

From The Rolf Luft Center for Diabetes Research
The Endocrine and Diabetes Unit
Department of Molecular Medicine
Karolinska Institutet, Stockholm, Sweden

**Molecular mechanisms underlying
the glucose-dependent
transcription of the insulin and
glucokinase genes in the
pancreatic β -cell**

Tilo Moede



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ABSTRACT

Background: Insulin is of vital importance in the maintenance of the glucose homeostasis in mammals. This necessitates a tight regulation of both insulin release and biosynthesis. Although pancreatic β -cells secrete only a fraction of the stored insulin upon glucose stimulation, insulin biosynthesis starts immediately in order to replenish the insulin store. It is well documented that glucose exerts its immediate effects at the posttranscriptional and translational levels by enhancing the stability of insulin-mRNA and up-regulating translation initiation and translation elongation. The glucose effect on transcription, however, is believed to occur only after several hours of exposure of the β -cell to high glucose. Because blood-glucose levels are only elevated for minutes rather than hours after food uptake, we hypothesized the existence of a short-term control of gene transcription by glucose. Since the β -cell transcription unit of the glucokinase gene has been shown to be regulated by the same stimuli as the insulin gene and has been discussed to utilize similar/same transcription regulatory elements, we postulated that also here glucose may affect transcription immediately.

Aim: The aims of this study were 1.) to evaluate the existence of a glucose-dependent short-term regulation of the transcription of the insulin and glucokinase genes and, if so, 2.) to identify the molecular mechanisms involved.

Findings: By studying mRNA steady-state levels, transcription initiation and by establishing a technique that allowed us to monitor gene transcription online, we demonstrated, that insulin gene transcription is indeed regulated by short-term exposure of pancreatic β -cells to glucose. Investigation of the mechanisms underlying the short-term control revealed a link between insulin exocytosis and insulin gene transcription: insulin, secreted in response to glucose stimulation, up-regulates insulin gene transcription through a pathway that involves the insulin receptor/PI3 kinase/p70 s6 kinase and CaM kinases. Analysis of insulin promoter *cis*-elements showed the involvement of A- and E-boxes in glucose-dependent up-regulation of insulin gene transcription. Because the β -cell specific transcription factor PDX-1, which binds to A-boxes in the insulin promoter, has been discussed to be involved in glucose-dependent up-regulation of insulin gene transcription by a mechanism involving cytoplasmic-nuclear translocation, we aimed to identify the amino acid sequences responsible for the nuclear import of PDX-1. Site-directed mutagenesis of putative phosphorylation sites and of positively charged amino acids in putative nuclear localization signal (NLS) motifs revealed that not a specific phosphorylation site, but the presence of the NLS motif RRMKWKK is necessary and, in conjunction with the integrity of the 'helix 3' domain of the PDX-1 homeodomain, sufficient for nuclear translocation of PDX-1.

Analysis of the glucose-dependent regulation of glucokinase gene transcription showed, as is the case for the insulin gene, secreted insulin being a key factor. Surprisingly, the molecular mechanisms involved in insulin-stimulated glucokinase gene transcription are different from those of insulin-stimulated insulin gene transcription. To study these differences in more detail we established a system that allowed us to monitor the activity of both the insulin and glucokinase promoters simultaneously within the same cell. By using this technique, we were able to demonstrate that insulin-stimulated insulin gene transcription is regulated through insulin receptor A/PI3 kinase class Ia/p70 s6 kinase, whereas glucokinase transcription is activated by a mechanism involving insulin receptor B/PI3 kinase class II like activity/PKB(c-Akt).

Conclusion: We were able to demonstrate that both the insulin and the glucokinase genes are regulated by short-term exposure of pancreatic β -cells to elevated glucose. The mechanism involves an insulin feedback loop, which utilizes different regulatory pathways for the activation of the two genes. Insulin gene transcription is activated by a mechanism involving insulin receptor A, PI3 kinase class Ia and p70 s6 kinase. Glucokinase gene expression, however, is regulated through insulin receptor B, PI3 kinase class II-like activity and PKB(c-Akt). These data demonstrate that selective insulin signaling can be gained through two different isoforms of the insulin receptor and report the first insulin receptor isoform-specific read-out. More importantly, the data demonstrate that the β -cell is a target for insulin action. This implicates that β -cell insulin resistance might contribute to the development of a β -cell dysfunction in type 2 diabetes.

PUBLICATIONS INCLUDED IN THIS THESIS

- I. Leibiger B, Moede T, Schwarz T, Brown GR, Kohler M, Leibiger IB, Berggren PO. **Short-term regulation of insulin gene transcription by glucose.** PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 95: 9307-9312, 1998.
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- III. Moede T*, Leibiger B*, Pour HG, Berggren PO, Leibiger IB. **Identification of a nuclear localization signal, RRMKWKK, in the homeodomain transcription factor PDX-1.** FEBS LETTERS 461: 229-234, 1999. *contributed equally
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- V. Leibiger B, Leibiger IB, Moede T, Kemper S, Kulkarni RN, Kahn CR, de Vargas LM, Berggren PO. **Selective insulin signaling through A and B insulin receptors regulates transcription of insulin and glucokinase genes in pancreatic beta cells.** MOLECULAR CELL 7: 559-570, 2001.

Some additional data, not published previously, are included in the results and discussion chapter.

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LIST OF ABBREVIATIONS

BGK	β -cell specific glucokinase
CaMK	Ca ²⁺ /calmodulin dependent kinase
CMV	cytomegalovirus
FRAP	FKBP rapamycin-associated protein
FRET	fluorescence resonance energy transfer
GLUT2	Glucose Transporter 2
GFP	Green fluorescent protein, in this thesis used as synonym for EGFP/GFP _{S65T} (enhanced green fluorescent protein/green fluorescent protein Mutant S65T)
hIR	Human insulin receptor
IGF-1	Insulin like growth factor 1
IGF-1R	Insulin like growth factor I receptor
IR	Insulin receptor
IRS	Insulin receptor substrate
JNK	Jun kinase
MAPK	Mitogen activated protein kinase
MODY	Maturity Onset Diabetes of the Young
mRNA	Messenger ribonucleic acid
mTOR	Human target of rapamycin
PDX-1	Pancreatic duodenal homeobox 1
PI3K	PI3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
RT-PCR	Reverse transcriptase polymerase chain reaction
SAPK	stress activated protein kinase
TBE	TRIS borate EDTA buffer

1 INTRODUCTION

Insulin, in adult mammals exclusively produced by and secreted from pancreatic β -cells of the islets of Langerhans, is vital for the maintenance of blood glucose homeostasis. Absence of insulin, as in type 1 diabetes, is lethal if untreated. Malfunction of either insulin secretion or insulin signaling causes the development of type 2 diabetes, the most common metabolic disorder in man (Taylor et al. 1995).

The unique role of insulin necessitates the strict and fast-acting regulation of its release and its biosynthesis in response to the complex set of stimuli resulting from food uptake, including neural as well as humoral components (for review see Sjöholm et al. 2001;), with elevated blood glucose levels being the major nutrient factor. Although β -cells only release a part of the stored insulin in response to glucose stimulation, biosynthesis of new insulin starts immediately. Glucose is well known to stimulate insulin biosynthesis in a relatively short period of time. In fact, within 30 min after start of glucose stimulation, newly synthesized (pro)insulin has been demonstrated (Permutt et al. 1972, 1975; Ashcroft et al. 1978). Data from over two decades of research demonstrate that glucose affects insulin biosynthesis immediately at the posttranscriptional and translational levels by enhancing insulin mRNA stability, translation initiation and elongation (Welsh et al. 1985, 1986; Gilligan et al. 1996). Although glucose is well appreciated as a positive stimulus at the level of insulin gene transcription (Docherty et al. 1994; Stein et al. 1993; German et al. 1990; Goodison et al. 1992; Melloul et al. 1993; German et al. 1994; Petersen et al. 1994; Sharma et al. 1994; Redmon et al. 1994; Sharma et al. 1995; Odagiri et al. 1996), the currently accepted view is that glucose affects insulin gene transcription as a long-term effect. More precisely, it is believed that pancreatic β -cells have to be exposed to elevated glucose concentrations for more than 2 hours to gain an effect at the transcriptional level (Bailey et al. 1992). As a consequence to that view, glucose-stimulated insulin mRNA translation (immediate effect) has to be uncoupled from glucose-stimulated insulin gene transcription (long-term effect), i.e. glucose-stimulated insulin gene transcription should have no impact on immediate glucose-triggered insulin biosynthesis. Moreover, no short-lived signals would contribute to glucose-stimulated insulin gene transcription. This concept, however, contradicts the physiological situation, where blood glucose levels either as the result of food uptake or in response to an oral glucose tolerance test (OGTT) decline to basal blood glucose values within 2 h. If, as in case of the OGTT, 2 h after glucose stimulation the glucose concentration is still elevated, the WHO defines this as a situation of either impaired glucose tolerance (>6.7 mM blood glucose), which is a risk indicator for the development of diabetes, or as a condition of diabetes mellitus (>10 mM blood glucose) (WHO consultation report 1999). Consequently, under physiological conditions, blood glucose concentration is

Introduction

elevated for minutes rather than hours. Therefore, we hypothesized the existence of a short-term control mechanism of insulin gene transcription by glucose and aimed to analyze the underlying mechanism. Noteworthy, the possible existence of such a short-term metabolic control of insulin gene transcription has been suggested indirectly before (Efrat et al. 1991), but has never been clearly demonstrated.

