in a glucose-dependent manner. Although compartmentation of glucokinase by association with tubulin filaments [33] and binding to insulin granules, a process which is controlled by interaction with nitric oxide synthase and S-nitrosylation [12, 23, 34] has been described, so far no counterpart to the glucokinase regulatory protein in liver has been identified in pancreatic beta cells.

Thus, the aim of this study was to search for glucokinase binding proteins by a systematic approach. To account for proteins specifically expressed in beta cells a rat pancreatic islet library was produced and a yeast two-hybrid library screening performed. Finally, with a protein sequence containing a fragment of midnolin [35] including its ubiquitin-like domain (ULD) a new glucokinase binding partner was identified and further investigated.

EXPERIMENTAL PROCEDURES

Material- All primers, including random hexamer primers, and chemicals for TaqMan assays were from Life Technologies (Karlsruhe, Germany). The RevertAid™ H Minus M-MuLV reverse transcriptase was from Fermentas (St. Leon-Rot, Germany). The GoTaq® Taq polymerase was from Promega (Mannheim, Germany) and dNTPs were purchased from Genecraft (Münster, Germany). Restriction enzymes for cloning were from New England Biolabs (Beverly, MA).

Yeast two-hybrid library screening- Yeast two-hybrid experiments were performed using pGBKT7 and pGADT7 vectors from the Matchmaker™ GAL4 Two-Hybrid System 3 (Clontech Laboratories, Inc., Palo Alto, CA). The cDNA library was constructed according to manufacturer's instructions (BD Matchmaker™ Library Construction & Screening Kit, Clontech) using pancreatic islets isolated from male Wistar rats. RNA was isolated [36] and used for cDNA first-stranded synthesis. Double-stranded cDNA (dscDNA) was produced by Long Distance-PCR and purified by a BD Chroma Spin TE-400 column (Clontech) to remove fragments smaller than 200 bp. Finally, recombination of dscDNA and pGADT7-Rec was performed in *S. cerevisiae* AH109 resulting in the GAL4-AD-library (prey proteins). The cDNA sequence of human beta cell glucokinase was amplified by PCR and subcloned in frame to the GAL4-DNA-BD (bait protein) into pGBKT7 and transformed into *S. cerevisiae* Y187. Mating of the two yeast strains was performed according to manufacturer's instructions and selected on SD-Leu, SD-Trp, SD-Leu/Trp, and finally on SD-Leu/Trp/His agar plates. As control, positive pGADT7-Rec-

library clones and pGBKT7-lamin were co-transformed into AH109 and selected on SD-Leu/Trp/His selection agar plates with or without addition of 3-Amino-1,2,4-triazol (3-AT, Sigma-Aldrich, Steinheim, Germany). Plasmid DNA from positive yeast colonies was isolated using the Y-DER® Yeast Extraction Reagent Kit (Thermo Scientific, Rockford, IL), expressed in *E.coli* TOP 10 and analyzed by PCR and sequencing (T7 5'-sequencing primer). For identification of the library inserts an NCBI-Blast search was performed based on the DNA or protein sequence. For further analysis the cDNA of sequence17 was removed from pGADT7-Rec-library (*NcoI* and *BamHI* restriction sites) and subcloned in frame into pGBKT7. To quantify beta-galactosidase activity in yeast lysates the Galacto-Star™ reporter assay (Life Technologies) was used with pGBKT7-lamin as control.

Plasmids- Generation of ECFP-glucokinase has been described previously [25]. Both, the cDNA of the complete sequence17 and of the ubiquitin-like domain (ULD) were amplified by PCR and subcloned in frame in EYFP-C1 and mCherry-N1 [37] (*SalI* and *BamHI* restriction sites). The cDNA sequence of midnolin was amplified by PCR using the midnolin-tGFP vector (MG208176, Origene) as template and subcloned in frame to the EYFP-C1 and N1 and mCherry-C1 and N1 vectors (*HindIII* and *KpnI* restriction sites). YFP-Parkin (addgene plasmid 23955) and mCherry-Parkin (addgene plasmid 23956) were generated and deposited by Richard Youle [38]. The ECFP-Nuc vector was from Clontech.

Cell culture and transient transfection- MIN6, HeLa and COS cells were grown in

Dulbecco's Modified Eagle Medium (DMEM, Biochrom AG, Berlin, Germany) supplemented with 25 mmol/l glucose, 10% (vol/vol) FCS, 10 U/ml penicillin, 10 µg/ml streptomycin, and 2 mmol/l glutamine in a humidified atmosphere at 37 °C and 5% CO₂. INS1E, RINm5F, MH7777A and HepG2 cells were grown in RPMI-1640 supplemented with 10 mM glucose, 10% (vol/vol) FCS, penicillin, and streptomycin in a humidified atmosphere at 37 °C and 5% CO₂. Medium of INS1E cells was additionally supplemented with 50 µmol/l 2-mercaptoethanol, medium of MH7777A cells with 10 µmol/l dexamethasone. Cells were transfected with the vector DNA by the use of jetPEI (Qbiogene, Montreal, Canada) or jetPrime (Polyplus-transfection SA, Illkirch, France). Stable MIN6 EYFP, MIN6 EYFP-ULD or MIN6 EYFP-parkin cell clones were selected through resistance against G418 (1,200 µg/ml). Expression of transfected plasmids was checked by qPCR, western blot and fluorescence microscopy analyses. mCherry fusion-constructs of all proteins served as an additional control.

Pancreatic islets and sections were from NMRI mice. Islets were isolated by collagenase digestion in bicarbonate-buffered Krebs-Ringer solution. Beta cells were obtained using calcium-free Krebs-Ringer solution and kept in RPMI-1640 medium supplemented with 5 mmol/l glucose for 24 h. Primary hepatocytes were isolated from C57/BL6 mice [25]. Isolated cells were suspended in Williams' medium E supplemented with 10 mmol/l glucose, 5% (vol/vol) fetal calf serum, 1 x 10⁻⁴ mmol/l dexamethasone, and 1 x 10⁻⁵ mmol/l insulin. Hepatocytes were seeded at a density of 4 x 10⁴ cells on glass cover slips and incubated for 24 h

in a humidified atmosphere at 37°C and 5% CO₂. Mice were housed at the central animal care facility of the faculties and cell and tissue isolation were approved by the state's Animal Care Committee.

Mammalian two-hybrid System (MMTHS)- Mammalian two-hybrid analyses were performed using modified vectors of the CheckMate/Flexi Vector Mammalian Two-Hybrid system (Promega Corp., Madison, WI). Generation of pACT-GK, pGL4.EYFP and pBIND.ECFP were described previously [30]. To generate pBIND.ECFP-sequence17 and pBIND.ECFP-ULD the cDNA of the complete sequence17 and of the ULD were amplified by PCR and subcloned in frame (*SgfI* and *PmeI* restriction sites) to the pBIND.ECFP-vector. MIN6 cells were seeded in six-well microplates at a density of 8 x 10⁴ per well and grown for 3 days. Thereafter cells were transfected with equimolar amounts of the vectors pGL4.EYFP, pBIND.ECFP-ULD or pBIND.ECFP-sequence17 and pACT-glucokinase or pACT as negative control and directly incubated with DMEM containing 3, 10 or 25 mmol/l glucose. Fluorescence intensities of ECFP and EYFP were determined every 2 h in the nuclei using a semiautomated microscope setup with the scan^R acquisition software (Olympus, Hamburg, Germany) as described previously [30]. The ratio EYFP/ECFP was calculated from single cells to quantify the interaction strength.

Cell viability assay- MIN6 cells were seeded in 24-well plates at a density of 100,000 cells and grown for 3 days. Cells were transiently transfected as indicated and grown for 24 h. Incubation with 30 µmol/l camptothecin (Sigma-Aldrich, Steinheim,

Germany) was performed in the final 5 h. Cell viability was determined using a microplate-based MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [39].

Recombinant glucokinase and ULD- The coding sequence for the ULD of sequence17 (midnolin) was amplified by PCR and subcloned in frame into the *BamHI* and *Sall* sites of pGEX-6P-1 expression vector and expressed in *E.coli* BL21 using the glutathione S-transferase (GST) Gene Fusion System (Amershan Pharmacia Biotech, Freiburg, Germany). Recombinant beta cell glucokinase [25] and ULD were expressed and purified as GST-tag protein. Cleavage of the GST-tag was achieved by PreScission protease (GE Healthcare, Freiburg, Germany).

Glucokinase enzyme activity-

Glucokinase enzyme activity was measured in an enzyme-coupled photometric assay [31]. MIN6, MIN6 EYFP, MIN6 EYFP-ULD or MIN6 EYFP-parkin cells were seeded in 10 cm dishes at a density of 3×10^6 cells and grown for three days. MIN6 cells were transiently transfected with EYFP-midnolin and grown for 48 h. Thereafter, cells were homogenized in phosphate buffered saline (pH 7.4) and the protein concentration was quantified by a Bio-Rad protein assay. To exclude the cellular hexokinase activity, glucokinase enzyme activities measured at 1 mmol/l glucose were subtracted from the values obtained at 3.125, 6.25, 12.5, 25, and 50 mmol/l glucose. Glucose phosphorylating activity of recombinant glucokinase was determined at 1, 3.125, 5, 6.25, 10, 12.5, 25, and 50 mmol/l glucose after 5 min incubation with 100 nmol/l recombinant ULD.

Measurement of insulin secretion- MIN6, MIN6 EYFP, MIN6 EYFP-ULD or MIN6

EYFP-parkin cells were seeded in six-well plates at a density of 3×10^5 cells and grown for three days. MIN6 cells were transiently transfected with EYFP-midnolin and grown for 48 h. Thereafter, the medium was replaced and cells were incubated in DMEM containing 3 or 25 mmol/l glucose for 48 h. Finally, cells were incubated for 1 h with bicarbonate-buffered Krebs-Ringer solution without glucose supplemented with 0.1% albumin and thereafter stimulated for 1 h with 3 or 25 mmol/l glucose. 1 ml of the incubation buffer from each well was carefully harvested and gently centrifuged to remove detached cells. In the final supernatants the secreted insulin was measured. Cells were homogenized by sonication in phosphate buffered saline (pH 7.4) and insulin content was measured in soluble fractions. Insulin was determined by RIA using a rat insulin standard or by ELISA and the protein concentration was quantified by Bradford protein assay.

Western Blot analysis- MIN6, INS1E, RINm5F, MH7777A, HepG2 and HeLa cells were seeded in 6 cm dishes at a density of 2.5×10^5 cells and incubated with standard DMEM for 24 h. Cells were homogenized in lysis buffer by sonication and insoluble material was pelleted by centrifugation. Total cellular protein was fractioned by reducing 10% SDS-PAGE and electroblotted to polyvinylidene difluoride (PVDF) membranes. Nonspecific binding sites of the membranes were blocked with 5% nonfat dried milk for 1 h at room temperature. Blots were incubated with glucokinase antibody (sc-7908, diluted 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), N-terminal midnolin antibody (251273, diluted 1:250, Abbiotec, San Diego, CA), C-terminal midnolin antibody (251274,

diluted 1:250, Abbiotec) at 4 °C overnight, followed by 2 h incubation with the appropriate peroxidase-labelled secondary antibody at room temperature. Blots were stripped with Re-blot plus (Millipore, Billerica, MA). The protein bands were visualized by chemiluminescence using the ECL detection system (GE Healthcare, Freiburg, Germany). Liver, brain, and muscle tissues were homogenized 2 x 20 sec at 6500 rpm in a Precellys 24 system (PEQLAB, Erlangen, Germany) using lysing matrix D (MP Biomedicals, Santa Ana, CA). MIN6 cells were transiently transfected as indicated and grown for 24 h. Cells were homogenized in lysis buffer by sonication and insoluble material was pelleted by centrifugation. Protein concentration was quantified by Bio-Rad protein assay. 40 µg of cellular protein were fractionated by reducing 10% SDS-PAGE and electroblotted to PVDF membranes. Nonspecific binding sites of the membranes were blocked with Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, NE) for 30 min at room temperature. Blots were incubated with midnolin antibody (251273, diluted 1:250, Abbiotec), parkin antibody (sc-32282, diluted 1:500 Santa Cruz Biotechnology) or glucokinase antibody (sc-7908, diluted 1:200, Santa Cruz Biotechnology), cleaved caspase-3 antibody (Asp 175, 9661, diluted 1:1000, New England Biolabs), caspase-3 antibody (8G10, 9665, diluted 1:1000, New England Biolabs) and GAPDH (sc-137179, diluted 1:2000, Santa Cruz Biotechnology) at 4 °C overnight, followed by incubation with the appropriate IRDye secondary antibodies (Li-Cor Biosciences) for 30 min at room temperature. Blots were stripped with Re-blot plus (Millipore). Specific protein bands were visualized in the Li-Cor Infrared Imaging System (Li-Cor Biosciences).

Quantification of specific protein bands was performed using Odyssey application software (Li-Cor Biosciences).

qPCR-analysis- MIN6 cells and NMRI islets were incubated with 3, 10 or 25 mmol/l glucose for 24 h. Total RNA was isolated from incubated MIN6 cells and NMRI islets, and from liver, brain, and muscle using the Qiagen RNeasy Kit (Qiagen, Hilden, Germany) and quality controlled. Random hexamer primers were used for reverse transcription. The gene expression was measured with TaqMan assays (Midn: Mm 00491444_m1, Park2: Mm 00450187_m1, Uba1: Mm00661812_m1, Ubl4a: Mm00455093_g1, Ubl4b: Mm00470977_s1) using the TaqMan Universal PCR Master Mix (Life Technologies) and expressed relative to Gapdh expression. Amplifications were performed in triplicates on a 7900HT real-time PCR system (Life Technologies).

Immunocytochemistry- MIN6, INS1E and HeLa cells, islets, primary beta cells and hepatocytes were seeded on cover glasses and grown for 48 h. Thereafter, cells were fixed with 4% paraformaldehyde in PBS at 4 °C overnight. Three-micrometer pancreas sections were deparaffinised in xylene and rehydrated in graded ethanol. High-pressure cooking in 0.01 mol/l citric acid buffer (pH 6.0) for 15 min was used for antigen retrieval. After cooling down the sections were incubated with 5% BSA in PBS (pH 7.4) for 30 min to block unspecific binding sites. Immunostaining was performed as described previously [40] with goat anti glucokinase antibody (sc-1979, diluted 1:50 or 1:100, Santa Cruz Biotechnology), rabbit N-terminal midnolin antibody (251273, diluted

1:50 or 1:250, Abbiotec), rabbit C-terminal midnolin antibody (251274, diluted 1:50 or 1:250, Abbiotec), goat tubulin antibody (sc-9935, diluted 1:50) or mouse parkin antibody (sc-32282, diluted 1:100, Santa Cruz Biotechnology) and the appropriate secondary antibody Alexa488 donkey anti-goat antibody (diluted 1:200, Dianova, Hamburg Germany), Alexa594 donkey anti-goat antibody (diluted 1:200, Dianova), Cy5 donkey anti-mouse and anti-rabbit antibodies (diluted 1:200 or 1:500, Dianova). For detection of the mitochondrial network cells were stained with MitoTracker® Deep Red FM (Invitrogen Molecular Probes, Eugene, OR). Finally cells were stained with DAPI and fixed onto slides.

Fluorescence microscopy- Analysis of transient transfected MIN6 cells and fluorescence resonance energy transfer (FRET) experiments were performed with a cellR/Olympus IX81 (Olympus) inverted microscope system equipped with a Cellcubator (Olympus) using 60% humidity, 37 °C and 5% CO₂. Glass bottom dishes or slides were fixed on the microscope stage and images were taken with an UPLSAPO 60 x 1.35 NA oil-immersion objective. HQ580/20, ET470/40, S484/15X, HQ620/60, and HC387/11 filter sets (AHF Analysentechnik, Tübingen, Germany) were used for excitation of mCherry, EGFP, Alexa488, Cy5, or MitoTracker® Deep Red FM, and DAPI, respectively. Emission was detected using HQ630/60M, a 510 (30)-630 (100) dual band filter, M517/30, HQ700/75, and 433(50)-517(40)-613(60) filter sets (AHF Analysentechnik). Image processing was done using xcellence software (Olympus) and AutoDeblur Gold WF software

(MediaCybernetics, Silver Spring, MD) for image deconvolution. The FRET setup has been described previously [30]. Sensitized emission based FRET efficiency (FRET_N) was calculated from the enhanced cyan fluorescence protein (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 [41]. Analyses of immunocytochemistry stained slides were performed with a Fluoview1000 (Olympus) or FV10i (Olympus) confocal microscope system and UPLSAPO 60 x 1.35 NA oil-immersion objective using sequential scanning mode. Image processing was done using FV10-ASW software (Olympus).