Glucokinase (hexokinase IV, ATP:D-hexose-6-phosphotransferase, EC 2.7.1.1.) catalyzes the first step in glucose utilization, the phosphorylation of glucose to glucose-6-phosphate. Because this is believed to be the rate-limiting step in the glucose-stimulus/insulin-secretion coupling in β -cell, glucokinase has often been referred to as the β -cell 'glucose sensor' (Matschinsky et al. 1990). Malfunction of glucokinase due to mutation of its gene has been shown to cause the MODY2-type of type 2 diabetes mellitus (Froguel et al. 1992; Gidh-Jain et al. 1993). The β -cell isoform of glucokinase differs from its counterpart in the liver only in the first 15 amino acids of the N-terminus (Magnuson et al. 1989; Iynedjian et al. 1989), which are encoded by two alternative first exons. For the expression of the respective isoforms in liver and pancreatic islets two different promoters are responsible (Magnuson et al. 1989, 1990, 1992). The β -cell active (upstream) promoter of the rat glucokinase gene (BGK) shows no sequence homologies with the liver (downstream) promoter, thus indicating differences in the use of *cis*- and *trans*-acting elements and allowing different modes in the nutrient- and hormone-dependent transcriptional regulation. The characterization of the 5'-flanking region of the β -cell transcription unit of the rat glucokinase gene by others (Shelton et al. 1992; Watada et al. 1996) and us (Leibiger et al. 1994a, 1994b, 1994c) revealed similar *cis*-elements with insulin gene promoters suggesting common mechanisms in transcriptional regulation. Therefore, we postulated that also here glucose might affect transcription immediately.

2 BACKGROUND

2.1 Insulin gene and the insulin gene promoter

In 1921 Banting and Best discovered, that intravenous injection of an extract from the islets of Langerhans into diabetic animals resulted in lowered blood glucose levels and reduced glucosuria (Banting et al. 1922). In 1922 this extract, called insulin, was used to successfully treat patients with diabetes mellitus. For this discovery Banting and Macleod received the Nobel price in 1923.

Shortly after it was discovered that insulin was a protein and the first crystalline insulin was obtained in 1926 by Abel et al.. Insulin was also the first protein to be completely sequenced, an achievement for which Sanger was awarded the Nobel price in 1959.

The insulin gene, cloned in 1979 by Bell and colleagues (Bell et al. 1979), was one of the first human genes to be cloned. Furthermore, in 1979 insulin became the first protein to be produced commercially by recombinant DNA techniques.

Insulin and the insulin gene have always been studied with great interest because of the enormous importance of the hormone in connection with the most common metabolic disorder in man – diabetes mellitus. Structural data for insulin and the insulin gene are available for a large number of species, demonstrating that the structure of the insulin gene is highly conserved throughout evolution. In general, the gene consists of 3 exons and 2 introns. While the length of the introns may vary from species to species, the relative positions and the genetic information encoded by the exons remain constant. Exon 1 always encodes the 5' untranslated region, exon 2 contains sequences encoding the signal peptide, the B-chain and part of the C-peptide and exon 3 encodes the remainder of the C-peptide, the A-chain and the 3' untranslated region. The rat I and the mouse I insulin gene however lack the intron 2.

The insulin gene is a single copy gene in all examined species, with three exceptions – rat, mouse and *Xenopus laevis*, where two non-allelic insulin genes are present (Lomedico et al. 1979; Wentworth et al. 1986, Shuldiner et al. 1989.).

Before discussing the mechanisms involved in the transcriptional control of β -cell specific insulin gene, current knowledge on the regulation of eukaryotic gene transcription will be briefly mention. Here we will concentrate on genes transcribed by the DNA-dependent RNA-polymerase II. Results of extensive work in the last decades demonstrate that regulation of transcription is dependent on the interaction of DNA-motifs (*cis*-acting elements) with protein factors (*trans*-acting elements). It has become common to classify the regulatory *cis*-elements according to their properties as promoters or enhancers.

Promoters are located close to the transcription initiation site and are directly involved in the assembly of the transcription initiation complex. Depending on the presence or absence of the conserved binding motif 5'-TATAAA-3' (the TATA-box, located 20 to 35 bp upstream of the

Background

transcription start site) binding the transcription factor TFIID, genes have been classified as “TATA-box-containing” and “TATA-box-lacking” (Kageyama et al. 1989). It is assumed that regulation of those two types of genes is different. TATA-box-lacking promoters are often found in so called “housekeeping” genes, which have usually multiple transcription initiation sites and in many cases possess multiple GC-rich elements in the 5'-flanking regions. Further DNA-motifs located upstream of the transcription start site, commonly 8-15 bp in length and often called “upstream promoter elements”, are thought to increase the rate of transcription. In contrast to promoters, enhancers show no orientation- and position-effect on transcription and may act as far as 12 kb from the transcription start site. It is thought that enhancers are composed of discrete elements interacting with nuclear factors to modulate promoter activity in a positive or negative (“silencer”) manner. For a physiologically temporal, cell-type specific and metabolically dependent transcriptional control the complex interaction of all *cis*- and *trans*-acting elements is necessary.

Trans-acting elements, such as transcriptional regulatory proteins, can be divided into classes according to structural motifs for recognition: (a) helix-turn-helix, (b) homeodomain, (c) zinc finger, (d) steroid receptor, (e) leucine zipper and helix-loop-helix, (f) β -sheet motifs and others (see Pabo et al. 1992 for review). These protein factors can bind as single proteins, homodimers and heterodimers. The architecture of the transcriptional machinery includes not only DNA/protein- but also protein/protein-interactions. The proper interaction of all these factors allows expression of a gene at the right place, at the right time, in an appropriate amount of the respective protein necessary to maintain cellular function.

Analysis of rat, mouse and human insulin promoters revealed that they contain common *cis*-elements, i.e. binding sites for transcription factors. These *cis*-elements and the corresponding identified *trans*-factors are schematically illustrated in figure 1.

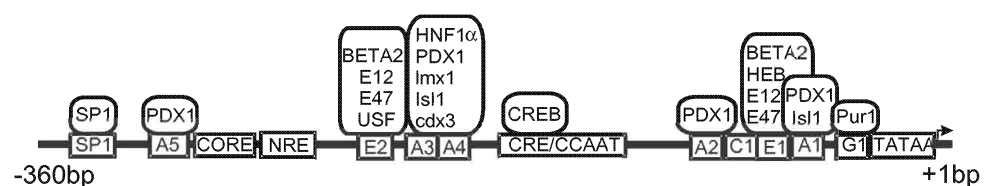


Figure 1 Schematic illustration of the insulin promoter composition Composite overview of the relative location of the so far described *cis*-elements in insulin promoters (rat I+II, mouse I+II and human) (boxes) and their identified *trans*-factors (adopted from Sander et al. 1997; for review see German et al. 1995)

Site-directed mutagenesis and deletion studies showed that the level of basal insulin gene expression is dependent to a varying degree on all of these *cis*-elements.

The mechanisms discussed in this thesis, however, concern the up-regulation of insulin gene expression by elevated, stimulatory glucose concentration, i.e. glucose concentration that leads to an insulin exocytosis. These mechanisms might be different from those contributing to basal transcription, resulting in situations where the insulin gene is still expressed, but the mechanisms associated with the stimulus secretion coupling (see 2.4) are disturbed.

It has been believed, that elevated glucose only has an effect on insulin gene transcription after hours of exposure (Baileys et al. 1992). To this end, the molecular mechanisms underlying glucose-stimulated insulin gene transcription have mainly been studied following incubation with high glucose concentrations over several hours or even days (Stein et al. 1993; German et al. 1990; Docherty et al. 1994; Melloul et al. 1993; German et al. 1994; Petersen et al. 1994; Sharma et al. 1994; Redmon et al. 1994; Sharma et al. 1995; Odagiri et al. 1996). According to this view, mechanisms regulating insulin gene transcription are dissociated from the immediate glucose effect and from short-term signaling associated with the regulation of translation or insulin secretion. However, from the physiological point of view pancreatic β -cells are exposed to elevated glucose levels for minutes rather than hours following food-uptake. Therefore, we hypothesized the existence of a short-term control, allowing glucose to exert physiological regulation at the level of insulin gene transcription.