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

RESULTS

Identification of sequence17 as a new glucokinase interaction partner in a yeast two-hybrid screening of a rat pancreatic islet library- A cDNA library was generated using RNA isolated from rat pancreatic islets with insert sizes ranging from 0.5 to 4 kb. The resulting dsDNA was cloned into pGADT7-Rec by recombination in yeast AH109 with a high efficiency of 1.2×10^6 transformants. Mating of AH109 pGADT7-library and Y187 pGBKT7-GK was established resulting in 5.5×10^5 library clones in three independent runs. 73 positive yeast clones showing His-reporter gene activity were selected, the plasmid DNA was isolated and sequence alignments were

performed. Because of maximal growth on SD-Leu/Trp/His selection agar plates, 14 yeast clones expressing protein sequences so far unknown to interact with glucokinase were taken for further investigations (Figure 1A). Four clones of those showed significant growth on SD-Leu/Trp/Ade and SD-Leu/Trp/His/Ade selection agar plates (Figure 1A). Only yeast expressing glucokinase and sequence17 exhibited a graduated growth on selection agar plates as a function of the selection stringency (Figure 1A). Yeast expressing glucokinase and sequence 32/71, 36 and 39 showed growth on selection agar plates independent of the stringency (SD-Leu/Trp/His < SD-Leu/Trp/Ade < SD-Leu/Trp/His/Ade) (Figure 1A) indicating their ability to induce transcription without glucokinase interaction. In fact, His-reporter gene expression could be detected after co-expression of pGADT7-sequence32/71, pGADT7-sequence36 and pGADT7-sequence39 with the negative control pGBKT7-lamin in AH109. In contrast, yeast with pGADT7-sequence17 showed growth on SD-Leu/Trp/His selection agar plates after co-expression with pGBKT7-glucokinase, but not with pGBKT7-lamin. This growth was sensitive to the specific inhibitor 3-AT (Figure 1B). Finally, a quantitative beta-galactosidase assay (Figure 1C) revealed significantly higher interaction strength for sequence17 with glucokinase compared to the control protein lamin.

Sequence17 contains a fragment of midnolin including the complete sequence of an ubiquitin-like domain- Sequence and protein database analyses of sequence17 revealed a 180 aa containing fragment which shows homology to the N-terminal part of midnolin (NCBI

Protein Accession Nr. NP_001178506.1) (Figure 1D). Interestingly, this fragment contains the full length sequence of an ubiquitin-like domain (ULD), consisting of 76 aa. Sequence alignment by MAFFT (v7.015b) [42] of rat, human, and mouse midnolin isoforms revealed a high homology between the different species. Notably, the 43 aa, which are missing in the observed sequence17 in comparison to rat midnolin (Figure 1D), are also lacking in human and mouse isoform1 of midnolin (Figure 1E).

Interaction analyses between (1) glucokinase and sequence17 and (2) glucokinase and the ULD of sequence17 (midnolin) in a mammalian two-hybrid system- The mammalian two-hybrid system (MMTHS) in a fluorescence-based semiautomated microscopy approach has been generated previously to investigate protein-protein interactions in mammalian cells [30]. The insulin-secreting MIN6 cells were triple-transfected with the MMTHS-vectors (Figure 2A, top) and incubated at different glucose concentrations. Whilst ECFP is constitutively expressed to control the transfection efficiency, the expression of EYFP is under control of an inducible promoter (Figure 2A, bottom). Thus, association of the VP16-activation domain (VP16-AD or VP16-AD-glucokinase) and the GAL4-DNA-binding domain (GAL4-DNA-BD-sequence17 or GAL4-DNA-BD-ULD) mediated by the protein-protein interaction will result in EYFP expression. Averaged data from 43 – 55 h post transfection revealed a significant interaction between sequence17 and glucokinase in comparison to the control independent from the glucose concentration in the culture medium (Figure 2B). In contrast, the interaction strength

between the ULD of sequence17 (midnolin) and glucokinase was glucose dependent (Figure 2C) and significantly higher at 3 mmol/l glucose compared to 10 or 25 mmol/l glucose (Figure 2C, D) suggesting that binding of glucokinase to an ULD containing protein occurs preferentially at low glucose in insulin-secreting cells.

Glucose-dependent gene regulation of ULD containing proteins in beta cells- Up-regulation of genes coding for ULD containing proteins or those involved in the ubiquitin-proteasome pathway at low glucose has been recently shown in insulin-secreting cells and islets [43]. To date around 30 ULD containing proteins have been described with at least in part cytoplasmic localization and, thus, can come into consideration as an interaction partner of glucokinase. Alignments of the ULDs revealed four genes with around 50% sequence similarity to the ULD of midnolin, namely Park2, Ubl4 (also known as Ubl4a or Gdx), Ubl4b and Ubac1. Ubl4b could not be detected in beta cells, which is in agreement with previous findings showing Ubl4b expression only in testis [44]. Therefore, beyond Midn, expression of Park2, Ubl4 and Ubac1 (Figure 3A) has been investigated in MIN6 cells and primary islets after 24 h incubation at 3, 10 and 25 mmol/l glucose.

Both in insulin-secreting MIN6 cells and islets Midn expression levels were highest at 3 mmol/l glucose (Figure 3B, F). A down-regulation was observed at 10 and 25 mmol/l glucose, which was significant at 25 mmol/l glucose in islets (Figure 3F). The same effect, but more pronounced, was observed for Park2 expression. In MIN6 cells and islets a significantly lower Park2 expression was

observed at 25 mmol/l glucose in comparison to 3 mmol/l glucose (Figure 3C, G). Ubl4 and Ubac1 expression levels were comparable at 3 and 10 mmol/l glucose in MIN6 cells and islets (Figure 3D, E, H, I). At 25 mmol/l glucose Ubl4 and Ubac1 expression was down-regulated in islets (Figure 3H, I). However, in MIN6 cells Ubl4 expression significantly increased at 25 mmol/l glucose (Figure 3D). Thus, only midnolin and parkin showed distinct glucose dependent gene expression.

Structural homologies of the ULDs of midnolin and parkin with ubiquitin- It has been shown that ULDs share strong homologies to ubiquitin [45, 46]. The 3D structures of ubiquitinD (Q921A3), the ULD of sequence17 (midnolin) and the ULD of parkin (Q9JK66) were constructed with SWISS-MODEL based on the protein sequences (Figure 4A) [47-51]. The ULD of sequence17 (midnolin) shows distinct homology to the 3D structure of ubiquitin, which adopts a compact, globular form [52]. In addition, the 3D structures revealed a strong homology between the ULDs of midnolin and parkin.

Interaction between glucokinase and midnolin or parkin- Using the MMTS the strongest interaction between glucokinase and the ULD of midnolin was obtained at 3 mmol/l glucose. Sensitized emission based fluorescence resonance energy transfer (FRET) analyses in COS cells confirmed our results. At 3 mmol/l glucose FRET was in ECFP-GK/ EYFP-ULD and ECFP-GK/ EYFP-midnolin co-expressing cells 3.5 and 4.7 times, respectively, higher than in negative control cells co-expressing ECFP-GK/ EYFP (Figure 4B). Cells co-expressing ECFP-GK/ EYFP-parkin showed only 2 times

higher interaction strength than the negative control (Figure 4B). Endogenous midnolin (Figure 4C) and parkin (Figure 4D) were detectable in the cytoplasm and nucleus. However, in line with the gene expression analyses (Figure 3B, C) the expression level of parkin appears to be lower compared to midnolin.

Effects of the ULD of midnolin, full-length midnolin and of parkin on glucokinase activity- To investigate the effect of the ULD of midnolin on glucokinase activity, glucose phosphorylating activities were determined. In *in vitro* experiments using recombinant glucokinase and ULD (100 nmol/l) no influence on glucokinase activity could be detected (Figure 5A). In contrast, overexpression of EYFP-ULD in insulin-secreting MIN6 cells significantly inhibited endogenous glucokinase activity by reducing both the maximal phosphorylating activity and the affinity to glucose (Figure 5B). Thus, either the ULD of midnolin was not properly folded in bacteria or the *in vitro* conditions were not adequate for interaction. The successful use of recombinant ubiquitin [53], which has the same size and high homology with the ULD of midnolin, however, argues against misfolding. Overexpression of full-length EYFP-midnolin significantly inhibited glucokinase enzyme activity and reduced the affinity to glucose (Figure 5C). Overexpression of EYFP-parkin significantly decreased glucokinase activity in MIN6 cells at 6.25 and 12.5 mmol/l glucose, thus affecting only the affinity of the enzyme to glucose (Figure 5D).

Effects of the ULD of midnolin, full-length midnolin and of parkin on glucose-

induced insulin secretion- Insulin-secreting MIN6 cells overexpressing EYFP as a transfection control showed a significant increase in insulin secretion after stimulation with 25 mmol/l glucose. The glucose responsiveness of MIN6 cells was comparable after 48 h pre-incubation at 3 or 25 mmol/l glucose and a 1 h final incubation period without glucose (Figure 5E). Glucose-induced insulin secretion was significantly reduced after overexpression of EYFP-ULD (Figure 5F). Glucose responsiveness was completely lost in MIN6 cells pre-incubated at 3 mmol/l glucose, whilst some glucose responsiveness remained after pre-incubation at 25 mmol/l glucose. Loss of glucose-induced insulin secretion was also observed after overexpression of EYFP-midnolin, both after pre-incubation with 3 or 25 mmol/l glucose (Figure 5G). Overexpression of EYFP-parkin disturbed insulin secretion in MIN6 cells (Figure 5H). Not only glucose-induced insulin secretion, but also the basal insulin secretion was significantly reduced (Figure 5H).

Midnolin overexpression and co-localization with glucokinase in insulin-secreting MIN6 cells- Overexpression of EYFP-ULD showed a homogenous distribution in insulin-secreting MIN6 cells with considerable fluorescence in the nucleus (Figure 6A). In the cytoplasm a distinct co-localization with ECFP-glucokinase was detectable (Figure 6A). EYFP-midnolin (Figure 6B) and midnolin-EYFP (Figure 6C) showed highest fluorescence in the cell nucleus. However, in around 50% of the transfected cells both midnolin EYFP-fusion-constructs were also detectable in the cytoplasm. Co-localization with ECFP-glucokinase was

observed in the cytoplasm for both midnolin EYFP-fusion-constructs (Figure 6B, C). In some cells, mostly with a high midnolin overexpression level, ECFP-glucokinase was also detectable in the nucleus (Figure 6B) and co-localized with the overexpressed midnolin EYFP-fusion-construct. Midnolin contains a nuclear localization signal. Based on overexpression analyses of midnolin GFP-fusion-constructs in CHO cells, Tsukahara et al. [35] postulated a mainly nucleolar localization of the overexpressed protein. In contrast co-expression experiments of EYFP-midnolin and a nucleoli localized fluorescence protein (ECFP-Nuc) in MIN6 cells clearly showed that midnolin is not located in the nucleoli (Figure 6D).

Effect of midnolin overexpression on cell viability and glucokinase protein expression- Overexpression of EYFP-ULD, EYFP-midnolin, midnolin-EYFP or EYFP-parkin did not reduce cell viability (Figure 6E) or induce apoptosis (Figure 6F). The cytotoxic quinoline alkaloid camptothecin served in our experiments as a positive control reducing cell viability by 55% in the MTT assay and induced a 10-fold increase in cleaved caspase-3. The glucokinase protein content determined in MIN6 cells after overexpression of EYFP, EYFP-ULD, EYFP-midnolin or EYFP-parkin was comparable (Figure 6G), indicating that neither overexpression of midnolin nor parkin influence glucokinase protein expression.

Midnolin expression and co-localization with glucokinase in insulin-secreting cells and primary cells – In insulin-secreting INS1E cells (Figure 7A), MIN6 cells (Figure 7B) and primary mouse beta cells (Figure 7C) midnolin

was detectable in the cytoplasm and the nucleus and showed distinct co-localization with glucokinase in the cytosol. In intact mouse islets midnolin expression was detected in the whole islet clearly co-localizing with glucokinase (Figure 7D). Staining of pancreatic sections showed midnolin expression in the islet with only negligible expression in the surrounding exocrine tissue and a distinct co-staining with glucokinase (Figure 7E). In hepatocytes (Figure 7F) both, midnolin and glucokinase were expressed in the cytosol and the nucleus and showed in part co-localization (Figure 7F).

Midnolin expression in different tissues, species and cell lines- The gene and likewise protein expression levels of midnolin (Figure 8A, C) in mouse liver, brain, and muscle appeared to be higher compared to parkin (Figure 8B, D). Midnolin expression was comparable in brain and liver, but significantly lower in muscle (Figure 8A, C). Midnolin was verified as a 50 kDa protein in all investigated mouse tissues (Figure 8E), in mouse insulin-secreting MIN6 cells, in rat insulin-secreting INS1E and RINm5F cells, in human MH7777A and rat HepG2 hepatoma cells, and in the human HeLa cell line (Figure 8G, H). The specific protein signal at 50 kDa was detectable using the N-terminal (Figure 8G) and C-terminal (Figure 8H) midnolin antibody. Interestingly, in cell lines of human origin a 35 kDa line was additionally detected particularly using the C-terminal antibody. In HeLa cells (Figure 8I-L), comparable to primary mouse beta cells and MIN6 cells (Figure 7H, I), midnolin was detectable in the nucleus and cytoplasm. The distribution pattern was independent of the antibody used (Figure 8I-L). Midnolin showed a

punctated cytoplasmic distribution, but was marginally co-localized with tubulin (Figure 8I, J) and not co-localized with mitochondria (Figure 8K, L).

DISCUSSION

Using yeast two-hybrid screening we elucidated a 180 aa protein fragment named sequence17 as a new interaction partner of glucokinase. This fragment is part of midnolin, a nucleolar protein, which has been described to be mainly expressed in the mesencephalon during mouse development [35]. By the gene trap method and the selection of embryonic stem cells midnolin has been identified to be higher expressed during cell differentiation than thereafter [35]. Although Tsukahara et al. described midnolin expression in adult tissues, especially in heart, lung, liver, and kidney, they finally concluded that midnolin is involved in regulation of genes related to neurogenesis [35]. Zielak et al. reported that midnolin is important to regulate mRNA transport in developing follicle in cattle [54]. Due to lack of any further investigations on midnolin it remains open if the protein could be an interaction partner of glucokinase in pancreatic beta cells.

We confirmed the interaction between glucokinase and sequence17 in insulin-secreting cells by mammalian two-hybrid analyses. Because sequence17 contains the full-length ULD of midnolin, binding of this ULD alone to glucokinase has also been studied. ULDs are domains which strongly resemble ubiquitin [46, 55]. The ULD of midnolin was found to have highest binding affinity to glucokinase if the cells were cultured at 3 mmol/l glucose. At this glucose concentration glucokinase exists mainly in its inactive super-open to open conformations

and insulin secretion is basal in pancreatic beta cells [4, 17, 19, 20, 30]. It is known that proteins containing a ULD are involved in intramolecular interactions and regulation of signal transduction [46, 56, 57]. In most cases proteins with a ULD act in close correlation with the ubiquitin-proteasome system [46]. In pancreatic islets up-regulation of genes encoding proteins of the ubiquitin-proteasome system at low glucose has been reported [43, 58]. Interestingly, RT-PCR analyses revealed highest midnolin expression at 3 mmol/l glucose in mouse pancreatic islets and insulin-secreting MIN6 cells. The expression level was stepwise reduced after 24 h incubation at 10 and 25 mmol/l glucose.

Parkin, containing a ULD with high homology to that of midnolin, showed in our study a comparable glucose dependent gene expression. This is in agreement with a previous study reporting up-regulation of parkin in rat pancreatic islets at low glucose culture [43]. Parkin is an E3 ubiquitin ligase with different expression patterns during development and in adult tissues [59-61]. Its function has been best investigated in the dopaminergic system, where loss-of-function mutations in the Park2 gene cause juvenile Parkinson's disease [38, 59]. Parkin binds via PINK1 to damaged mitochondria and thereby triggers their autophagy [38, 62, 63]. Little is known about the role of parkin in pancreatic beta cells. FRET analyses showed some interaction between glucokinase and parkin. Overexpression of parkin in insulin-secreting MIN6 cells resulted in reduced glucokinase enzyme activity at low glucose suggesting some relevance of the parkin:glucokinase interaction. However, this cannot explain the loss of glucose-induced and

the reduction of basal insulin secretion, which we observed in parkin overexpressing MIN6 cells. Interestingly, Park2 gene expression in MIN6 cells was much lower compared to mouse pancreatic islets, and thus, possibly down-regulated in an adaptive process to maintain insulin secretion. Assuming that parkin in pancreatic beta cells is also important to regulate mitochondrial integrity, further work is necessary to characterize this pathway.

Overexpression of the ULD of midnolin and the full-length midnolin in MIN6 cells resulted in reduced glucokinase activity and glucose-induced insulin secretion. This effect was not induced by diminished cell viability or reduced glucokinase protein, but more pronounced in low glucose culture which strengthens our hypothesis that inactive glucokinase binds to the ULD of midnolin. To our knowledge midnolin expression has not been investigated on the protein level. Quite recently polyclonal antibodies against the N- and C-terminal part of midnolin became available. Both antibodies revealed that midnolin is significantly produced in insulin-secreting cells and localized not only in the nucleus but also in the cytoplasm. Furthermore, we observed distinct co-localization between glucokinase and midnolin in the cytoplasm of isolated primary mouse pancreatic beta cells as well as intact islets and in pancreatic sections. The localization of midnolin in the cytoplasm was not restricted to insulin-secreting cells, but also found in primary hepatocytes and HeLa cells. In our fluorescence microscopy experiments neither the endogenous nor the overexpressed midnolin showed localization in nucleoli. Thus, these results question the

exclusively nuclear and nucleolar localization of midnolin, proposed by Tsukahara et al. on the basis of the identified NLS sequence of midnolin and the use of GFP-chimera of the protein [35]. Notably, Tsukahara et al. did not perform immunocytochemistry analyses.

The first protein entry of midnolin (NCBI Protein Accession No. XP_234902) [35] has been somewhat revised recently (NCBI Protein Accession No. NP_001178506.1). Because the identified sequence17 did not contain the 43 aa specific for isoform 2 and 3 of midnolin and in all investigated tissues and cell lines of mouse and rat origin a single line of the protein at 50 kDa has been observed, isoform1 is most likely the predominant midnolin expressed in adult mouse and rat tissues. Notably, the midnolin expression level found in liver, brain and muscle was significant and to a considerable degree higher than the parkin expression. Interestingly, only in human cell lines we detected in addition another shorter isoform of midnolin of around 37 kDa. Because this isoform became apparent using the C-terminal antibody a truncation closer to the N-terminus can be assumed.

Together, our findings suggest an important role of midnolin in adult tissues. Because midnolin contains a ULD as an integral component the protein could play a role in protein quality control or cellular signalling. In pancreatic beta cells evidence has been obtained that the ULD of midnolin serves as an anchor domain mediating binding to the glucose sensor enzyme glucokinase. This interaction seems to occur preferentially at low glucose and inhibits glucokinase activity. In light of the fact that glucokinase is regulated by the ubiquitin

proteasome system [21-24] it has to be the aim of future work to elucidate if midnolin acts for example as known for parkin as an E3 ubiquitin ligase. Characterization of the midnolin isoforms, especially in human tissues, and functional analyses in primary pancreatic beta cells remain another important issue of future work. Furthermore the results of our study indicate a specific function of parkin in pancreatic beta cells.

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Abbreviations- ULD, ubiquitin-like domain; GK, glucokinase; dsDNA, double-stranded cDNA; SD, synthetic defined; Leu, Leucine; Trp, Tryptophan; His, Histidine; Ade, Adenine; 3-AT, 3-Amino-1,2,4-triazol; aa, amino acid; MMTHS, Mammalian two-hybrid system; AD, activation domain; BD, binding domain; Park2, parkin; Ubl4 (Gdx), ubiquitin-like 4a; Uba1, ubiquitin associated domain containing 1; FRET, fluorescence resonance energy transfer efficiency; NLS, nucleolar localization signal; PCR, polymerase chain reaction; RT-PCR, Real-time polymerase chain reaction; PINK1 mitochondrial serine/threonine-protein kinase 1; kDa, kilo Dalton; dNTP, deoxyribonucleotide triphosphate; bp, base pair(s); FCS, fetal calf serum; GST, glutathione S-transferase; ELISA, enzyme-linked immunosorbent assay; RIA, radioimmuno assay; PVDF, polyvinylidene difluoride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SDS-PAGE, sodiumdodecylsulfate polyacrylamide gel electrophoresis;

FIGURE LEGENDS

Figure 1. Identification of a new glucokinase interaction partner in a yeast two-hybrid library screening. (A) 13 different library inserts were identified in a yeast two-hybrid library screening showing different growth on SD-selection agar plates containing Leu/Trp/His (open bars), Leu/Trp/Ade (grey bars) or Leu/Trp/His/Ade (black bars). (B) Growth of AH109 co-expressing pGADT7-sequence17 and pGBKT7-glucokinase (left side of agar plates) or pGBKT7-lamin (right side of agar plates) on SD-Leu/Trp/His selection agar containing 2.5 (1), 5 (2), 7.7 (3), 10 (4), 12.5 (5), or 15 (6) mmol/l 3-AT. (C) Beta-galactosidase reporter gene activity in yeast expressing pGADT7-sequence17 and pGBKT7-glucokinase (black bar) or pGBKT7-lamin (open bar). ** $p < 0.05$ (Student's t-test). (D) Sequence alignment of sequence17 with midnolin (NCBI Protein Accession No. NP_001178506.1) with homologies marked in grey. The black box frames the ULD. (E) Shown is a section of a MAFFT [42] alignment of sequence17 with the rat isoform (D4AE48), mouse isoform1 (Q3TPJ7-1) and isoform2 (Q3TPJ7-2) and human isoform (Q504T8) of midnolin.

Figure 2. Glucokinase interacts with the ULD of midnolin preferentially at low glucose. (A) The MMTS plasmids pGL4.EYFP, pBIND.ECFP-ULD or pBIND.ECFP-sequence17, and pACT-glucokinase or pACT were cotransfected and MIN6 cells cultured at 3, 10 or 25 mmol/l glucose. Whereas ECFP is constitutively expressed, EYFP expression indicates protein-protein interaction. The EYFP/ECFP fluorescence ratio was determined every two hours in the cell nucleus in a semi-automated microscopy approach. (B) Interaction of BD-sequence17 and AD-glucokinase (black bars) or AD (open bars). (C) Interaction between BD-ULD and AD-glucokinase (black bars) or AD (open bars). (D) Time course of interaction between BD-ULD and AD-glucokinase in MIN6 cells cultured at 3 mmol/l (closed circles, solid line) or 10 mmol/l (open circles, dashed line). Shown are normalized mean nuclear EYFP/ECFP ratios \pm SEM obtained 43 – 55 h after transfection of 4 - 8 individual experiments with a total of 624 – 6434 nuclei analyzed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control; ### $p < 0.01$, #### $p < 0.001$ compared to 3 mmol/l glucose (ANOVA/Bonferroni's multiple comparison test).

Figure 3. Glucose-dependent gene expression of ULD containing proteins. (A) Sequence alignment of the ULD of sequence17 with the ULD of parkin (Q9WVS6), UBL4A (P21126) and UBAC1 (Q8VDI7). MIN6 cells (B-E) and NMRI islets (F-I) were cultured at 3, 10 or 25 mmol/l glucose for 24 h. Gene expression levels of Midn (B, F), Park2 (C, G), Ubl4 (D, H) and Uba1 (E, I) were determined. Relative expression levels normalized to housekeeping gene Gapdh are shown. Data are expressed as means \pm SEM of 3 individual experiments. * $p < 0.05$, ** $p < 0.01$ compared to 3 mmol/l glucose; # $p < 0.05$, ## $p < 0.01$ compared to 10 mmol/l glucose (ANOVA/Bonferroni's multiple comparison test).

Figure 4. Homologies and differences between midnolin and parkin. (A) Comparison of the 3D structures of ubiquitinD (Q921A3), the ULD of sequence17 (midnolin) and the ULD of parkin (Q9JK66). Images were constructed with SWISS-MODEL [47, 48, 50]. (B) FRET efficiencies were calculated in COS cells transfected with ECFP-glucokinase and EYFP (white bar), EYFP-ULD (black bar) EYFP-midnolin (grey bar) or EYFP-parkin (white striped bar). Shown are means \pm SEM of 3 individual experiments, * $p < 0.05$, *** $p < 0.001$ compared to control (ANOVA/Bonferroni's multiple comparison test). MIN6 cells (C, D) were fixed and stained for midnolin (N-terminal part) (red, C) or parkin (red, D) and DAPI (blue, C, D). Representative images shown were obtained from z-stacks after deconvolution. Scale bars, 10 μm .

Figure 5. Effects of the ULD of midnolin, full-length midnolin and of parkin on glucokinase activity and glucose-induced insulin secretion in MIN6 cells. (A) Activity of recombinant beta cell glucokinase was determined at 1, 3.125, 5, 6.25, 10, 12.5, 25, and 50 mmol/l glucose without (closed circles, solid line) and after incubation with 100 nmol/l recombinant ULD for 5 min (open circles, dashed line). Shown are means \pm SEM in U/mg protein of 6 individual experiments. Activity of endogenous glucokinase was determined in MIN6 EYFP cells (closed circles, solid line, B, C, D), MIN6 EYFP-ULD cells (open circles, dashed line, B), MIN6 cells transiently transfected with EYFP-midnolin (open circles, dashed line, C) and MIN6 EYFP-parkin cells (open circles, dashed line, D). Glucokinase enzyme activity measured at 1 mmol/l glucose was subtracted from the values obtained at 3.125, 6.25, 12.5, 25, and 50 mmol/l glucose to exclude the cellular hexokinase activity. Shown are means \pm SEM in mU/mg cellular protein of 3 individual experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control at the same glucose concentration (ANOVA/Bonferroni's multiple comparison test). MIN6 EYFP cells (E), MIN6 EYFP-ULD cells (F), MIN6 cells transiently transfected with EYFP-midnolin (G) and MIN6 EYFP-parkin cells (H) were cultured at 3 mmol/l (grey bars) or 25 mmol/l (black bars) glucose for 48 h. After starvation for 1 h, cells were incubated at 3 mmol/l (open bars) or 25 mmol/l (closed bars) glucose. Data are means \pm SEM of 4 - 11 individual experiments. *** $p < 0.001$ compared to 3 mmol/l glucose; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to MIN6 EYFP cells (ANOVA/Bonferroni's multiple comparison test).

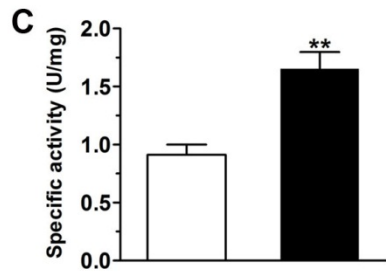
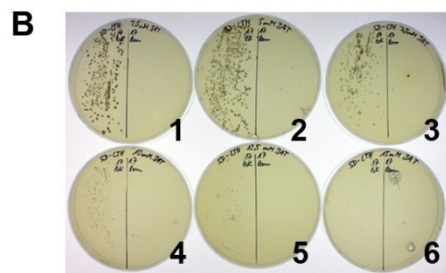
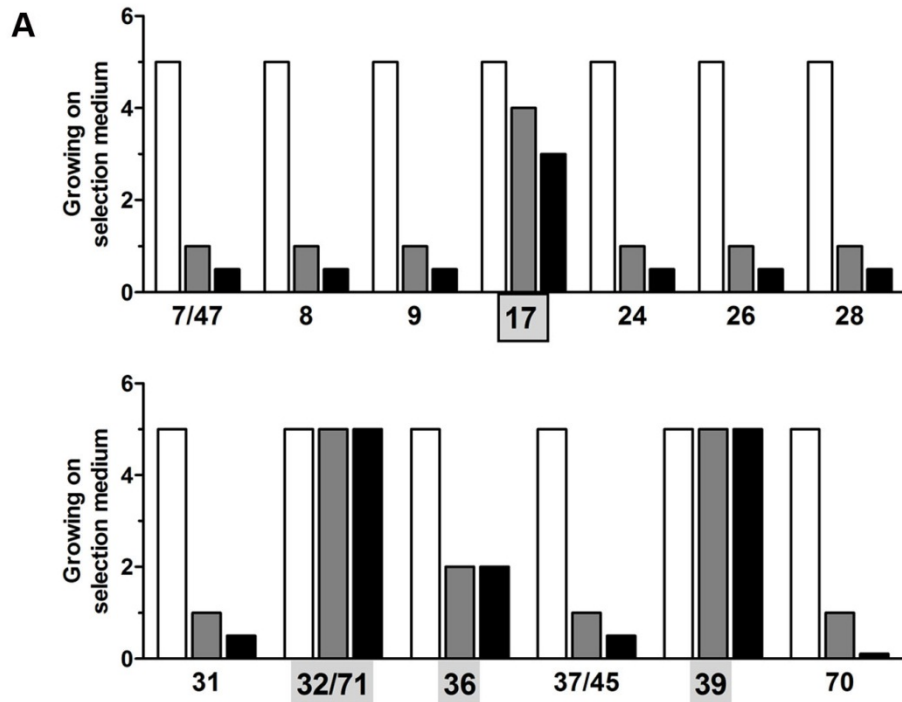
Figure 6. Subcellular localization of midnolin after overexpression in MIN6 cells and its effect on glucokinase expression and cell viability. (A-D) MIN6 cells were transiently co-transfected with EYFP-ULD (A left panel), EYFP-midnolin (B, D left panel), midnolin-EYFP (C left panel) and ECFP-glucokinase (A-C middle panel) or ECFP-Nuc (D middle panel). EYFP fusion proteins in red, ECFP fusion proteins in green and the merge images (A-D right panel) are shown. Representative images shown were obtained from z-stacks after deconvolution. Scale bars 10 μm . (E-F) MIN6 cells were transiently transfected with

EYFP (white bar and white lengthwise-striped bar, E, F), EYFP-ULD (black bar, E, F), EYFP-midnolin (grey bar, E, F), midnolin-EYFP (cross-striped grey bar, E) and EYFP-parkin (white striped bar, E, F) and treated with 30 $\mu\text{mol/l}$ camptothecin (white lengthwise-striped bar, E, F). Cell viability was determined by MTT assay (E) or quantification of cleaved caspase-3 in relation to caspase-3 and GAPDH expression (F). (G) Glucokinase expression is shown in relation to GAPDH expression. Data are means \pm SEM of 3 - 4 individual experiments. ** $p < 0.01$, *** $p < 0.001$ compared to EYFP transfected MIN6 cells (ANOVA/Bonferroni's multiple comparison test).

Figure 7. Co-localization of midnolin and glucokinase in insulin-secreting cells, primary beta cells and hepatocytes. INS1E cells (A), MIN6 cells (B), primary mouse beta cells (C), mouse pancreatic islets (D) and hepatocytes (F) were fixed and stained for midnolin (N-terminal part) (red, A-C, F), (C-terminal part) (red, D), glucokinase (green, A-D, F), and with DAPI (blue, A-D, F). (G) Pancreatic sections were stained for midnolin (N-terminal part) (red), glucokinase (green), and with DAPI (blue, A-D, F). Representative images shown were obtained from z-stacks after deconvolution (B, C, F) or processed with FV10-ASW software (A, D, E). Scale bars, 5 (C), 10 (A, B, F), 20 (E) or 30 (D) μm , respectively.