Interestingly, the finding that elevated glucose up-regulates insulin gene transcription rather quickly is not new. In fact, data reported by Efrat et al. in 1991 (Efrat et al. 1991) shows that in nuclear run-off assays insulin gene transcription is elevated as early as 10 min following glucose stimulation, with a maximum at 30 min and followed by a decline in transcriptional activity. Interestingly, the decline occurred despite the fact, that stimulatory glucose concentrations were maintained for up to 2 hours (Efrat et al. 1991).

2.2 Glucokinase gene and the glucokinase promoter

The enzyme glucokinase, also known as hexokinase IV (EC 7.7.1.1., ATP:D-hexose-6-phosphotransferase), is expressed almost exclusively in liver and β -cells. It is characterized, compared to other hexokinases, by a lower affinity to glucose, a higher substrate specificity for glucose, increased K_m -values and the absence of product-inhibition (Printz et al. 1993). Glucokinase is an important factor in the maintenance of glucose homeostasis. Mutations in the glucokinase gene are linked to a form of diabetes, MODY2 (maturity onset diabetes of the youth type 2) (Froguel et al. 1992; Gidh-Jain et al. 1993).

The liver and the β -cell glucokinase proteins are identical, except for 11 of the first 15 amino acids (Magnuson et al. 1989; Iynedjian et al. 1989). This is the result of the use of alternative first exons and consequently alternative promoters in those two tissues (Magnuson et al. 1989, 1990, 1992).

The β -cell specific promoter and first exon are located more than 15 kb upstream of the liver specific glucokinase gene promote. A first characterization of the glucokinase promoter was performed by Shelton et al. 1992 (Shelton et al. 1992) and Leibiger et al. 1994 (Leibiger et al. 1994a). The analysis of the glucokinase promoter so far revealed a number of *cis*-elements that are similar to that of the insulin promoter, i.e. A- and E-box elements (figure 2). But it also lacks some of the *cis*-elements of the insulin promoter, most notably the TATA-box. It also contains two TGGT-motifs, which greatly influence the basic transcriptional activity of the glucokinase promoter (Leibiger et al. 1994a; Moates et al. 1996).

Furthermore, it has been shown that the BGK promoter fragment up to nucleotide –278 contains all elements necessary for both cell-specific and glucose dependent glucokinase gene transcription (Jetton et al. 1994, 1998).

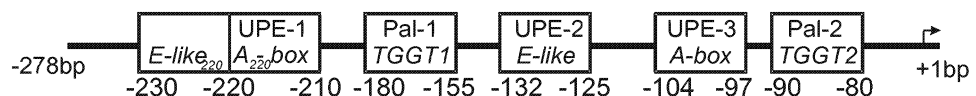


Figure 2 **The glucokinase promoter** Schematic illustration of the locations of the *cis*-elements within the β -cell specific glucokinase (BGK) promoter up to –278 bp. The glucokinase promoter lacks a TATAA-box. The nomenclature of *cis*-elements used by Magnuson et al. (Magnuson et al. 1993) is given in the top line; our nomenclature is given in italics below. (adopted and modified from Magnuson et al. 1993)

Previous studies regarding glucose-dependent transcription of the glucokinase gene in β -cells have been performed under conditions similar to those used for the insulin gene, i.e. with exposure to the sugar for hours to days, and generated contradictory results (Tiedge et al. 1991; Liang et al. 1994; Jetton et al. 1994; Schuit et al. 1997; Jetton et al. 1998; Kajimoto et al. 1999; da Silva Xavier et al. 2000).

2.3 PDX-1

Among all transcription factors discussed to be involved in glucose-dependent transcriptional control in pancreatic β -cells, the most favored one is the pancreatic duodenal homeobox containing factor-1, PDX-1, (for review see McKinnon et al. 2001). PDX-1 is identical or homologous to transcription factors described as IPF-1 (Ohlsson et al. 1993), STF-1 (Leonard et al. 1993), IDX-1 (Miller et al. 1994), GSF (Marshak et al. 1996), IUF-1 (Macfarlane et al. 1994,1996,1997) and GSTF (Olson et al. 1993). PDX-1 is a 283 amino acid protein with a predicted molecular weight of 31 kD and belongs to the class of homeodomain-containing transcription factors. Like most transcription factors, PDX-1 has a modular structure with separate functional domains (fig. 3). Its middle region contains an antennapedia-like homeodomain, which is involved in protein-DNA interaction. The N-terminus of PDX-1 contains the trans-activation domain, which allows the interaction with other transcription factors (Lu et al. 1996).

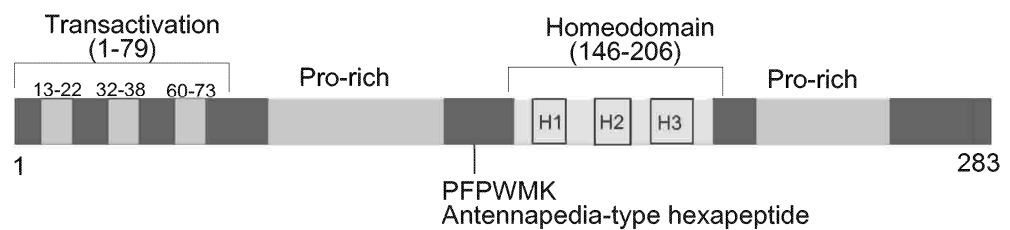


Figure 3 Structure of PDX-1 Schematic overview of the structure of PDX-1. The NH₂-terminus of PDX-1 consists of the transactivation domain, that contains 3 conserved subdomains, marked in a lighter gray. The antennapedia-like homeodomain stretches from 146-206 amino acids and contains 3 helices, marked H1, H2 and H3. The homeodomain is flanked on both sides by a Pro-rich domain. (adopted from McKinnon et al. 2001)

PDX-1 is expressed in β - and δ -cells of the islets of Langerhans and in dispersed endocrine cells of the duodenum (Peshavaria et al. 1994) and plays a crucial role in pancreas development and the transcriptional control of several β -cell specific genes (reviewed in Edlund et al. 1999,2001; McKinnon et al. 2001). Homozygous disruption of PDX-1 expression results in pancreatic agenesis and is consequently lethal (Jonsson et al. 1994; Offield et al. 1996). Heterozygous mutations of the PDX-1 gene are associated with diabetes in animals and with MODY4 in human (Ahlgren et al. 1998; Stoffers et al. 1997a). In the adult, PDX-1 is discussed to be one of the transcription factors involved in the transcriptional control of β -cell specific genes encoding insulin, β -cell glucokinase and amylin as well as in the transcription of the δ -cell specific somatostatin gene (Leonard et al. 1993).

Electrophoretic mobility shift assays revealed that PDX-1 binds to A-box motif sequences in promoters of the rat insulin-I gene (Ohlsson et al. 1991), rat insulin-II gene (Leibiger et al. 1995), human insulin gene (Boam et al. 1990), glucose transporter 2 (GLUT2) gene (Waeber et al. 1996), glucokinase gene (Watada et al. 1996) and amylin gene (Serup et al. 1996; Carty et al. 1997; Bretherton-Watt et al. 1996).

In the context of stimulus-induced gene transcription it was shown that DNA-binding of PDX-1 is dependent on phosphorylation (Macfarlane et al. 1994), and that PDX-1 binds to its *cis*-elements in a glucose-dependent manner (Macfarlane et al. 1994, 1996, 1997, German et al. 1990; Goodison et al. 1992). Macfarlane et al. suggested a glucose-dependent translocation into the nucleus as a possible mechanism of activation of gene transcription by PDX-1 (Macfarlane et al. 1999), while Rafiq et al. suggested an intranuclear trafficking of PDX-1 upon glucose stimulation, which results in the translocation of PDX-1 from the nuclear periphery to the nucleoplasm (Rafiq et al. 1998).

Background

2.4 The pancreatic β -cell and the regulation of insulin secretion

Pancreatic β -cells have to synthesize and release insulin in appropriate rates to keep the blood glucose concentrations within narrow physiological limits. This basic principle is modulated by a variety of metabolic, neural, and hormonal factors with glucose being the major nutrient component.