Figure 8. Midnolin expression in different tissues. (A-B) Brain (open bars), liver (grey bars), and muscle (black bars) were obtained from NMRI mice. Total RNA was isolated and Midn (A) and Park2 (B) gene expression determined. Shown are relative expression levels normalized to Gapdh. (C-F) 40 μg of isolated protein were analyzed by SDS-PAGE and immunoblotted using antibodies against the N-terminal part of midnolin (E), GAPDH (F) and parkin. Quantifications of the 50 kDa midnolin line (C) and the 50 kDa parkin line (D) are shown in relation to the 37 kDa GAPDH line. Shown are means \pm SEM of 3 individual experiments. * $p < 0.05$, ** $p < 0.01$ compared to muscle (ANOVA/Bonferroni's multiple comparison test); # $p < 0.05$, ## $p < 0.01$ compared to muscle (Student's t-test). (G-H) 40 μg protein isolated from MIN6, INS1E, RINm5F, MH7777A, HepG2, and HeLa cells were analyzed by SDS-PAGE and immunoblotted using antibodies against the N-terminal part (G) and C-terminal part (H) of midnolin. (I-L) HeLa cells were fixed and stained for midnolin using the N-terminal (green, I, K) or C-terminal (green, J, L) midnolin antibody, and for beta-tubulin (red, I, J) or with MitoTracker[®] Deep Red FM (red, K, L), and with DAPI (blue, I-L). Representative images shown were obtained from z-stacks after deconvolution. Scale bars 10 μm .

Figure 1



D

```

(1) 1 10 20 30 40 50 60 70 80
Translation of Sequenz 17 (1) -----FCRGAPGGACELSTTAESAARMSLAIHSTTGTRYDLSVPHDETVEGLRRLSORLKVPERLALLHKDTR
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Section 2
(81) 81 90 100 110 120 130 140 150 160
Translation of Sequenz 17 (71) LSSGKLQEFVGVGGSKLTLVPTVEAGLMSQASREQSVMQALESLTETQ-----
NP_001178506.1 (81) LSSGKLQEFVGVGGSKLTLVPTVEAGLMSQASREQSVMQALESLTETQPPATPGPRAAGGGFRKYRLILFKRPWHRQG
Section 3
(161) 161 170 180 190 200 210 220 230 240
Translation of Sequenz 17 (120) -----VSDFLSGRSPLTLALRVGDHMMFVQLQAAQHPLOHRRHVLAAAAAARGDSSIAFPVSS-----
NP_001178506.1 (161) PQSPFERGGERPQVSDFLSGRSPLTLALRVGDHMMFVQLQAAQHPLOHRRHVLAAAAAARGDSSIAFPVSSPCRFV
Section 4
(241) 241 250 260 270 280 290 300 310 320
Translation of Sequenz 17 (183) -----
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Section 5
(321) 321 330 340 350 360 370 380 390 400
Translation of Sequenz 17 (183) -----
NP_001178506.1 (321) SGTFSGLTFHENCQDSSGRPRRDIGTILQILNDLLSATRHYQGMFASLTQLRCHAQCSFASPAPDLTPKTTSCFKLATPSL
Section 6
(401) 401 410 420 430 440 450 460 470 480
Translation of Sequenz 17 (183) -----
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(481) 481 490 505
Translation of Sequenz 17 (183) -----
NP_001178506.1 (481) LGLDFEDSVWKFVNPDIQSEFVVA
    
```

E

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Sequenz17      ALESLTET-----QVSDFLSGR
rMidn (D4AE48) ALESLTETQPPATPGPRAAGGGFRKYRLILFKRPWHRQGPQSPERGGERPQVSDFLSGR
mMidn (Q3TPJ7-1) ALESLTET-----QVSDFLSGR
mMidn (Q3TPJ7-2) ALESLTETQPPATPGPRAAGGGFRKYRLILFKRPWHRQGPQSPERGGERPQVSDFLSGR
hMidn (Q504T8)  ALESLTET-----QVSDFLSGR
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Figure 2

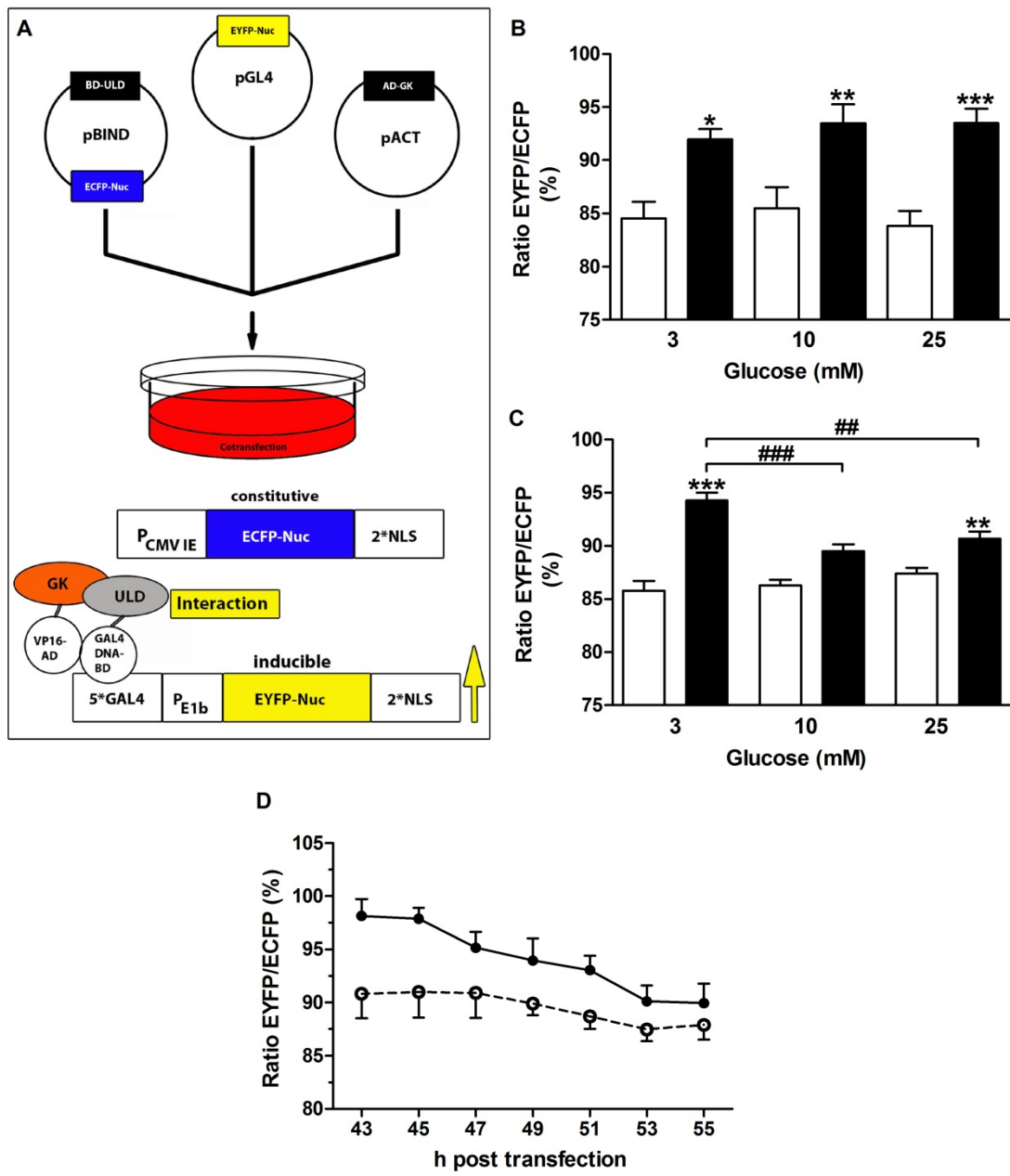


Figure 3

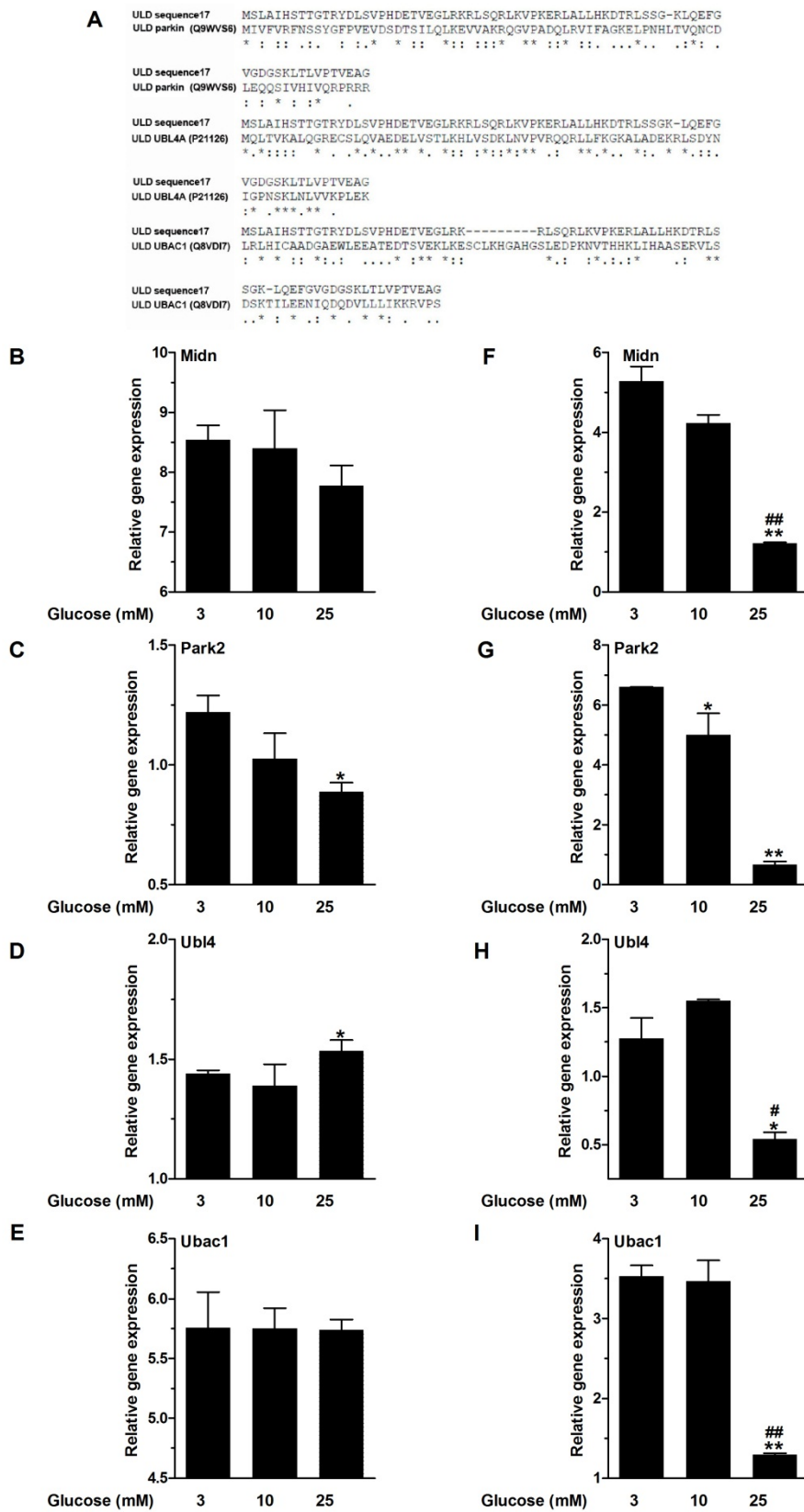
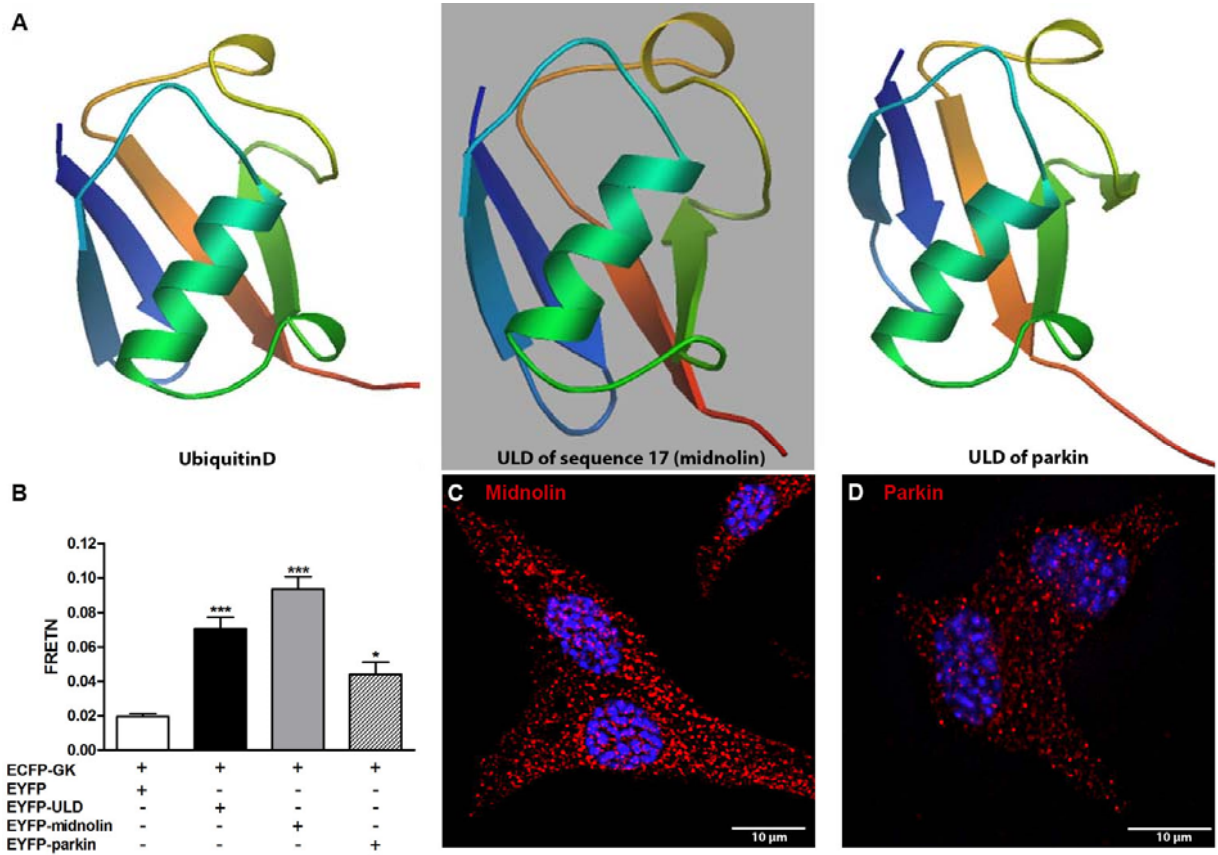


Figure 4



Figure

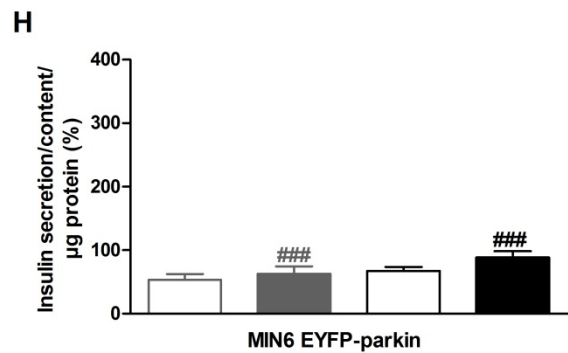
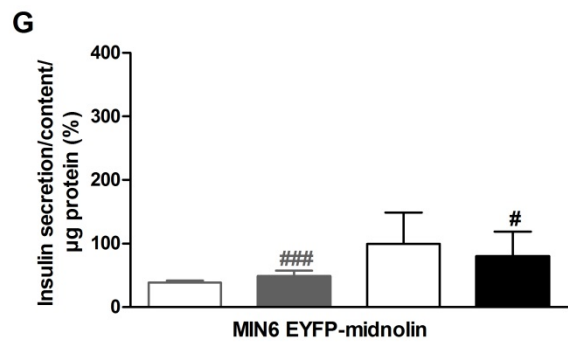
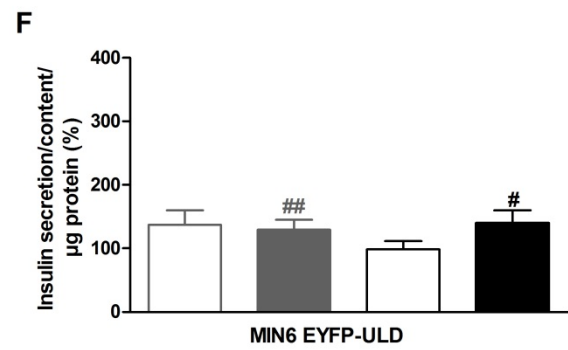
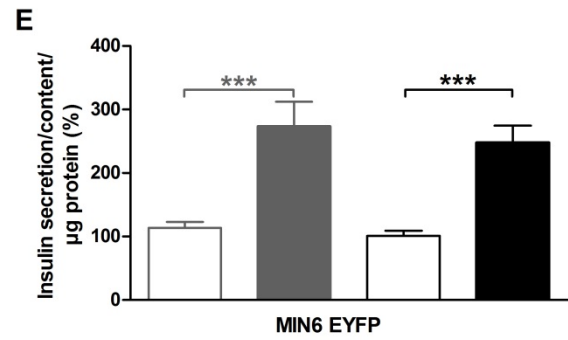
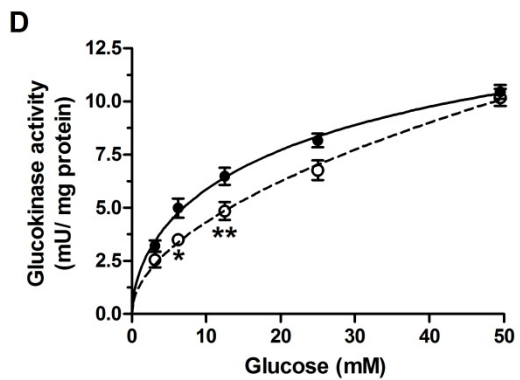
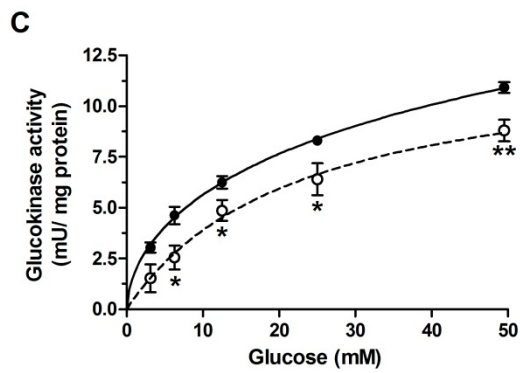
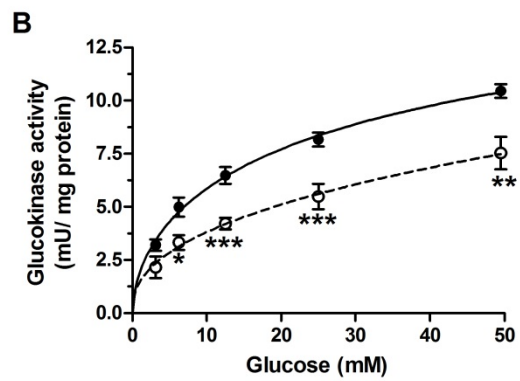
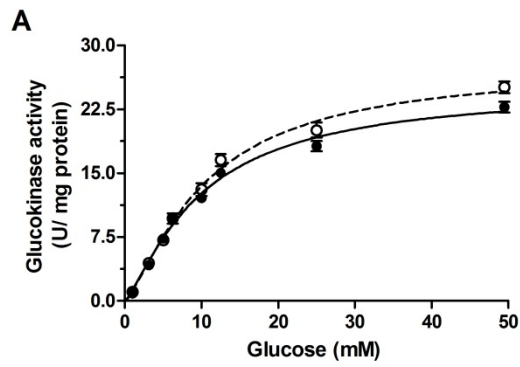


Figure 6

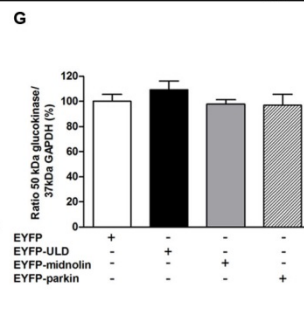
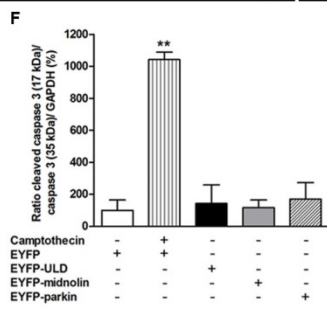
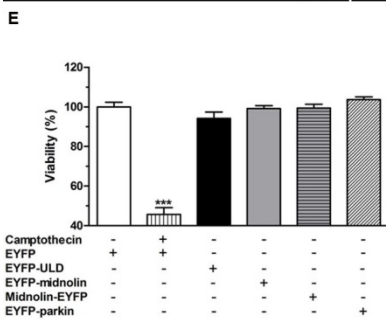
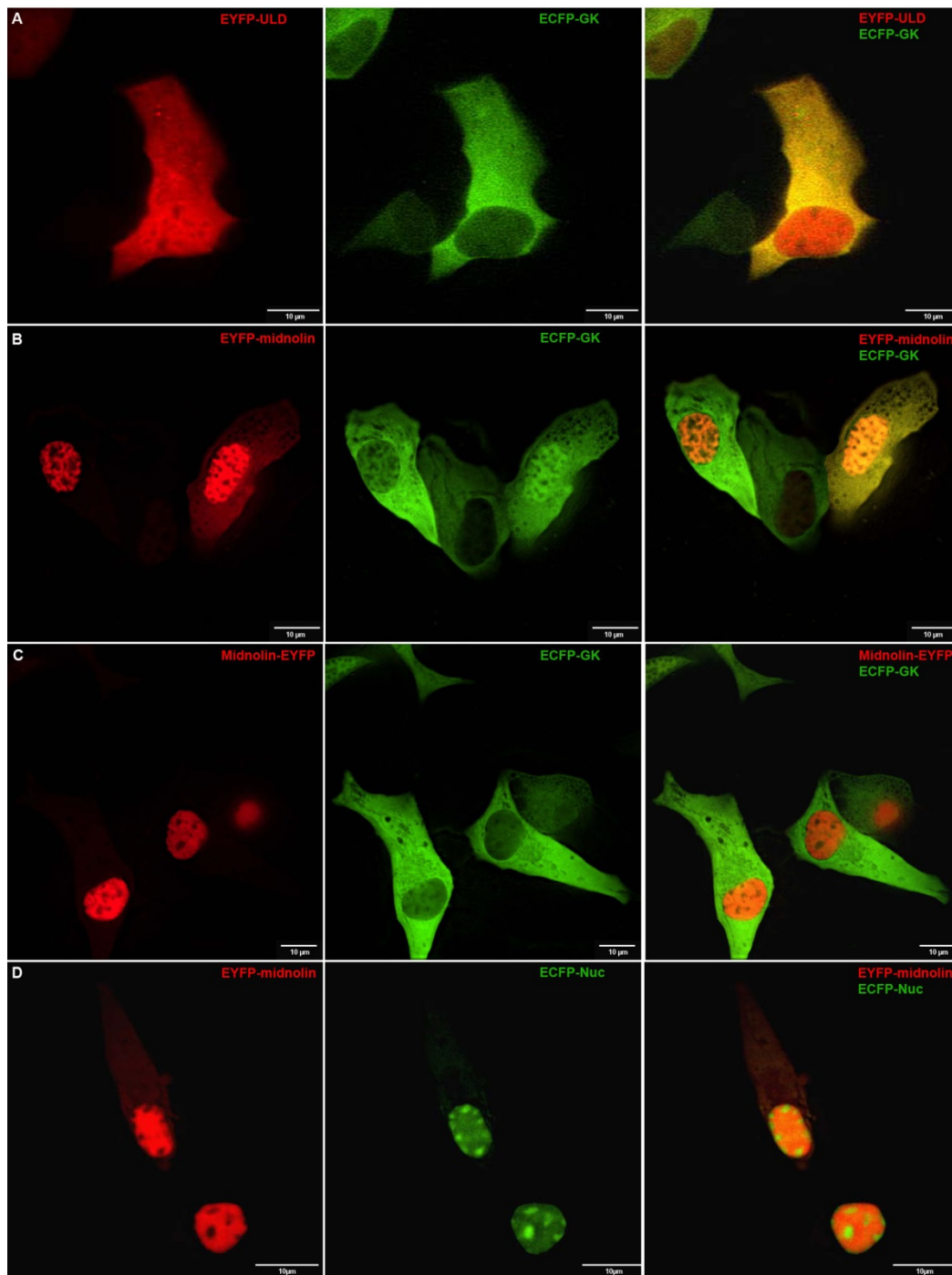


Figure 7

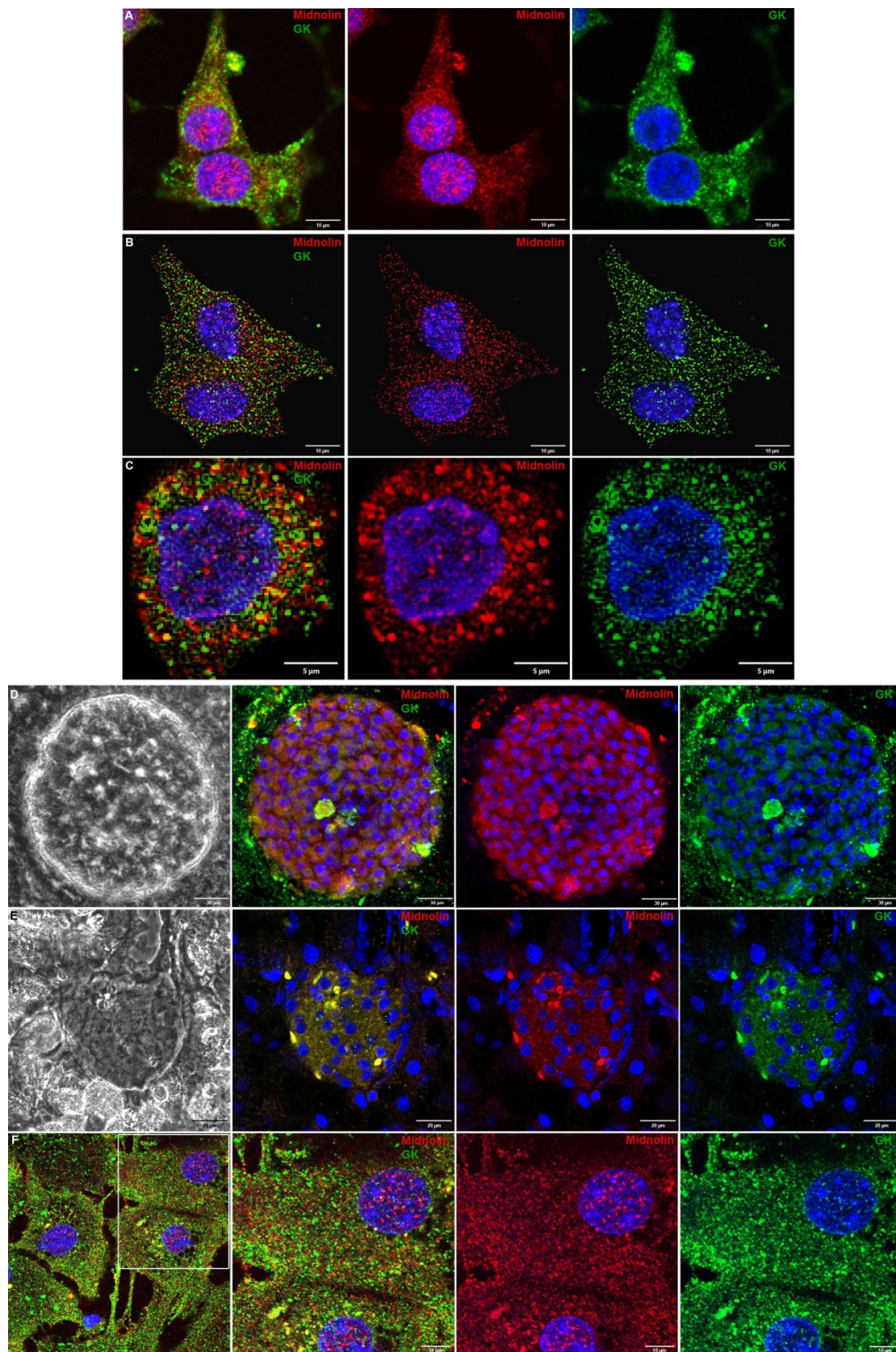
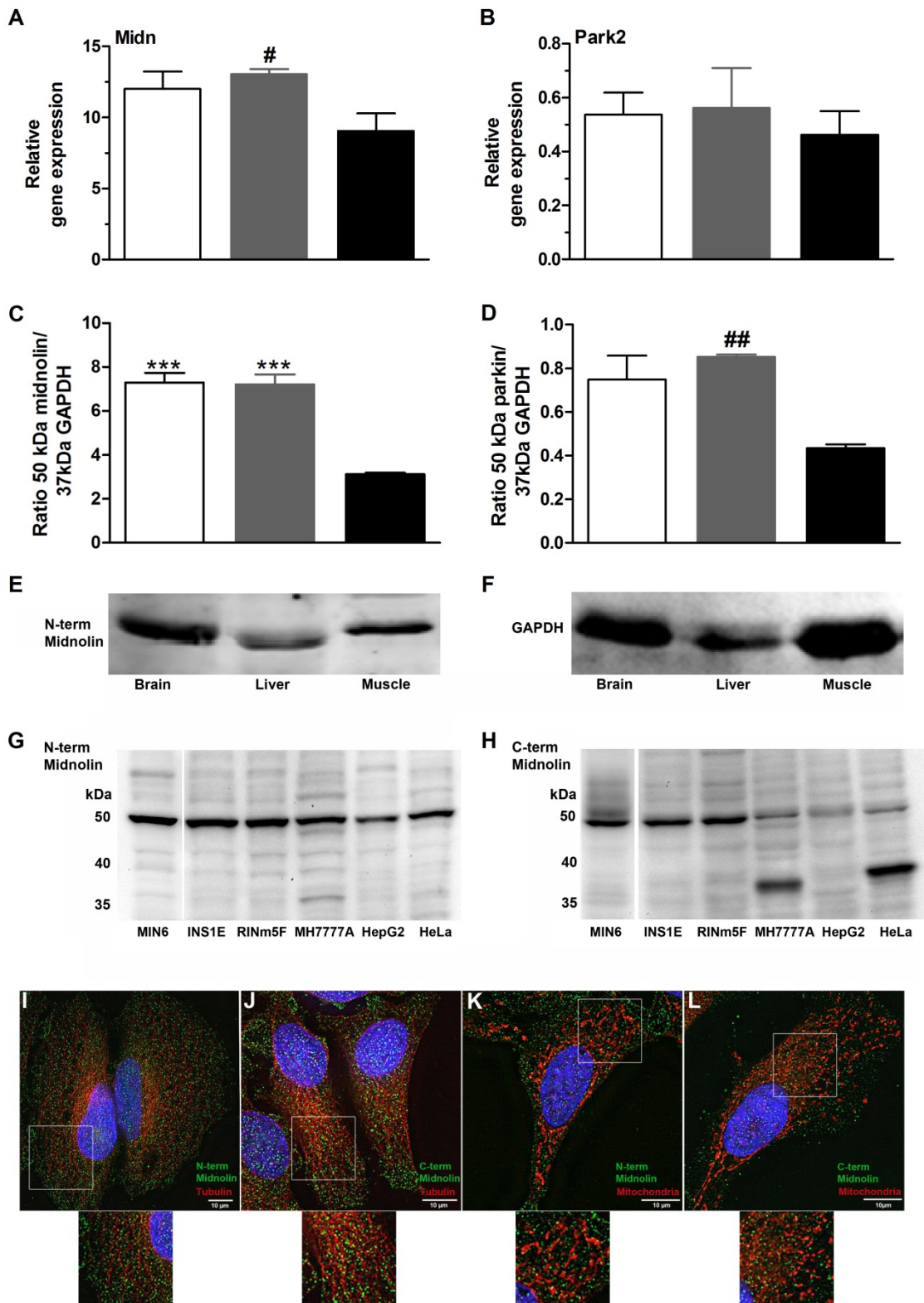


Figure 8



3 Diskussion

Die Zahl der an Typ 2 Diabetes mellitus erkrankten Patienten ist in den letzten Jahren stark angestiegen und nimmt weiterhin zu. Charakteristisch ist neben einer Insulinresistenz der Zielgewebe vor allem die gestörte glucoseinduzierte Insulinsekretion der pankreatischen Beta-Zellen. Hierbei ist die Glucokinase von besonderer Bedeutung, da sie in der Stimulus-Sekretionskopplung der glucoseinduzierten Insulinsekretion die Rolle des Glucosesensors einnimmt (Matschinsky *et al.* 1993). Das Enzym koppelt zusammen mit dem Glucosetransporter GLUT2 den intrazellulären Metabolismus an die extrazelluläre Glucosekonzentration, sorgt somit für die Initiation der Insulinsekretion und ist damit für die Aufrechterhaltung der Blutglucosehomöostase essentiell (Lenzen & Panten 1988; Bell *et al.* 1990; Lenzen & Tiedge 1994). Bei der Regulation der Glucokinaseaktivität spielen neben der Glucose selbst posttranslationale Mechanismen eine wesentliche Rolle (Van Schaftingen 1989; Tiedge *et al.* 1999; Baltrusch *et al.* 2001; Rizzo *et al.* 2002; Baltrusch & Lenzen 2007). Das Verständnis der Glucokinaseregulation ist eine wichtige Grundlage, um die gestörte Insulinsekretion durch Entwicklung neuer Therapeutika wiederherzustellen. Seit etwa einem Jahrzehnt sind chemische Glucokinaseaktivatoren verfügbar, die einen Ansatzpunkt in der Behandlung der diabetischen Hyperglykämie darstellen (Grimsby *et al.* 2003; Efanov *et al.* 2005; Leighton *et al.* 2005). Allerdings konnten diese Substanzen aufgrund unerwünschter Nebeneffekte bislang noch nicht in der Therapie eingesetzt werden (Matschinsky 2013).