The search for the “glucose receptor” of the pancreatic β -cell started in the 1960s and rapidly led to three key findings. First, glucose must be metabolized by the β -cell to stimulate insulin secretion. This conclusion was based on evidence that showed that the insulinotropic effect of glucose was mimicked by other metabolizable carbohydrates and was inhibited, if glucose metabolism was disturbed (Grodsky et al. 1963, Coore et al. 1964). Secondly, Ca^{2+} was a key factor in insulin secretion, since absence of extracellular Ca^{2+} prevented the stimulatory effect of glucose on insulin secretion (Grodsky et

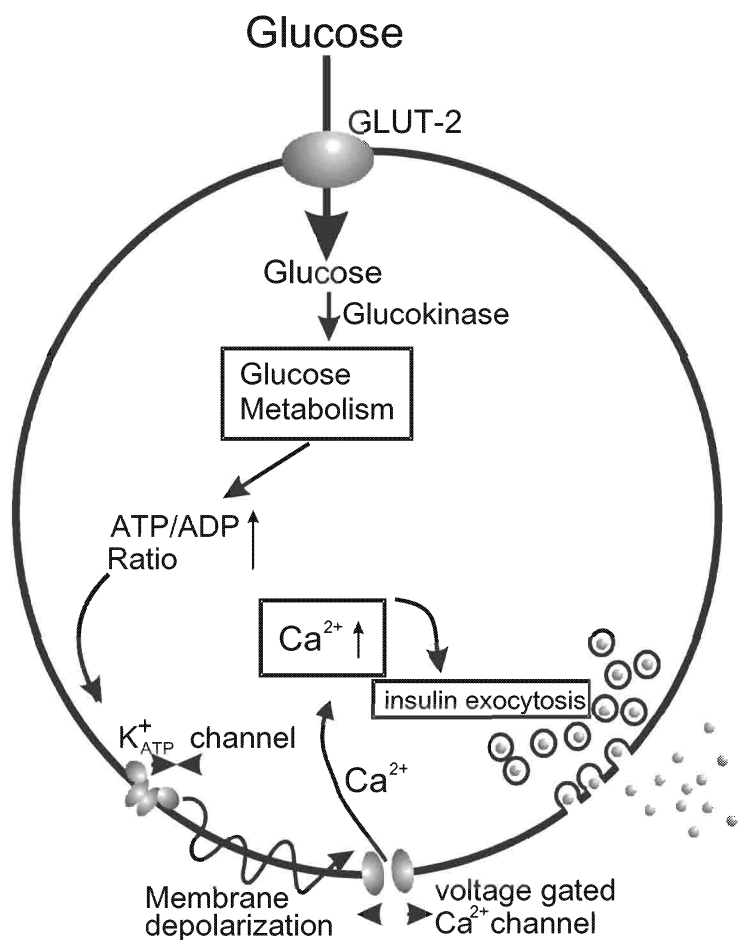


Figure 4 **Schematic model of the events involved in insulin secretion**

This figure give a schematic overview of the basic events involved in the process commonly called stimulus secretion coupling.

al. 1966, Milner et al. 1967). And finally, β -cells were electrically excitable, as shown by recordings of action potentials (Dean et al. 1968).

These three findings were the cornerstones of a puzzle called “stimulus-secretion-coupling” that, although the understanding of this process at the molecular level is much increased, is not yet completed.

The current view of this process is summarized in figure 4.

Briefly, this complex of processes starts with the uptake of glucose by the beta cell high- K_m /low affinity glucose transporter GLUT2 and proceeds with the conversion of glucose into glucose-6-phosphate by the beta cell isoform of glucokinase. The following metabolism of glucose in glycolysis and the Krebs cycle results in the generation of ATP. Elevation in the ATP/ADP ratio leads to closure of ATP-sensitive K^+ channels, which in turn results in depolarization of the plasma membrane. The subsequent opening of voltage-gated L-type Ca^{2+} channels leads to an increase in the cytoplasmic free Ca^{2+} concentration, $[Ca^{2+}]_i$, which promotes insulin secretion (reviewed in Berggren et al. 1994; Ashcroft et al. 1994).

2.5 Insulin receptor signaling pathways

Insulin has been shown to exhibit pleiotropic effects involving mitogenic and/or metabolic events. Moreover, the effect of insulin is tissue- as well as development-dependent. The fact that insulin may transduce its signal through a variety of pathways has been discussed in extensive detail (White et al. 1994). The two major pathways described to date, which employ insulin receptors as the primary target, include signaling via mitogen activated protein (MAP) kinases and phosphoinositol 3-kinase (PI3K). A schematic overview of the processes involved in insulin receptor signaling leading to an increase in gene expression or protein synthesis is shown in figure 5.

The most interesting pathways in the context of the present thesis work include the activation of PI3K and the downstream located p70 S6 kinase, PDK1 and PKB/Akt, and the Ras/Raf/Mek/Erk and p38(MAPK) pathways, since all these pathways have also been shown to be involved in the activation of gene transcription (reviewed in Saltiel et al. 2001).

The insulin receptor (IR), the first step in these cascades, exists in two isoforms as a result of alternative mRNA splicing of the 11th exon of the insulin pro-receptor transcript (Seino et al. 1989). The A type (IR-A), or

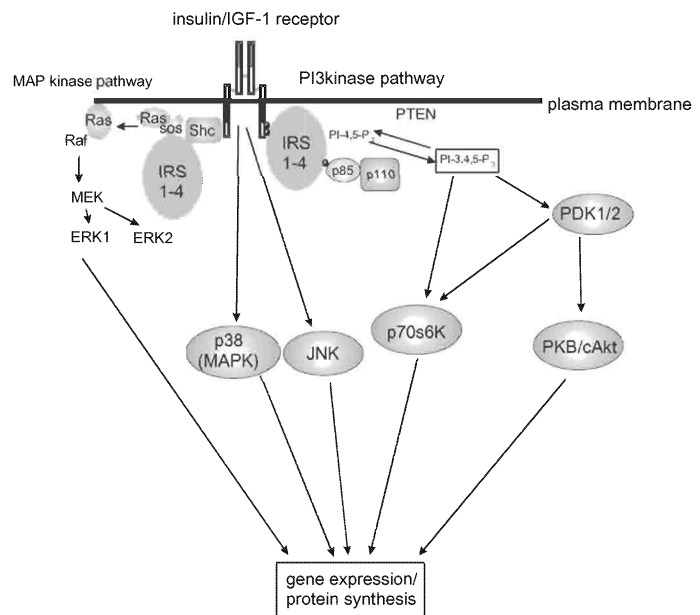


Figure 5 Schematic overview over processes involved in IR dependent gene expression. Brief and simplified overview over processes involved in the activation of gene expression or protein synthesis through the insulin or IGF-1 receptor. JNK = Jun kinase, MEK = MAP kinase kinase. (adopted and simplified from Bevan et al. 2001 and Dupont et al. 2001)

Ex11-, (Ullrich et al. 1985) lacks whereas the B type (IR-B), or Ex11+, (Ebina et al. 1985) contains the respective sequence coding for 12 amino acids in the C-terminus of the α -chain of the receptor (fig. 6)

Little is known about insulin signaling via IR-A and IR-B. Whereas IR-B, which shows a 2-fold less affinity for insulin in comparison to IR-A (Mosthaf et al. 1990; Yamaguchi et al. 1993;

McClain et al. 1991), is predominantly expressed in liver and muscle, IR-A is mainly expressed in brain (Moller et al. 1989; Seino et al. 1989; Mosthaf et al. 1990). Besides the affinity for insulin, differences in their kinase activity (Kellerer et al. 1992) as well as internalization and recycling (Vogt et al. 1991; Yamaguchi et al. 1991) have been described. These data implied differences in the function of either IR isoform, but no isoform-specific insulin-induced effect has been reported so far. Attempts to correlate tissue-specific expression of IR isoforms with diabetes mellitus have

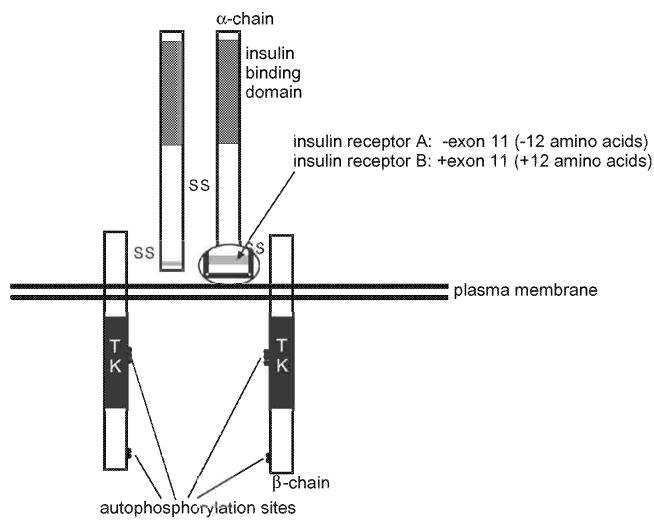


Figure 6 The insulin receptor. Schematic overview of the structure of the insulin receptor. The difference between A and B type, the 12 amino acids near the C-terminus of the α -chain is highlighted. TK = tyrosin kinase domain SS = disulfide bonds (adopted from Siddle et al. 1992)

generated conflicting results (Mosthaf et al. 1991; Benecke et al. 1992; Norgren et al. 1993) that do not clarify the functional role of either isoform. In fact, the functional significance of these IR isoforms remains unclear.