3.1 Einfluss der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase und des chemischen Aktivators LY2121260 auf die Insulinsekretion

Das Postulat der Bindung der Glucokinase an einen intrazellulären Regulator mit Proteincharakter in Anwesenheit erhöhter Glucosekonzentrationen (Tiedge *et al.* 1999) konnte durch die Identifizierung der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase als Interaktionspartner bestätigt werden. Das bifunktionelle Enzym ist der einzige bekannte endogene Aktivator der Glucokinase (Baltrusch *et al.* 2001; Massa *et al.* 2004). Die Bindung erfolgt über die Fructose-2,6-bisphosphatasedomäne und führt bei stimulatorischen Glucosekonzentrationen zu einer Erhöhung der maximalen Glucosephosphorylierungsrate (Baltrusch *et al.* 2001; Baltrusch *et al.* 2006; Langer *et al.* 2010). Die Glucoseaffinität der Glucokinase wird durch die Interaktion nicht beeinflusst (Massa *et al.* 2004). Im Gegensatz dazu steigern alle bekannten chemischen Aktivatoren neben der Aktivität auch die Affinität

der Glucokinase zur Glucose (Grimsby *et al.* 2003; Efanov *et al.* 2005; Matschinsky *et al.* 2006).

In der vorliegenden Arbeit sollte der Einfluss des endogenen Aktivators 6-Phosphofructo-2-kinase/Fruktose-2,6-bisphosphatase und der des synthetischen Aktivators LY2121260 auf die glucoseinduzierte Insulinsekretion vergleichend untersucht werden. Hierfür wurde in insulinsezernierenden MIN6 Zellen die 6-Phosphofructo-2-kinase/Fruktose-2,6-bisphosphatase überexprimiert. Das bifunktionelle Enzym steigerte die glucosestimulierte Insulinsekretion in Anwesenheit von 10 mmol/l Glucose, während die Basalsekretion bei 3 mmol/l unverändert blieb. Im Gegensatz dazu konnte die Insulinsekretion durch den chemischen Aktivator LY2121260 auch bei 3 mmol/l gesteigert werden. Das Ergebnis spiegelt deutlich die Erhöhung der Glucoseaffinität der Glucokinase durch den chemischen Aktivator wider, während diese durch das bifunktionelle Enzym nicht beeinflusst wird. Der stimulatorische Effekt des Aktivators LY2121260 auf die Insulinsekretion bestätigt das Ergebnis einer vorherigen Studie, in der eine gesteigerte Sekretion in einem Bereich von 4 – 8 mmol/l Glucose gezeigt werden konnte (Efanov *et al.* 2005). Des Weiteren konnte im Rahmen der vorliegenden Dissertation erstmals ein additiver Effekt der sekretionssteigernden Wirkungen beider Aktivatoren in Gegenwart einer stimulatorischen Glucosekonzentration von 10 mmol/l gezeigt werden. Da das bifunktionelle Enzym nur bei hoher Glucosekonzentration bindet (Langer *et al.* 2010), ist bei Stimulation mit 3 mmol/l lediglich die Interaktion des synthetischen Aktivators zu erwarten. Tatsächlich war die Basalsekretion vergleichbar erhöht wie in Zellen, die nur mit dem chemischen Aktivator behandelt wurden, nicht aber das bifunktionelle Enzym überexprimierten. Interessant ist weiterhin der Befund, dass die Bindung des endogenen Aktivators 6-Phosphofructo-2-kinase/Fruktose-2,6-bisphosphatase an die Glucokinase für den chemischen Aktivator notwendig ist, um die maximale Phosphorylierungskapazität der Glucokinase zu steigern. Dies konnte durch Verwendung einer Mutante der 6-Phosphofructo-2-kinase/Fruktose-2,6-bisphosphatase, die nicht zur Interaktion mit der Glucokinase befähigt ist, bestätigt werden. Die Überexpression dieser Mutante anstelle des Wildtyp-Proteins in MIN6 Zellen steigerte die Insulinsekretion nicht, sondern führte sogar noch zu einer leichten Reduktion. Diese lässt sich dadurch erklären, dass MIN6 Zellen endogen die Isoformen 2 und 3 der 6-Phosphofructo-2-kinase/Fruktose-2,6-bisphosphatase exprimieren (Arden *et al.* 2008). Durch Überexpression der Mutante kommt es zur Störung des basalen Interaktionsgleichgewichts zwischen den endogenen Proteinen 6-Phosphofructo-2-kinase/Fruktose-2,6-bisphosphatase und Glucokinase. In Gegenwart der Mutante der 6-

Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase steigerte der chemische Aktivator LY2121260 tatsächlich lediglich die Basalsekretion, während der stimulatorische Effekt bei 10 mmol/l Glucose ausblieb.

Die Bindung eines chemischen Aktivators erfordert die Anwesenheit von Glucose, da nur dann das allosterische Bindungszentrum in der Glucokinase zugänglich ist (Kamata *et al.* 2004; Zelent *et al.* 2005). Allerdings reichen hier Konzentrationen von 1 – 4 mmol/l aus (Grimsby *et al.* 2003; Kamata *et al.* 2004; Efanov *et al.* 2005). Übereinstimmend konnte eine Beteiligung der Aminosäuren Arg63, Val62, Met210, Met235 und Tyr214 an der Interaktion der Glucokinase mit dem Aktivator LY2121260 bereits gezeigt werden (Efanov *et al.* 2005). Während innerhalb der Bisphosphatasedomäne der 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase eine Sequenz identifiziert werden konnte, die für die Interaktion mit der Glucokinase verantwortlich ist (Baltrusch *et al.* 2001), konnte die Bindungsstelle innerhalb der Glucokinase bislang nicht gefunden werden. Es ist denkbar, dass der endogene Aktivator die Glucokinase in den aktiven Konformationen stabilisiert, aber nicht fixiert (Zhang *et al.* 2006; Antoine *et al.* 2009; Larion & Miller 2010; Larion *et al.* 2012), so dass der chemische Aktivator zusätzlich an das Enzym binden kann. Die Bindung des synthetischen Aktivators alleine führt immer zu einer Fixierung der vollständig geschlossenen Form der Glucokinase, welches wahrscheinlich aufgrund fehlender Flexibilität in der Glucokinase zu der gesteigerten Glucoseaffinität führt und mit einem Verlust der Kooperativität einhergeht (Kamata *et al.* 2004; Efanov *et al.* 2005). Die Stabilisierung der Glucokinase durch einen chemischen Aktivator in Anwesenheit von Glucose resultiert nicht nur in einer Steigerung der Enzymaktivität, sondern auch in einem gesteigerten Proteingehalt (Efanov *et al.* 2005). Eine 32-stündige Inkubation mit 3 mmol/l Glucose in Anwesenheit des chemischen Aktivators LY2121260 verdoppelte die Glucokinase Proteinkonzentration in INS1E Zellen, welches möglicherweise auf einer Stabilisierung der Glucose-gebundenen Konformation beruht (Efanov *et al.* 2005).

3.2 Regulation der Glucokinase Proteinstabilität über das Ubiquitin-Proteasom-System

Die Glucokinase zeigt vor allem bei erhöhter Glucosekonzentration eine hohe Stabilität. Dies konnte nicht nur in den Rattenzelllinien INS1E und RINm5F (Efanov *et al.* 2005; Schmitt *et al.* 2011), sondern auch in primären Beta-Zellen der Ratte (Chen *et al.* 1994) gezeigt werden. Der Proteingehalt einer Zelle wird reguliert durch das Zusammenspiel von Proteinsynthese und Proteinabbau. Während bekannt ist, dass die Glucokinaseexpression in

der Beta-Zelle über Glucose reguliert wird (Tiedge & Lenzen 1991; Chen *et al.* 1994; Tiedge *et al.* 1999; Efanov *et al.* 2005), ist relativ wenig über ihren intrazellulären Abbau bekannt. Die Halbwertszeit der Glucokinase beträgt in Hepatozyten etwa 12 Stunden (Kopitz *et al.* 1990). Des Weiteren gibt es Hinweise, dass die Glucokinase mit Ubiquitin interagiert oder selbst ubiquitiniert wird und über das Ubiquitin-Proteasom-System abgebaut wird (Bjorkhaug *et al.* 2007; Yen *et al.* 2008; Negahdar *et al.* 2012).

Daher wurde im Rahmen der vorliegenden Dissertation der Einfluss des Ubiquitin-Proteasom-Systems auf die Stabilität der Glucokinase untersucht. Hierfür wurden insulinproduzierende MIN6 Zellen mit dem Proteasominhibitor MG132 (Z-Leu-Leu-Leu-al) behandelt. Zusätzlich wurde zur Inhibierung der Translation Cycloheximid eingesetzt. Um sicherzustellen, dass die ermittelten Effekte nicht auf eine Toxizität der Inhibitoren zurückzuführen sind, wurde zunächst die Zellvitalität untersucht. Hierbei wurde sowohl bei einzelner Verwendung der Inhibitoren als auch bei kombinierter Inhibition lediglich eine Reduktion der Vitalität um 30% festgestellt.

Für das bessere Verständnis der Vorgänge nach Hemmung der Translation und des Proteinabbaus war es wichtig, ein Protein vergleichend miteinzubeziehen, welches eine längere Lebensdauer aufweist und so von den Inhibitoren über einen Zeitraum von 12 Stunden unbeeinflusst bleibt und eine konstante Expression zeigt. Im Rahmen einer Proteinstabilitätsstudie wurden ca. 8000 humane Proteine bezüglich ihrer Stabilität in vier Kategorien mit verschiedenen Halbwertszeiten eingestuft (Yen *et al.* 2008). Hierfür wurden für die Proteine entsprechende Proteinstabilitätsindizes vergeben. Die Glucokinase hat mit einem Proteinstabilitätsindex von $\sim 4,3$ eine mittlere Lebensdauer. Die Glycerinaldehyd-3-phosphatdehydrogenase (GAPDH), wie die Glucokinase ein Enzym der Glykolyse, kann hingegen mit einem Index von $\sim 6,2$ der Gruppe der extra-langlebigen Proteine zugeordnet werden (Yen *et al.* 2008). Daher wurde dieses Protein als Vergleich in die Analysen integriert. Die Proteinexpression der Glucokinase konnte durch Hemmung des proteasomalen Abbaus um 50% gesteigert werden, wohingegen die Expression des langlebigen Proteins GAPDH unbeeinflusst blieb. Der Translationsinhibitor Cycloheximid hingegen senkte wie erwartet die Proteinexpression der Glucokinase. Durch das Zusammenspiel von Proteintranslation und -degradation ergibt sich bei gleichbleibenden Bedingungen ein relativ konstanter Proteingehalt. Dies konnte für die Glucokinaseexpression durch die gleichzeitige Verwendung beider Inhibitoren gezeigt werden. Die Expression der GAPDH blieb hingegen bei allen Inkubationsversuchen relativ konstant. Auf

Genexpressionsebene wurde nach Hemmung der Proteindegradation eine verminderte Transkription beobachtet, welche möglicherweise auf einem adaptiven Effekt beruht. Damit ist jedoch sichergestellt, dass der beobachtete Anstieg auf der Proteinebene nicht einer erhöhten Transkriptionsrate zuzuordnen ist. Ein Anstieg der Glucokinase Proteinexpression nach Hemmung des Proteasoms wurde bereits in einem *in vitro* System (Bjorkhaug *et al.* 2007) sowie nach 3-stündiger Inkubation von MIN6 Zellen gezeigt (Negahdar *et al.* 2012). Zudem konnten Negahdar *et al.* (2012) zeigen, dass die Hemmung des lysosomalen Abbaus nur zu einer geringen Steigerung der Glucokinaseexpression führt (Negahdar *et al.* 2012). Dieses spricht im Einklang mit den Ergebnissen der vorliegenden Arbeit für den proteasomalen Abbau des Enzyms. Um den Einfluss der Proteasom- und Translationshemmung auf die Glucokinase näher zu charakterisieren, wurde die endogene Enzymaktivität in MIN6 Zelllysaten gemessen. Hierbei resultierte die Hemmung des Proteasoms in einer Reduktion der Glucokinaseaktivität um etwa 50%. Gleichzeitige Inkubation mit Cycloheximid konnte die Enzymaktivität wiederherstellen. Im Zusammenhang mit der gesteigerten Proteinexpression weist dies darauf hin, dass die Hemmung des Proteasoms zu einer Anreicherung von inaktiver, fehlgefalteter Glucokinase führt. Die gleichzeitige Hemmung der Translation erhöht die Verfügbarkeit langlebiger Chaperone, die dieser Fehlfaltung entgegenwirken können und die Menge an aktivem Enzym erhöhen. Die parallelen Analysen der Insulinsekretion zeigten, dass die Hemmung des Proteasoms zu einem Verlust der glucosestimulierten Insulinsekretion führt. Dieses ist in Übereinstimmung mit anderen Studien (Kitiphongspattana *et al.* 2005; Yan *et al.* 2005; Kawaguchi *et al.* 2006; Lopez-Avalos *et al.* 2006; Hartley *et al.* 2009), bei denen der Einfluss der Hemmung auf die Glucokinase jedoch unbeachtet blieb. Auch wenn der beobachtete Verlust der Insulinsekretion nicht allein durch die Reduktion der Glucokinaseaktivität erklärbar ist, so konnte im Rahmen der vorliegenden Dissertation jedoch gezeigt werden, dass die Aktivitätsreduktion dieses Enzyms dabei eine entscheidende Rolle spielt.