2.6 Fluorescent proteins as dynamic and semi-quantitative reporters for promoter activity

The use of fluorescent proteins as a 'protein-tag' in cell biology, is not only very popular but, in the purest sense of the word, did and does shed new light on the mechanisms that determine key-processes in cell function: sub-cellular localization of proteins, co-localization of proteins, physical interaction of proteins, protein translocation, etc. Used in a rational experimental set-up and in combination with techniques that allow high resolution and/or high dynamics of the obtained images, fluorescent proteins provide a tool that makes the phrase 'seeing is believing' become true in cell physiology. This is because for the first time cellular processes can be monitored in living cells without the interference and/or limitation set by the availability of a substrate, which needs to be administrated or provided by the cell, as generally used in enzyme-based approaches. Moreover, the generation of color-different members of the GFP-family (see for review Tsien et al. 1998), i.e. blue (BFP, EBFP), cyan (ECFP), green (GFP, EGFP), yellow (EYFP), as well as the cloning of new fluorescent proteins such as the red fluorescent protein DsRed from *Discosoma Sp.* (Matz et al. 1999), provide fluorescent probes exhibiting discrete as well as combinatory spectra of light/energy excitation and emission. This allows at the one hand the combinatory use of different 'colors' to monitor discrete proteins at the same time. On the other hand it opens the platform to develop costume-designed molecular probes to monitor protein interactions, substrate concentrations, enzyme activities etc. at the sub-cellular level, partially based upon fluorescence resonance energy transfer (FRET)(Mitra et al. 1996; Miyawaki et al. 1997; Xu et al. 1998c). A timely review on the potential application of fluorescent proteins, based on the GFP-family, was published by Tsien (Tsien et al. 1998).

The use of fluorescent proteins as 'reporter genes', although propagated as genetic markers from the beginning by Chalfie et al. (Chalfie et al. 1994), is employed quite rarely. This statement does not mean to reflect the use of GFP (and its family members) as a 'marker' in transfection experiments, stable or transient, where it is widely used. This statement concerns more the use of fluorescent proteins as dynamic 'reporters' when compared to the classical reporter genes chloramphenicolacetyltransferase (CAT), neomycinphosphotransferase-II (neo), β -galactosidase (β -gal), luciferase (luc), etc. In the context of the present thesis work, the use of the fluorescent proteins GFP and DsRed was instrumental because of the following reasons:

- 1) this approach allows us to overcome the limitations set by the amount of tissue as well as the transfection efficiency when working with primary islet cells due to the possibility to monitor gene expression at the single cell level;
- 2) this approach allows direct evaluation of stimulus-induced promoter activation without external normalization procedures because GFP/DsRed expression is monitored in the same cell before, while and after stimulation without the additional need of extracellular substrates to generate fluorescence;

3) the combination of discretely detectable fluorescent proteins, here GFP and DsRed, enables to monitor stimulus-induced transcription by two different promoters simultaneously in the same cell;

4) this approach gives the advantage to monitor stimulus-induced promoter-driven GFP/DsRed expression online.

To test fluorescent proteins as suitable reporters for semi-quantitative as well as for short-term-induced gene transcription, we first studied whether GFP can be used as a quantitative reporter gene. Therefore, we employed the widely accepted Tet-On system. Here we placed the GFP gene under the control of the Tet-On promoter and monitored GFP expression in transfected cells using increasing amounts of doxycycline (0-2000 ng/ml) as the stimulator. Indeed, GFP expression, measured by digital imaging fluorescence microscopy, was elevated in a dose-dependent manner (paper I fig. 4; paper IV, fig. 1a). When estimated as 'fluorescence/pixel of monitored cell cluster', where the 'cell cluster' reflects the sum of both GFP-expressing and non-expressing cells, GFP-fluorescence at maximum was increased more than 100-fold (see paper I, fig. 4a). When measured as 'fluorescence/pixel of GFP-expressing cell', GFP-expression was elevated more than 4-fold (paper I, fig. 4b; paper IV, fig. 1a).

To test whether fluorescent proteins can be used as reporters to study short-term regulation of gene transcription, we analyzed the immediate early gene *c-fos* promoter to drive GFP or DsRed expression upon stimulation with phorbol 12-myristate 13-acetate (PMA). The formation of the fluorophore of GFP_{S65T} has been reported to take approximately 30-60 min after translation of the GFP mRNA (Heim et al. 1995, Heim et al. 1994). Considering this and the time necessary for transcription initiation, transcription elongation, transcript processing, nuclear-cytoplasmic mRNA transport and translation, we expected a significant increase in GFP-fluorescence 80 to 120 min after start of stimulation. In transient expression studies using a variety of cell lines, we observed that the expression of GFP by individual cells within a transfected cell cluster is heterogeneous. This is because the basal GFP-fluorescence in each individual cell is a function of the copy number of the respective expressing plasmids, which differs in individual cells in a transient expression system. To quantify GFP expression, we set the intensity of GFP-fluorescence of each individual cell at minute 20 as the basal value 1.0. Further on, the intensity of GFP-fluorescence of each individual cell monitored was related to its basal value. By employing digital imaging fluorescence microscopy we were able to show that the PMA stimulation resulted in an increase in GFP-fluorescence between 80 and 100 min after start of stimulation and reached a maximum at 240 to 260 min (paper I, fig. 5b; paper IV, fig. 1b). A non-specific effect of PMA on GFP-fluorescence can be excluded because PMA stimulation in combination with a pharmacological inhibitor of protein kinase C, i.e. bisindolylmaleimide-I, blocked PMA-stimulated *c-fos* promoter-driven GFP.

Background

2.7 Simultaneous monitoring of BGK- and insulin-promoter activities in the same cell

To study the differences between insulin-stimulated BGK and insulin gene transcription, we established a reporter gene system that allowed us to monitor the promoters of the two genes in the same cell simultaneously. The basics of using GFP as a dynamic and semi-quantitative reporter for promoter activity are described in Background 2.6, paper I and paper IV. In order to monitor a second promoter simultaneously, we had to choose a fluorescent protein that could be clearly distinguished from GFP. Because excitation/emission profiles shifted towards the blue/UV spectrum would result in either harming the cells by UV-light or, at least, provoke the activation of respective MAP-kinase pathways (Pandey et al. 1996, Price et al. 1996), we did chose DsRed as a candidate, which exhibits excitation/emission properties that are shifted towards the green/red spectrum.

DsRed, cloned from *Discosoma sp.* (Matz et al. 1999), has spectroscopic characteristics (excitation maximum 558 nm, with a minor maximum at 485, emission maximum 595 nm) that are very distinct from the parameters of GFP_{S65T} or enhanced GFP (excitation maximum 485 nm, emission maximum 515 nm). In fact, GFP and DsRed can be detected without interference with each other as exemplified in (paper IV, fig. 3) by expressing nuclear-standing PDX-1~DsRed and plasma membrane-standing insulin receptor ~GFP in the same cell.

A second prerequisite for using DsRed as a reporter in combination with GFP was the fact that stimulus-induced DsRed expression followed the same dynamics as GFP expression when expressed under control of the same promoter (paper IV, fig. 4a,b). Finally, co-transfection of HIT-T15 cells with BGK promoter-driven GFP and insulin promoter-driven DsRed, showed that glucose-stimulated up-regulation of the two promoters can be measured by both constructs without interference (paper IV, fig. 4c).

3 AIMS

1. To investigate whether short-term glucose stimulation activates insulin gene transcription.
2. To identify the mechanism(s) underlying short-term glucose-stimulated insulin gene transcription.
3. To analyze transcription factors, that might be involved in the short-term glucose-dependent regulation of insulin gene transcription
4. To investigate whether short-term glucose-stimulation activates glucokinase gene transcription.
5. To investigate the mechanism(s) underlying the short-term regulation of glucokinase gene transcription by glucose.
6. To investigate differences in the mechanism(s) underlying the up-regulation of insulin and glucokinase gene transcription by glucose.

4 MATERIALS AND METHODS

4.1 Cell Culture

4.1.1 HIT Cells

HIT-T15 cells were obtained from ATCC (Manassas, VA). HIT cells were reported to show glucose responsiveness at sub-physiological concentrations, i.e. between 0.1 mM and 2.0 mM of the sugar (Sharma et al. 1994). The cells were grown in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and 10% fetal calf serum at 5% CO₂ and 37°C. After transfection and before stimulation the HIT cells were cultured in RPMI at sub-stimulatory glucose concentrations, i.e. 0.1 mM, for at least 12 hours.

4.1.2 Pancreatic islets and islet cells

Pancreatic islets from normally fed rat or mice were isolated by collagenase digestion as described in (Lacy 1967). Isolated islets and cells of disaggregated islets were incubated overnight at 5% CO₂ and 37°C in RPMI 1640 medium supplemented as above and containing 5.6 mM glucose.

4.1.3 MIN6 cells

MIN6 cells (Miyazaki et al. 1990) were obtained from Dr. J. Miyazaki (Dept. Nutrition and Physiol. Chemistry, Osaka University School of Medicine, Osaka, Japan) and were adopted to culture at 11.1 and 5.5 mM glucose in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum, 100 µg/ml streptomycin and 100U/ml penicillin.

4.1.4 Treatment of cells in stimulation experiments and application of pharmacological inhibitors and antibodies

HIT T-15 cells were incubated over night in RPMI 1640 containing sub-stimulatory, i.e. 0.1 mM, glucose concentration. Islet cells and islets were pre-incubated with RPMI 1640 containing 5.6 mM glucose over night.

After stimulation with 16.7 mM glucose for 15 min, 50 mM KCl, 1 µM glibenclamide or 5 µU/ml to 5mU/ml insulin for 5 min the cells were washed and further incubated in RPMI containing sub-stimulatory glucose concentrations.

Pharmacological inhibitors and antibodies were applied to the fully supplemented medium with sub-stimulatory glucose concentrations in the appropriate concentrations 30 min before the start of stimulation and were kept present during the stimulation. The following inhibitors were used: 10 µM nimodipine (L-type Ca²⁺ channel), 25 µM to 100 µM LY 294002 (PI3 kinases), 20 nM

to 150 nM Wortmannin (PI3 kinases), 20 μ M PD 98059 (ERK), 20 μ M SB 203580 (p38MAPK/RK), 150 nM Bisindolylmaleimide I (PKC), 100 μ M Rp-isomer of cAMPS (PKA), 10 μ M KN-62 (CaMK), 10 nM Rapamycin (mTOR/p70s6k), 100 μ M HNMPA-(AM)₃ (insulin receptor tyrosine kinase), 10 μ M PD 169316 (p38/RK/SAPK2a+SAPK1/JNK), 400 nM autocalcine-2 related inhibitory peptide (CaMK II). Blocking antibodies against the insulin receptor type B (α IR-B), insulin receptor type A and B (α IR-AB) and the IGF-1 receptor (α IGF-1R) were used at 0.67 μ g/ml. Actinomycin D (RNA polymerase) was used at a concentration of 5 μ g/ml.

4.2 Plasmids and Adenoviruses

4.2.1 Plasmids

A detailed description of all used plasmids and their construction can be found in the respective sections of the five papers.

4.2.2 Adenovirus constructs

The adenovirus constructs Ad-rIns1-GFP, Ad- β GK-GFP and Ad-CMV-GFP were provided by Dr. Moitoso de Vargas (School of Medicine, Boston University, Boston, Massachusetts)

4.3 Transfections and Infections

Transfections were carried out 48-72h prior to experiments using the lipofectamine technique. HIT-T 15 cells, MIN6 cells and islet cells were seeded on 24 mm cover glasses in 35 mm dishes 24 h prior to transfection. The transfection was performed using 2 to 3 μ g plasmid-DNA and 6 to 9 μ l lipofectamine (Gibco/Invitrogen, Carlsbad, California) in unsupplemented RMPI 1640 or DMEM, respectively, per 35 mm dish for 12 to 14 hours.

Large-scale transfections for batch-type experiments were performed by using the calcium phosphate/co-precipitation technique as described in (Leibiger et al. 1994a).

Infections of whole pancreatic islets with the adenovirus constructs were performed as described by Dr. Moitoso de Vargas in (de Vargas et al. 1997).

4.4 Quantification of (prepro) insulin and glucokinase mRNA amounts

Materials and Methods

4.4.1 Quantification by RNase-protection assay

For RNase-protection analysis radiolabeled cRNA were generated on the respective linearized cDNA-containing plasmids by employing the SP6/T7 *in vitro* transcription kit (Boehringer Mannheim/Roche Biosciences, Mannheim, Germany) and [α - 32 P]CTP (Amersham/Amersham Biosciences, Uppsala, Sweden). After purification by polyacrylamide gel electrophoresis (6% acrylamide/ 7M urea in 1xTBE), equal cpm of the labeled cRNA probes (8×10^4 cpm/ μ l, final activity) were mixed with the total RNA in hybridization solution, incubated for 5 min at 90°C, and hybridized at 45°C overnight. RNase protection was performed by using the RPA II kit (Ambion, Austin, Texas). Values obtained for insulin mRNA were normalized by β -actin mRNA values.

4.4.2 Quantification by comparative RT-PCR

The primers used for determining the levels of insulin mRNA and β -actin mRNA can be found in paper I, while the primers used for BGK mRNA are mentioned in paper V of this thesis. Total RNA from either 10^6 HIT cells or 10 pancreatic islets was reverse-transcribed by using moloney murine leukemia virus revertase. Aliquots of the generated cDNA were used for PCR-mediated amplification using the RT-PCR kit (Stratagene, La Jolla, California) and [α - 32 P]dCTP (Amersham/Amersham Biosciences, Uppsala, Sweden). PCR conditions were chosen that guaranteed that the amplification of insulin or glucokinase and β -actin fragments was in the linear range (compare paper I, fig. 1c). PCR was performed in an AutogeneII thermocycler (Grant, U.K.). Labeled PCR products were separated on a 6% polyacrylamide sequencing gel and analyzed by phosphorimaging. Quantification was performed with TINA software (Raytest), using co-amplified β -actin RT-PCR products as an internal standard.

4.5 Nuclear Run-Off Analysis

5×10^7 HIT-T15 cells were pre-incubated overnight at sub-stimulatory glucose concentrations (0.1 mM) in fully supplemented RPMI 1640 medium. After stimulation with 16.7 mM glucose for 15 min, cells were washed and further incubated, until isolation of nuclei, in sub-stimulatory RPMI. Nuclei were isolated and run-off reactions were performed as described in (Greenberger al. 1997). The labeled RNA was hybridized to 2.5 μ g insulin, glucokinase, β -actin and control pBluescript DNA, which were immobilized on nitrocellulose filters. Hybridization was performed as described with an equal amount of cpm to each filter from all experimental condition. An RNase A digestion step was performed after hybridization, to digest the unhybridized RNA. The filters were dried and analyzed by phosphorimaging. Values obtained for Insulin- and BGK-mRNA were normalized by β -actin mRNA values.

For nuclear run-off assays on islets, nuclei from 2000 islets per experiment were used. The islets were treated similarly to HIT-T15 cells, except that the sub-stimulatory RPMI 1640 medium contained 5.6 mM glucose.

4.6 Online monitoring of GFP and DsRed fluorescence

4.6.1 *Detection of fluorescence by digital imaging fluorescence microscopy*

Transfected cells were grown on 24 mm glass cover glasses. After stimulation (either 15 min 16.7 mM glucose, or for 5 min various concentrations of insulin, 50 mM KCl, 100 μ M tolbutamide or 1 μ M glibenclamide at sub-stimulatory glucose concentrations, i.e. 0.1 mM for HIT-T15 cells and 5.6 mM for islet cells), the cover glasses were placed into a temperature controlled perfusion chamber, that was mounted on an inverted microscope (Zeiss Axiovert 135TV, Carl Zeiss GmbH, Göttingen, Germany). During the experiments the cells were kept at 37°C and were perfused with fully supplemented RPMI 1640 medium at sub-stimulatory glucose concentrations. The lens used was a Zeiss plan NEOFLUOAR x25/0.8 Imm Korr (Carl Zeiss). Fluorescence was imaged using a cooled charged-coupled device camera (CH250 with KAF 1400; Photometrics, Tucson, AZ) connected to an imaging system (Inovision, Durham, NJ). Excitation light was obtained from a SPEX fluorolog-2 MM1T11I spectrofluorometer (Spex Industries, Edison, NJ). The following settings were used: for GFP_{S65T}, excitation at 485 nm, a 505 nm dichroic mirror and an emission band-pass filter of 500-530 nm; for DsRed, excitation at 558 nm, a 565 nm dichroic mirror and a 580 nm band-pass filter for emission. Data acquisition and calculation of fluorescence intensity was performed using the ISEE software for Unix (Inovision).

Cells were exposed to the excitation light for 1 min every 20 min during experiments. The correlation of fluorescence with cells was verified by overlaying the fluorescence and the phase contrast image. For the calculation the fluorescence intensity of an individual cell at the beginning of the experiment ($t = 20$ min or $t = 60$ min after start of stimulation respectively) was set as 1. The fluorescence intensity of each monitored cell was followed over time and calculated relative to its intensity at $t = 20$ min or $t = 60$ min.

4.6.2 *Detection of fluorescence by laser scanning confocal microscopy*

Transfected cells on 24 mm cover glasses were treated as described in section 4.6.1.

Laser scanning confocal microscopy was performed for paper I, III and IV by using the Leica CLSM confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany). For the detection of GFP in transfected cells the following settings were used: x40/1.30 oil Leitz Fluotar or 100x/1.30 oil Leitz Fluotar objective lens, excitation wavelength 488nm (argon/krypton

Materials and Methods

laser), a 450-490 excitation filter, a 495 dichroic mirror and a 500-550 nm band-pass emission filter. The same settings were used for monitoring GFP in Ad-rIns1-GFP infected islets, except that an x25/0.75 oil Leitz NPL Fluotar objective lens was used.