Der Verlust der Enzymaktivität und die gesteigerte Proteinexpression nach Hemmung des Proteinabbaus deuten auf eine Anreicherung von fehlgefaltetem Glucokinaseprotein hin. Es ist bekannt, dass fehlgefaltete Proteine in der Zelle aggregieren können, wenn sie nicht schnell genug über das Proteasom abgebaut werden können (Johnston *et al.* 1998). Zur Analyse einer möglichen Aggregation der Glucokinase nach Behandlung mit den Inhibitoren wurde eine Immunfluoreszenzfärbung mit einem Aggresom-Detektions-Assay etabliert. Anhand dieser Färbung können fehlgefaltete und aggregierte Proteine innerhalb

von Aggresomen detektiert werden (Shen *et al.* 2011). Tatsächlich wurde in MIN6 Zellen und primären Beta-Zellen eine Aggregation der Glucokinase im Zytoplasma beobachtet. Diese Aggregate kolokalisierten mit dem Aggresommarker, so dass von einer spezifischen Aggregation des Proteins ausgegangen werden kann. Besonders deutlich konnte die Aggregation der Glucokinase in Hepatozyten gezeigt werden, welches durch die dort höhere Expressionsrate des Proteins bedingt ist (Iynedjian *et al.* 1986). Durch Hemmung der Translation konnte die Aggregation der Glucokinase sowohl in MIN6 Zellen als auch in Hepatozyten vermindert werden. Die Abnahme der Aggregation durch Hemmung der Translation deutet darauf hin, dass die fehlgefaltete Glucokinase durch Chaperone rekonstituiert werden kann. Durch Translationshemmung werden mehr Chaperone verfügbar, die einer Fehlfaltung entgegenwirken können, da diese nicht für die Faltung neu translatierter Proteine benötigt werden. Zudem können Chaperone sogar die Zahl bereits gebildeter Proteinaggregate reduzieren, indem sie die aggregierten Proteine neu falten (Hartl & Hayer-Hartl 2002; Liberek *et al.* 2008). Es wird angenommen, dass etwa 30% der neu synthetisierten Proteine defekte ribosomale Produkte sind, die aufgrund von Fehlern in der Translation oder in posttranslationalen Prozessen nicht ihre native Form erhalten (Schubert *et al.* 2000). Unter physiologischen Umständen werden solche defekten oder fehlgefalteten Proteine direkt über das Ubiquitin-Proteasom-System abgebaut. Durch Hemmung des Proteasoms steht dieser Weg nicht zur Verfügung und es kommt zur Anreicherung fehlgefalteter Proteine und schließlich zur Aggregation. Interessanterweise konnten über den Computer-Algorithmus TANGO (Fernandez-Escamilla *et al.* 2004) β -Faltblatt Regionen innerhalb der Glucokinase mit einer hohen Tendenz zur Aggregation identifiziert werden. Da die Glucokinase aufgrund ihrer katalytischen Flexibilität in mehreren Konformationen vorliegt (Kamata *et al.* 2004; Antoine *et al.* 2009; Larion *et al.* 2012), ist die korrekte Faltung des Enzyms besonders wichtig. Die dargestellten, unter Verwendung von MG132 erhaltenen Befunde konnten durch weitere, spezifische Proteasominhibitoren wie Epoxomicin und Lactacystin bestätigt werden.

Zum besseren Verständnis der Aggregationsvorgänge wurde die Glucokinase gekoppelt an das photokonvertierbare Fluoreszenzprotein Dendra2 in MIN6 Zellen überexprimiert. Dendra2 zeichnet sich dadurch aus, dass das grün fluoreszierende Protein durch Photokonvertierung mit Licht im UV-Bereich irreversibel in ein rot fluoreszierendes Protein überführt werden kann (Gurskaya *et al.* 2006). Somit kann während der Aggregationsbildung zwischen bereits existierender (rote Dendra2-Glucokinase Form) und neu synthetisierter Glucokinase (grüne Dendra2-Glucokinase Form) differenziert werden.

Sowohl in transient transfizierten als auch in stabil überexprimierenden Zellen konnte nach 12-stündiger Inkubation mit den Proteasominhibitoren MG132, Epoxomicin und Lactacystin eine starke Aggregation der Glucokinase detektiert werden, wie sie zuvor für die endogene Glucokinase gezeigt wurde. Analysen des zeitlichen Verlaufs der Aggregation in stabil überexprimierenden Zellen zeigten, dass durch die Behandlung mit MG132 bereits nach 5 Stunden erste kleinere Aggregate im Zytoplasma detektierbar waren. Mit zunehmender Inkubationszeit nahm die Aggregation deutlich zu und nach 9 Stunden zeigte sich eine starke Lokalisation im perinukleären Bereich. Durch eine dreidimensionale Darstellung des Aggresoms konnte zudem die kompakte Form gezeigt werden. Dieses Aggregationsverhalten entspricht einem von Kopito *et al.* (2000) beschriebenen Aggregationsweg, der davon ausgeht, dass sich zunächst kleine Aggregationsmonomere in der Zellperipherie bilden. Diese bilden dann einen oligomeren Kern, an den sich weitere Monomere anlagern (Kopito 2000). Anhand der Fluoreszenzaufnahmen war es nun möglich, altes Glucokinaseprotein (rote Fluoreszenz) im Zentrum des Aggresoms zu detektieren, während sich neue fehlgefaltete Glucokinase (grüne Fluoreszenz) anlagerte. Interessant war die Feststellung, dass sich das Hauptaggresom im perinukleären Bereich befindet, welches für einen Mikrotubuli-assoziierten Transport zum Mikrotubuli-organisierenden Zentrum (MTOC) spricht (Johnston *et al.* 1998; Garcia-Mata *et al.* 1999). Gleichzeitige Inkubation mit Nocodazol, einem Inhibitor der Mikrotubulopolymerisation, verhinderte tatsächlich die Akkumulation am MTOC. Stattdessen konnte eine Verteilung kleinerer Aggregate innerhalb des Zytoplasmas beobachtet werden. Physiologisch werden fehlgefaltete oder aggregierte Proteine in der Zelle durch Chaperone neu gefaltet oder durch das Proteasom abgebaut (Hartl & Hayer-Hartl 2002). Der pathogene Mechanismus der Aggresombildung wurde bereits bei einigen neurodegenerativen Erkrankungen wie Alzheimer oder Parkinson beobachtet (Garcia-Mata *et al.* 1999). Hierbei wurde auch diskutiert, dass der Mikrotubuli-assoziierte Transport der Aggregate Teil eines protektiven Mechanismus ist, um die Zelle vor toxischen Aggresomen zu schützen (Kopito 2000). Solange die Menge an aggregiertem Protein nicht zu hoch ist, ist dies ein denkbarer Mechanismus, da gezeigt wurde, dass auch das Proteasom und Chaperone zu den MTOC lokalisierten Aggresom rekrutiert wurden (Garcia-Mata *et al.* 2002). Andererseits sind stark aggregierte Proteine schwerer zu entfalten und könnten das 26S Proteasom inhibieren, welches in einer Abnahme der proteolytischen Aktivität resultiert (Johnston *et al.* 1998). In einer vor kurzem veröffentlichten Studie wurde gezeigt, dass bei Patienten mit Typ 2 Diabetes die Proteasomaktivität in den Beta-Zellen reduziert (Bugliani *et al.* 2013) und eine Anreicherung von ubiquitinierten Proteinen

detektierbar ist (Costes *et al.* 2011; Bugliani *et al.* 2013). Daher ist es aufgrund der im Rahmen der vorliegenden Dissertation erhobenen Befunde denkbar, dass die Glucokinase in Folge dieser reduzierten Proteasomaktivität im Typ 2 Diabetes Teil der entstehenden Aggregate ist und es demzufolge zu einer Abnahme der Insulinsekretion kommt.

3.3 Interaktion der Glucokinase mit Ubiquitin

Die Glucokinase besitzt 22 Lysin-Reste, die für eine Ubiquitinierung in Frage kommen. In *in vitro* Mutationsversuchen von Bjorkhaug *et al.* (2007) konnte bestätigt werden, dass mehrere dieser Lysin-Reste als Ubiquitin-Akzeptor dienen können. Tatsächlich wurden ubiquitinierte Formen der Glucokinase in dieser Studie gefunden, wobei keine spezifische Bindungsstelle identifiziert werden konnte (Bjorkhaug *et al.* 2007). Zusätzlich wurde in der Glucokinase eine Sequenz identifiziert, die eine Homologie zu der Kernsequenz des Ubiquitin-interagierenden Motivs besitzt. Diese Sequenz befindet sich in der C-terminalen α -Helix und umfasst die Aminosäuren 442 - 456 (Bjorkhaug *et al.* 2007). Da Ubiquitin-interagierende Motive an der Bindung von Polyubiquitin oder der Interaktion mit Ubiquitin-ähnlichen Proteinen beteiligt sind, aber auch die Ubiquitinierung fördern können (Hicke *et al.* 2005; Hurley *et al.* 2006; Bjorkhaug *et al.* 2007), spricht dieses Motiv ebenfalls für die Ubiquitinierung bzw. für die Interaktion der Glucokinase mit Ubiquitin. Im Rahmen der vorliegenden Dissertation konnte anhand von FRET-Experimenten die Interaktion der Glucokinase mit Ubiquitin bestätigt werden. Als Vergleich wurde eine Ubiquitinmutante verwendet, der alle Lysin-Reste fehlen und in welcher das C-terminale Glycin mutiert ist (Dantuma *et al.* 2006). Durch diese Mutationen ist eine Konjugation des Ubiquitins nicht mehr möglich und eine Interaktion mit der Glucokinase war nicht detektierbar. Diese Ergebnisse bestätigen die bereits *in vitro* postulierte Interaktion der Glucokinase mit Ubiquitin und sprechen für eine Regulation der Glucokinase durch das Ubiquitin-Proteasom-System.

3.4 Interaktion der Glucokinase mit der Ubiquitin-ähnlichen Domäne des Midnolins und mit der E3-Ligase Parkin

Einen weiteren Hinweis für die Beteiligung des Ubiquitin-Proteasom-Systems in der Regulation der Glucokinase lieferte die Identifikation eines neuen Interaktionspartners. In einem Hefe *Library Screening* wurde ein 180 Aminosäuren großes Proteinfragment identifiziert, welches eine Homologie zum N-Terminus des Midnolins aufweist (Kollmann 2004). Interessanterweise beinhaltet dieses Fragment die komplette Sequenz der Ubiquitin-ähnlichen Domäne des Midnolins, welche aus 76 Aminosäuren besteht. Diese Domäne

ähnelt in ihrer Struktur stark dem Ubiquitin und nimmt ebenfalls die beschriebene Ubiquitinsuperfaltung ein (Kiel & Serrano 2006; Grabbe & Dikic 2009).

Midnolin wurde erstmals von Tsukahara *et al.* (2000) im Rahmen einer Analyse von Genen, die in der embryonalen Entwicklung der Maus eine Rolle spielen, identifiziert. Basierend auf dem Expressionsort im Mausembryo und der intrazellulären Lokalisation entstand der Name *Midbrain nucleolar protein*, kurz Midnolin (Tsukahara *et al.* 2000). Da es bisher keine cDNA-Sequenz des Midnolins gibt, konnte eine direkte Interaktion des Proteins mit der Glucokinase nicht untersucht werden. Daher wurde im Rahmen der vorliegenden Dissertation die Interaktion des gefundenen Proteinfragments sowie der Ubiquitin-ähnlichen Domäne mit der Glucokinase charakterisiert. Die Interaktion mit der Glucokinase konnte sowohl für das Gesamtfragment als auch für die Ubiquitin-ähnliche Domäne in einem fluoreszenzbasierten *Mammalian Two-Hybrid System* (Langer *et al.* 2010) bestätigt werden. Während die Interaktion des Gesamtfragments glucoseunabhängig war, zeigte die Ubiquitin-ähnliche Domäne eine stärkere Interaktion bei niedriger Glucosekonzentration. Viele Proteine, die eine Ubiquitin-ähnliche Domäne besitzen, sind Teil des Ubiquitin-Proteasom-Systems und haben unterschiedliche Funktionen (Grabbe & Dikic 2009). Bei erhöhter Glucosekonzentration liegt die Glucokinase in der geschlossenen bis offenen Konformation vor, während bei niedrigen Glucosekonzentrationen hauptsächlich die weit-offene bis offene Konformation vorkommt (Kamata *et al.* 2004; Baltrusch *et al.* 2006; Antoine *et al.* 2009). Das Ergebnis, welches mit dem *Mammalian Two-Hybrid System* erzielt wurde, spricht daher für eine Interaktion mit der offenen bis weit-offenen Konformation. Aufgrund der möglichen Zuordnung der Ubiquitin-ähnlichen Domäne zum Ubiquitin-Proteasom-System ist dies ein Hinweis für einen Regulationsmechanismus der Glucokinase über dieses System. Die Hypothese, dass die Glucokinase durch eine Ubiquitin-ähnliche Domäne reguliert werden kann, wird durch Experimente zur glucosestimulierten Insulinsekretion unterstützt. Diese war in MIN6 Zellen nach Überexpression der Ubiquitin-ähnlichen Domäne, vor allem bei Vorinkubation mit 3 mmol/l Glucose, signifikant reduziert. Zudem konnte gezeigt werden, dass die Glucokinaseaktivität bei Überexpression dieser Domäne in MIN6 Zellen inhibiert wird. In Experimenten mit rekombinanter Glucokinase und der Ubiquitin-ähnlichen Domäne hingegen konnte kein Einfluss auf die Enzymaktivität beobachtet werden. Dieses spricht zudem für einen komplexeren Regulationsmechanismus wie das Ubiquitin-Proteasom-System, bei dem weitere intrazelluläre Faktoren benötigt werden.

In den Beta-Zellen des Pankreas werden Komponenten des Ubiquitin-Proteasom-Systems glucoseabhängig reguliert. Es konnte gezeigt werden, dass einige Gene, die für Proteine aus diesem Bereich codieren, bei hoher Glucose vermindert transkribiert werden (Lopez-Avalos *et al.* 2006; Maris *et al.* 2010; Costes *et al.* 2011). Dazu zählt unter anderem die E3-Ligase Parkin, die eine Ubiquitin-ähnliche Domäne besitzt (Lopez-Avalos *et al.* 2006), die der des Midnolins strukturell sehr ähnlich ist. In insulinproduzierenden MIN6 Zellen und primären Beta-Zellen konnte im Rahmen der vorliegenden Dissertation gezeigt werden, dass Midnolin und Parkin bei ansteigender Glucosekonzentration vermindert exprimiert werden, während andere Proteine, die ebenfalls eine Ubiquitin-ähnliche Domäne besitzen, auf Genexpressionsebene nicht reguliert wurden.

Aufgrund der analogen Genregulation sowie der strukturellen Ähnlichkeit der Ubiquitin-ähnlichen Domänen von Midnolin und Parkin wurde Parkin als möglicher Interaktionspartner der Glucokinase weiterführend untersucht. In FRET-Analysen konnte ebenfalls eine Interaktion der Glucokinase mit Parkin detektiert werden, jedoch fällt diese im Vergleich zur Ubiquitin-ähnlichen Domäne des Midnolins oder auch zu anderen Interaktionspartnern wie dem Glucokinase Regulatorprotein oder der 6-Phosphofructo-2-kinase/Fruktose-2,6-bisphosphatase (Langer *et al.* 2010) deutlich geringer aus. Die Genexpressionsanalysen gaben zusätzlich einen Hinweis darauf, dass die Parkinexpression in MIN6 Zellen und primären Beta-Zellen deutlich geringer ausfällt als die Expression des Midnolins. Messungen der Gen- und Proteinexpression in Geweben aus Muskel, Leber und Gehirn zeigten zusätzlich eine deutlich geringere Parkinexpression bezogen auf das Kontrollprotein GAPDH. Durch Überexpression von Parkin in insulinproduzierenden MIN6 Zellen konnte eine signifikante Inhibierung der endogenen Glucokinaseaktivität bei niedrigen Glucosekonzentrationen beobachtet werden. Interessanterweise führte diese Überexpression auch zu einer signifikanten Reduktion der glucosestimulierten und einer Herabsetzung der basalen Insulinsekretion. Die E3-Ligase Parkin spielt eine Rolle bei der Autophagie von Mitochondrien (Narendra *et al.* 2008). Die Ergebnisse der vorliegenden Dissertation deuten somit auf eine wesentliche, bislang aber noch unbekannt Rolle von Parkin in der Funktion der Beta-Zellen des Pankreas hin.

3.5 Expressions- und Lokalisationsanalysen des Midnolins in verschiedenen Geweben und Zelllinien

Aufgrund der exakten Homologie der identifizierten Ubiquitin-ähnlichen Domäne und der des Midnolins war es von Interesse, die Expression des Proteins in Beta-Zellen

nachzuweisen und zu charakterisieren. Hierfür wurden kürzlich synthetisierte polyklonale Antikörper verwendet, die gegen den N- und C-Terminus des Midnolins gerichtet sind. Somit war es möglich, in Immunfluoreszenzanalysen die Expression des Proteins in MIN6 und primären Beta-Zellen nachzuweisen. Überraschenderweise beschränkte sich die punktförmige Midnolinexpression nicht auf den Zellkern, sondern war auch im Zytoplasma detektierbar, welches für die Interaktion mit der Glucokinase in den Beta-Zellen des Pankreas essentiell ist. In primären Beta-Zellen konnte zudem eine Kolo-kalisation des Midnolins mit der Glucokinase im Zytoplasma gezeigt werden. Die zusätzlich zytosolische Lokalisation des Midnolins steht im Widerspruch zu der von Tsukahara *et al.* (2000) beschriebenen exklusiven, nukleären Lokalisation (Tsukahara *et al.* 2000). Neben der N-terminalen Ubiquitin-ähnlichen Domäne wurde eine C-terminale nukleoläre Lokalisationssequenz postuliert. Aufgrund dieser Sequenz und anhand von Experimenten mit GFP-Chimären des Midnolins wurde eine nukleäre bzw. nukleoläre Expression postuliert (Tsukahara *et al.* 2000). Hierbei wurde eine Funktion des Midnolins in der Regulation von Genen, die in der Neurogenese (Tsukahara *et al.* 2000) sowie in der Kontrolle der Entwicklung von Rinderfollikeln durch Regulation des mRNA-Transports eine Rolle spielen, angenommen (Zielak *et al.* 2008). Während in der embryonalen Entwicklung die Midnolinexpression vor allem im Mesencephalon detektierbar war, konnte in einigen adulten Geweben eine Expression durch *Northern Blot* Analysen gezeigt werden. Dieses betraf vor allem Herz, Lunge, Leber und Niere, wobei eine mögliche Funktion des Midnolins in adultem Gewebe nicht bekannt ist (Tsukahara *et al.* 2000). Analysen auf Proteinebene sind in diesen Studien nicht durchgeführt worden. Im Rahmen der vorliegenden Dissertation konnte sowohl auf Protein- als auch auf Genexpressionsebene Midnolin in Muskel, Gehirn und Leber erstmals nachgewiesen werden. In beiden Analysen war im Muskel die geringste Expression detektierbar. Die gezeigte Proteinexpression gibt Hinweise darauf, dass Midnolin auch im adulten Gewebe eine wichtige Rolle spielt. Zusätzlich konnte das Protein durch *Western Blot* Analysen in verschiedenen Zelllinien nachgewiesen werden. In der Mauszelllinie MIN6 und den Rattenzelllinien INS1E, RINm5F und MH7777A sowie den humanen Zelllinien HepG2 und HeLa konnte bei Verwendung des N- und C-terminalen Antikörpers eine deutliche Bande bei 50 kDa detektiert werden. Während in der Maus drei Isoformen bekannt sind (Q3TPJ7-1: ~ 49 kDa, Q3TPJ7-2, -3: ~54 kDa), ist beim Menschen nur eine Isoform aufgeführt (Q504T8: ~49 kDa). Daher scheint die Isoform1 die hauptsächlich exprimierte Form zu sein. Zusätzlich konnte bei Verwendung des C-terminalen Antikörpers in den humanen Zelllinien eine Bande bei etwa 37 kDa

detektiert werden. Bei dieser Isoform ist eine Deletion am N-Terminus wahrscheinlich. Auch in humanen HeLa Zellen konnte in Immunfluoreszenzanalysen eine nukleäre und zytoplasmatische punktförmige Verteilung des Midnolins beobachtet werden. Dieses Ergebnis stellt die von Tsukahara *et al.* (2000) diskutierte exklusive Verteilung des Midnolins im Nukleus und den Nukleoli in Frage (Tsukahara *et al.* 2000) und eröffnet neue Aspekte der Funktion des Midnolins in adultem Gewebe. Während für Parkin eine Assoziation mit den Mitochondrien bekannt ist (Narendra *et al.* 2008), konnte für Midnolin in HeLa Zellen eine geringfügige Kolokalisation mit β -Tubulin, jedoch keine Kolokalisation mit den Mitochondrien detektiert werden. Parkin ist ein Multidomänenprotein, das für seine Funktion als E3-Ligase eine RING-Domäne besitzt. Ob Midnolin neben der Ubiquitin-ähnlichen Domäne weitere funktionelle Domänen besitzt, die dem Protein ebenfalls eine Funktion als E3-Ligase zukommen lassen, ist fraglich. In weiterführenden Studien ist daher zu klären, welche Funktion das Midnolin in den Beta-Zellen hat und vor allem welchen Einfluss es auf die Glucokinase hat. Zusätzlich stellt auch die Interaktion der Glucokinase mit Parkin einen interessanten Ansatzpunkt für weitere Analysen dar. Allerdings weisen die Ergebnisse dieser Dissertation deutlicher auf eine Rolle des Midnolins als inhibitorischen Glucokinase-Interaktionspartner hin. Dies ist besonders deshalb interessant, da das Glucokinase Regulatorprotein, welches die Glucokinase in der Leber bei niedrigen Glucosekonzentrationen inhibiert (Van Schaftingen 1989), in den Beta-Zellen nicht exprimiert wird. Somit könnte eine Inhibierung der Glucokinaseaktivität durch Midnolin ein an die Beta-Zellen des Pankreas adaptiertes Regulationsprinzip darstellen.

4 Zusammenfassung

Als Glucosesensorenzym ist die Glucokinase zentraler Bestandteil der glucoseinduzierten Insulinsekretion in den Beta-Zellen des Pankreas. Zusammen mit dem Glucosetransporter GLUT2 koppelt das Enzym die extrazelluläre Glucosekonzentration an den intrazellulären Metabolismus und sorgt somit durch Initiation der Insulinsekretion für die Aufrechterhaltung der Blutglucosehomöostase. Zur Anpassung der Glucokinaseaktivität an Schwankungen der Glucosekonzentration sind posttranslationale Regulationsmechanismen wie Protein-Protein-Interaktionen und eine intrazelluläre Kompartimentierung von essentieller Bedeutung. In der Leber erfolgt die Regulation hauptsächlich über das Glucokinase Regulatorprotein, welches bei niedrigen Glucosekonzentrationen für die Bindung und Inhibierung der Glucokinase sowie für eine Translokation in den Nukleus verantwortlich ist. Sowohl in der Leber als auch in den Beta-Zellen des Pankreas wird die Glucokinase durch das bifunktionelle Enzym 6-Phosphofructo-2-kinase/Fruktose-2,6-bisphosphatase aktiviert. Das als Dimer vorliegende Enzym interagiert bei hohen Glucosekonzentrationen über die Bisphosphatasedomäne mit der Glucokinase. Dabei bleibt die Glucoseaffinität der Glucokinase unverändert. Zur Behandlung von Hyperglykämien, die das Leitsymptom des Typ 2 Diabetes mellitus darstellen, wurden vor etwa einem Jahrzehnt chemische Glucokinaseaktivatoren synthetisiert, die bislang jedoch noch nicht therapeutisch eingesetzt wurden. Neben der Erhöhung der Glucokinaseaktivität wird durch diese synthetischen Aktivatoren auch die Glucoseaffinität gesteigert, welches in einem erhöhten Glucoseumsatz bei niedriger Blutglucosekonzentration und damit einem Hypoglykämierisiko einhergeht.

In der vorliegenden Arbeit konnte gezeigt werden, dass der endogene Aktivator 6-Phosphofructo-2-kinase/Fruktose-2,6-bisphosphatase und der synthetische Aktivator LY2121260 in insulinsezernierenden MIN6 Zellen einen additiven Effekt auf die glucosestimulierte Insulinsekretion haben. Zusätzlich konnte durch die Verwendung einer Mutante der 6-Phosphofructo-2-kinase/Fruktose-2,6-bisphosphatase, die nicht zur Interaktion befähigt ist, nachgewiesen werden, dass die Anwesenheit des endogenen Aktivators für die Steigerung der maximalen Phosphorylierungskapazität der Glucokinase durch den synthetischen Aktivator LY2121260 bei einer stimulatorischen Glucosekonzentration essentiell ist. Bei basaler Glucosekonzentration hingegen findet die Interaktion der Glucokinase mit dem synthetischen Aktivator ohne Beteiligung der 6-

Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase statt. Es ist daher davon auszugehen, dass die beiden Aktivatoren die Glucokinase über verschiedene Mechanismen aktivieren.

Neben den bereits bekannten posttranslationalen Regulationsmechanismen der Glucokinase in der Beta-Zelle ist eine Regulation über die Proteinstabilität des Enzyms durch das Ubiquitin-Proteasom-System weitgehend unerforscht. Die Bedeutung des Ubiquitin-Proteasom-Systems für die Glucokinasestabilität und -aktivität konnte im Rahmen der vorliegenden Dissertation mit Hilfe von Proteasom-Inhibitoren und eines Translationsinhibitors gezeigt werden. Die Hemmung des proteasomalen Abbaus resultierte in insulinsezernierenden MIN6 Zellen in einer reduzierten Glucokinaseaktivität und einem Verlust der glucoseinduzierten Insulinsekretion, bedingt durch eine Anreicherung von inaktivem fehlgefaltetem Glucokinaseprotein. Die Zunahme an fehlgefaltetem Protein führte sowohl in primären Beta-Zellen als auch in Hepatozyten zu einer Aggregation des Glucosesensors im Zytoplasma. Durch Etablierung einer Immunfluoreszenzfärbung mit einem Aggresom-Detektions-Kit konnte die Anwesenheit der Glucokinase in Aggresomen gezeigt werden. Die gleichzeitige Hemmung von Translation und proteasomalem Abbau verminderte die Aggregation des Glucosesensors und konnte die Aktivität des Enzyms wiederherstellen. Zusätzlich wurde durch Verwendung von MIN6 Dendra2-Glucokinase Zellen ein Mikrotubuli-assoziiertes Transport der Aggregate zum Mikrotubuli-organisierenden Zentrum gezeigt. Das Ubiquitin-Proteasom-System spielt somit in der Regulation der Glucokinasestabilität und damit einhergehend auch in der Regulation der Enzymaktivität eine wichtige Rolle. Zusätzlich konnte die Interaktion der Glucokinase mit Ubiquitin durch FRET-Analysen bestätigt werden, welches ebenfalls für einen proteasomalen Abbau der Glucokinase spricht.

Gestützt wird die Hypothese der Glucokinaserregulation durch das Ubiquitin-Proteasom-System durch die Identifizierung eines Proteinfragments mit Homologie zum N-Terminus des Midnolins. Von besonderer Bedeutung ist hierbei die Ubiquitin-ähnliche Domäne des Proteins, die ebenfalls Bestandteil des identifizierten Proteinfragments ist. Im Rahmen dieser Dissertation konnte die Interaktion der Ubiquitin-ähnlichen Domäne sowie des Proteinfragments, welches diese Domäne enthält, mit der Glucokinase in einem *Mammalian Two-Hybrid* System gezeigt werden. Hierbei wurde zudem deutlich, dass die Bindung der Domäne an die Glucokinase, im Gegensatz zum gesamten Proteinfragment, durch niedrige Glucosekonzentrationen verstärkt wird. Genexpressionsanalysen zeigten, dass die Expression des Midnolins ebenfalls glucoseabhängig erfolgt und die Transkription bei niedriger Glucosekonzentration erhöht ist. Eine solche glucoseabhängige Genexpression

konnte auch für die E3-Ligase Parkin gezeigt werden, die eine strukturell vergleichbare Ubiquitin-ähnliche Domäne besitzt. In FRET-Experimenten wurde eine Interaktion der Glucokinase mit der Ubiquitin-ähnlichen Domäne des Midnolins bestätigt sowie in geringerem Ausmaß auch mit Parkin nachgewiesen. Die Überexpression der Ubiquitin-ähnlichen Domäne des Midnolins resultierte in einer Reduktion der Glucokinaseaktivität und der glucosestimulierten Insulinsekretion. Auch für Parkin konnte eine Interaktion mit der Glucokinase bei niedriger Glucosekonzentration gezeigt werden. Die Glucokinaseaktivität hingegen wurde durch Parkin bei niedrigen Glucosekonzentrationen deutlich weniger inhibiert. Zusätzlich zu der Reduktion der glucosestimulierten Insulinsekretion wurde auch eine Hemmung der Basalsekretion durch Parkin gefunden. Weiterhin konnte gezeigt werden, dass die Genexpression von Parkin in insulinsezernierenden Zellen und Geweben wie Leber, Muskel und Gehirn deutlich geringer ausfällt als die Midnolin-Expression. Dies ließ sich zusätzlich auf Proteinebene bestätigen. Für Midnolin wurde ergänzend eine Expression in verschiedenen Zelllinien der Maus und Ratte sowie humanen Ursprungs gezeigt. Hierbei konnte in den humanen Zelllinien die Expression einer weiteren Isoform des Midnolins gezeigt werden, die vermutlich einer N-terminal deletierten Form entspricht. Midnolin wurde bisher nur als ein nukleolär lokalisiertes Protein beschrieben. In humanen HeLa und insulinsezernierenden MIN6 Zellen konnte jedoch zusätzlich eine zytosolische Lokalisation detektiert werden. Des Weiteren wurde in primären Beta-Zellen eine Kolo-kalisation des Midnolins mit der Glucokinase festgestellt. Die vorliegenden Befunde deuten somit auf eine direkte Interaktion des Midnolins mit dem Glucosesensor Glucokinase im Zytosol der Beta-Zellen des Pankreas hin. Vergleichend zu Parkin kann dem Midnolin eine besondere Rolle in der Regulation der Glucokinase in den Beta-Zellen zugeordnet werden. Da das inhibierende Glucokinase Regulatorprotein der Leber in den Beta-Zellen des Pankreas nicht exprimiert wird, könnte hier das Midnolin im Zusammenspiel mit der 6-Phosphofructo-2-kinase/Fruuctose-2,6-bisphosphatase die Funktion übernehmen, die Glucokinaseaktivität glucoseabhängig bedarfsgerecht zu modulieren.

Die durchgeführten Studien belegen eine Rolle des Ubiquitin-Proteasom-Systems und des eine Ubiquitin-ähnliche Domäne enthaltenden Proteins Midnolin in der Regulation der Glucokinaseaktivität und der glucoseinduzierten Insulinsekretion in den Beta-Zellen des Pankreas. Die Ergebnisse der vorliegenden Dissertation stellen in Zusammenhang mit der kürzlich gezeigten Reduktion der proteasomalen Aktivität in Beta-Zellen des Pankreas bei Patienten mit Typ 2 Diabetes einen Bezug zur Pathogenese dieser Krankheit dar.

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6 Publikationen

Teilergebnisse der vorliegenden Dissertation sind bereits veröffentlicht.

Kurzbeiträge:

Brix, A., Kollmann, K., Langer, S., Lenzen, S., Baltrusch, S. (2010) Identification of an ubiquitin-like domain as a potential new interaction partner of glucokinase in pancreatic beta cells. *Diabetologia* **53**, Suppl. 1, S 202, 496, 46. EASD Annual Meeting, Stockholm, 2010

Brix, A., Kollmann, K., Langer, S., Lenzen, S., Baltrusch, S. (2011) Identifizierung einer ubiquitin-ähnlichen Domäne als neuer potentieller Interaktionspartner der Glucokinase in Beta-Zellen des Pankreas. *Diabetologie und Stoffwechsel* **20**, Suppl. 1, S 9, FV 16, 46. Jahrestagung der Deutschen Diabetes-Gesellschaft, Leipzig, 2011

Brix, A., Lenzen, S. Baltrusch, S. (2012) Einfluss des Ubiquitin-Proteasom-Systems auf das Glucosesensorenzym Glucokinase in MIN6 Beta-Zellen. *Diabetologie und Stoffwechsel* **7**, Suppl. 1, S 21, FV 55, 47. Jahrestagung der Deutschen Diabetes-Gesellschaft, Stuttgart, 2012

Hofmeister-Brix, A., Lenzen, S. Baltrusch, S. (2012) Influence of the ubiquitin-proteasome-system on the glucose sensor glucokinase in pancreatic beta cells. *Diabetologia* **55**, Suppl. 1, S 167, 390, 48. EASD Annual Meeting, Berlin, 2012

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Baltrusch, S., Schmitt, H., Brix, A., Langer, S., Lenzen, S. (2012) Additive activation of glucokinase by the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and the chemical activator LY2121260. *Biochemical Pharmacology*, **83** (2012): 1300-1306

Hofmeister-Brix, A., Lenzen, S., Baltrusch, S. The ubiquitin proteasome system regulates the stability and activity of the glucose sensor glucokinase in pancreatic beta cells. *Biochemical Journal*, **465** (2013): 173-84

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Die vorliegende Dissertation wurde im November 2009 unter Anleitung von Herrn Professor Dr. S. Lenzen am Institut für Klinische Biochemie der Medizinischen Hochschule Hannover begonnen und im Juni 2013 abgeschlossen.

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