In paper V, confocal microscopy was performed by using a Leica TCS SP2 (Leica Lasertechnik GmbH) with the following settings: Leica HCX PL APO x63/1,20/0,17 UV objective lens, excitation wavelength 488 nm (Ar Laser) and 543 nm (HeNe laser), a 488/543 double-dichroic mirror, and detection of GFP at 505-525 nm and DsRed at 605-670 nm.

Ad- β GK-GFP infected islets were monitored with the following settings: Leica x10 objective lens, excitation wavelength 488 nm (Ar Laser), a 488/543 double dichroic mirror, and detection of GFP at 505-525 nm. For the detection of fluorescence in infected islets, after stimulation the islet was placed into the perfusion chamber and fixed with a metallic mesh to stabilize its position during the experiment.

4.7 Analysis of tyrosine phosphorylation of insulin receptors (IR) and insulin receptor substrate (IRS) and of protein kinase activities

4.7.1 Analysis of tyrosine phosphorylation of IR and IRS

HIT T15 cells were treated as described in 4.1.4. Antibodies used for immunoprecipitation were Anti-rat carboxiterminal IRS-1 (Upstate Biotechnology), Anti-mouse IRS-2 (Upstate Biotechnology) and Insulin R β (C-19) (Santa Cruz Biotechnology). Cell lysis and immunoprecipitation was performed following the manufacturer's instructions. The immunoblot was incubated with antiphosphotyrosine antibody PY20 (Transduction Laboratories). Level of tyrosine phosphorylation was detected by ECL (Amersham) and quantified by scanning densitometry using the TINA software. To determine protein levels the Immunoblots were reprobed with the respective immunoprecipitation antibodies.

4.7.2 Analysis of p70s6K Activity

HIT T15 cells were treated as described in 4.1.4. Analysis of p70s6 kinase was performed employing the S6 kinase kit (Upstate Biotech.) according to the manufacturers instructions after immunoprecipitation of p70s6 kinase from cell lysates using a p70s6k antibody (Upstate Biotech).

4.7.3 Analysis of PI3K Activity

HIT-T15 cells were transfected with either pRcCMVi.hIR(A)-GFP or pRcCMVi.hIR(B)-GFP and treated as described in 4.1.4. Cell lysates containing 2mg of protein were subjected to

immunoprecipitation using anti-GFP antibody A11122 (Molecular Probes). PI3 kinase activity was measured in the GFP-immunoprecipitates as described in (Krook et al. 1997) using L- α -phosphatidylinositol (Avanti Polar-Lipids) as substrate.

For the determination of IRS associated PI3 kinase activity the immunoprecipitation was performed with either Anti-rat carboxiterminal IRS-1 (Upstate Biotechnology) or Anti-mouse IRS-2 (Upstate Biotechnology) antibodies, while the PI3 kinase assay itself was performed similarly.

4.7.4 Analysis of PKB activity

HIT T15 cells were treated as described in 4.1.4. Analysis of PKB activity was performed by using the Akt1/PKB α Immunoprecipitation Kinase Assay kit (Upstate Biotechnology) according to the manufacturer's instructions.

Results and Discussion

5 RESULTS AND DISCUSSION

5.1 Short-term regulation of insulin gene transcription by glucose

To investigate the short-term regulation of the insulin gene transcription by glucose we began to study the steady-state levels of insulin mRNA by incubating β -cells with elevated glucose for only 15 min. The chosen 15 min for stimulation correspond more closely to the duration of elevated blood-glucose levels after a meal or an oral glucose tolerance test. Stimulation of HIT-T15 cells from 0.1 mM to 16.7 mM glucose increased the insulin mRNA levels about 2.5 fold at 60 to 120 min after start of stimulation (paper I, fig. 1b). This was measured by both RNase protection assay and comparative RT-PCR. We established the latter approach to measure steady-state mRNA levels in limited amounts of material, such as pancreatic islets and islet cells. Stimulation of pancreatic islets pre-incubated at sub-stimulatory glucose concentrations (5.6 mM) with 16.7 mM glucose for 15 min resulted in an approximately 5-fold rise in steady-state insulin mRNA levels (paper I, fig. 2).

Two processes contribute to the steady-state mRNA levels in a cell: mRNA synthesis (measured as transcription initiation) and mRNA degradation (measured as mRNA stability). Nuclear run-off analysis revealed that transcription initiation was elevated as early as 15 min after start of glucose stimulation, reached a maximum (about 2-fold elevation) 30 min after start of stimulation and declined to basal levels at minute 120 (paper I, fig. 1a).

When looking at the dynamics of total insulin mRNA amounts, we found that insulin mRNA levels started to decrease 120 min after start of stimulation and almost reached baseline levels after 180 min (paper I, fig. 3). This suggested an increase in degradation of insulin mRNA shortly after stimulation of transcription. Analysis of insulin mRNA stability by employing actinomycin D-treatment demonstrated that the stability of pre-existing insulin mRNA remained unchanged upon glucose stimulation, while newly synthesized plus pre-existing, insulin mRNA was degraded more rapidly (paper I, fig. 3). These data led us to suggest that short-term glucose stimulation does not lead to a stabilization of insulin mRNA but leads to the preferential degradation of newly synthesized insulin mRNA.

The results of these mRNA studies demonstrated that glucose exerted its effect at the level of transcription initiation. In order to study the mechanism(s) involved in the glucose-dependent up-regulation of insulin gene transcription, we established a reporter gene assay that allowed us to monitor gene expression at the single cell level. Therefore, we fused the rat insulin-I gene promoter to the reporter gene coding for the S65T-mutant of the green fluorescent protein, GFP (Chalfie et al. 1994, Heim et al. 1995) and analyzed GFP-expression in transiently transfected islet cells and HIT cells by either digital imaging fluorescence microscopy or confocal microscopy.

Results and Discussion

The applicability of GFP as a semi-quantitative and as a dynamic reporter for promoter activity has been discussed in 2.6 and has been shown in (paper I and paper IV).

Stimulation with 16.7 mM glucose (15 min) resulted in an elevation of GFP-fluorescence in transfected islet cells and HIT cells, beginning from approximately 80 min following start of stimulation. The specificity of the glucose effect on the insulin promoter-driven GFP expression was shown by the lack of effect, when GFP expression is driven by the human cytomegalovirus (CMV) promoter (paper I, fig. 5a).

Taken together, this data demonstrate the existence of a short-term response of insulin gene transcription to elevated glucose concentrations.

5.2 Identification of the mechanism(s) involved in the short term regulation of insulin gene transcription

Incubation of pancreatic β -cells with stimulatory glucose concentrations leads to the activation of a cascade of reactions, which ends in the exocytosis of stored insulin. Briefly, this complex of processes, often referred to as the stimulus-secretion-coupling, starts with the uptake of glucose by the pancreatic β -cell via high- K_m /low affinity glucose transporter GLUT2 and proceeds with the metabolism of glucose in glycolysis and Krebs cycle and, hereby, ATP generation. The elevation in the ATP/ADP ratio leads to closure of ATP-sensitive K^+ channels, which in turn results in depolarization of the plasma membrane. The subsequent opening of voltage-gated L-type Ca^{2+} channels leads to an increase in the cytoplasmic free Ca^{2+} concentration, which promotes insulin secretion.

To determine where in this chain of events the signal that is responsible for the up-regulation of insulin gene transcription originates from, we analyzed both insulin mRNA levels and insulin promoter-driven GFP expression in response to various stimuli.

To decide whether glucose metabolism is sufficient to stimulate insulin gene transcription or if more distal processes in the stimulus-secretion-coupling are involved in the up-regulation of insulin gene transcription, we stimulated the cells either with glucose in the presence of the L-type Ca^{2+} -channel blocker nimodipine or stimulated them only with insulin secretagogues that work independently of glucose metabolism, such as depolarizing KCl-concentrations, or the sulphonylurea compounds glibenclamide and tolbutamide.

Allowing glucose metabolism but preventing Ca^{2+} influx (and insulin secretion) by 10 μ M nimodipine abolished glucose-stimulated up-regulation of insulin gene transcription (paper II, fig. 1a). On the other hand, stimulating Ca^{2+} influx (and insulin secretion) with secretagogues (50mM KCl, 100 μ M tolbutamide or 1 μ M glibenclamide) at basal glucose levels led to an increase in insulin gene transcription measured both as increase in insulin mRNA levels (paper II, fig. 1a) and in insulin promoter-driven GFP expression (paper II, fig. 1b).

These data demonstrate that insulin gene transcription can be triggered without augmentation of glucose metabolism. This suggests that the signal responsible for the increased insulin gene transcription must be coupled to processes leading to or resulting from insulin exocytosis.

To analyze, which intracellular signals contribute to the stimulation of insulin gene transcription we employed inhibitors of protein kinase A (PKA), protein kinase C (PKC), PI3 kinase (PI3K), p70 s6 kinase (p70s6k), CaM kinases, ERK kinase- and p38MAPK/RK-pathways together with stimulation with either KCl or glucose. Whereas inhibition of PKA, PKC, p70s6k, PI3K and CaM kinases abolished the up-regulation of insulin gene transcription by glucose, only inhibition of PI3K, p70s6k and CaM kinases affected KCl-stimulated insulin gene transcription (paper II, fig. 2a).

PI3K has been shown to be activated by receptor tyrosine kinases, such as insulin- and IGF-I receptors (Myers et al. 1996). Interestingly, pancreatic β -cells have been reported to express insulin receptors, and to activate PI3K through IRS in response to glucose stimulation (Velloso et al. 1995; Harbeck et al. 1996; Sun et al. 1997; Zhang et al. 1998). These findings and our own data suggesting a link between exocytosis and stimulated insulin gene expression, led us to suggest that insulin might be the signal that triggers the stimulation of insulin gene transcription in response to glucose stimulation.

In order to test this hypothesis we studied whether addition of exogenous insulin to the culture medium, at sub-stimulatory glucose concentrations, is sufficient to trigger insulin gene transcription. Indeed, addition of 50 μ U/ml (0.3 nM) insulin for 5 min to fully supplemented culture medium at sub-stimulatory glucose concentration is enough to increase endogenous insulin mRNA levels (paper II, fig. 2c) and insulin promoter-driven GFP expression (paper II, fig. 2d) in HIT-T15 and also pancreatic β -cells. Inhibition of PI3K, p70s6k or CaM kinase in combination with insulin-stimulation abolished the stimulatory effect, thus confirming the involvement of these pathways (paper II, fig. 2b).

To test the involvement of IR in this signaling pathway, we first established the presence of both insulin receptor A and B isoforms (IR-A and IR-B) in pancreatic β -cells and HIT-15 by RT-PCR. Over-expression of the IR-A led to a pronounced stimulation of insulin promoter-driven GFP expression, thereby strengthening the argument in favor for an involvement of insulin receptors in this signaling pathway (paper II fig. 3a). Furthermore, inhibition of the insulin receptor with HNMPA-(AM)₃, a reported selective inhibitor of the IR tyrosine kinase (Saperstein et al. 1989, Baltensperger et al. 1992), abolished the stimulatory effect of both glucose and insulin on insulin transcription (paper II, fig. 3a).

Signal transduction via PI3K has been shown to result in different downstream located effects, probably due to the activation of at least two different and independent signaling pathways, i.e. via the rapamycin-sensitive pathway, which leads to the activation of p70s6k (Price, et al. 1992), and via the rapamycin-insensitive pathway, which leads to activation of protein kinase B, also known as RAC kinase or c-Akt (Burgering et al. 1995, Alessi et al. 1996). Inhibition of the positive effects of both insulin and glucose by rapamycin suggested the involvement of p70s6k,

Results and Discussion

but was not conclusive. This is because not p70s6k itself but the upstream located mTOR/FRAP is the primary target of rapamycin (Hara et al. 1998). Further support for the direct involvement of p70s6k was given when studying the effect of over-expression of p70s6k on insulin-stimulated insulin promoter-driven GFP expression. In this case there was a more pronounced elevation in stimulus-derived GFP expression (paper II, figure 5D), similar to that obtained when over-expressing insulin receptors (paper II, figure 3b). This effect was abolished, when over-expression and stimulation was combined with rapamycin treatment (paper II, fig. 3b). Even more conclusive was the experiment, where we employed the rapamycin-insensitive mutant p70 Δ 2-46/ Δ CT104 (Weng et al. 1995, Hara et al. 1997) for over-expression in HIT cells. If p70s6k is necessary for up-regulation of insulin gene transcription, expression of this mutant should rescue, at least part of, the lost endogenous p70s6k effect as result of rapamycin treatment. Indeed, over-expression of p70 Δ 2-46/ Δ CT104 in islet cells and HIT cells combined with rapamycin treatment led to levels of insulin promoter-driven GFP expression which were similar to those obtained following insulin-stimulation in mock-transfected cells and those obtained in cells over-expressing wild-type p70s6k, both without rapamycin treatment (paper II, fig. 3b).

In conclusion, we were able to demonstrate that the short-term stimulation of insulin gene transcription by glucose is in fact a positive feedback to secreted insulin, regulated by a mechanism involving the insulin receptor/PI3K/p70s6k and CaM kinase pathways (figure 7).

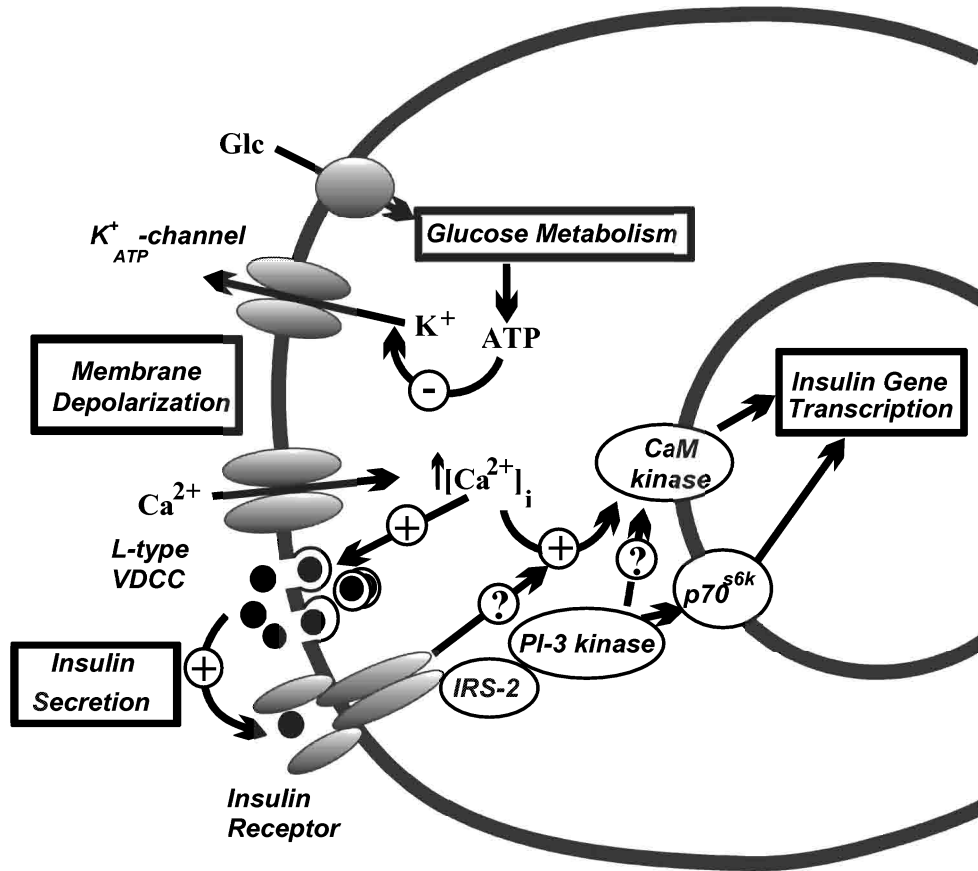


Figure 7 **Insulin-secretion/insulin-gene-transcription coupling.** The scheme illustrates the coupling between the insulin exocytosis and insulin gene transcription. K⁺_{ATP}-channel - ATP-sensitive K⁺ channel; L-type VDCC - L-type voltage-dependent Ca²⁺ channel; Glc - glucose (adopted from Leibiger et al. 1998a; paper II, fig. 5)

5.3 Identification of a nuclear localization signal, RRMKWKK, in the homeodomain transcription factor PDX-1

The rat insulin-I promoter, used in the experiments described above, contains a number of *cis*-elements that have been suggested to be involved in the glucose regulation of insulin gene transcription (Myers et al. 1996; Stein et al. 1993; Docherty et al. 1994; German et al. 1994; Sharma et al. 1994; Odagiri et al. 1996; Stoffers et al. 1997b). We therefore mutated separately these *cis*-elements, i.e. A1, E1, CAAT-box, CRE-box, A3/4 and E2 in the promoter of the prInsI.GFP construct and studied the impact of these mutations on both insulin- and glucose-stimulated insulin promoter-driven GFP expression. Insulin-stimulated insulin promoter activity was sensitive to mutation of A3/4, E2 and E, but not to mutation of A1, CAAT and CRE-box (paper II, fig. 4). However, when stimulating with glucose, also the A1 element became sensitive to mutation, whereas CRE and CAAT-box remained insensitive. These data lead us to suggest, that the insulin-dependent pathway of activation of insulin gene transcription involves the E-box elements and the A3/4 element, while a glucose-specific signaling might operate through a pathway involving PKA and/or PKC and the A1 *cis*-element.

PDX-1, a homeodomain-containing transcription factor that binds to A-box elements in the insulin promoter, has been discussed to be involved in the glucose-dependent activation of insulin gene transcription (Macfarlane et al. 1994, 1996, 1997, German et al. 1990; Goodison et al. 1992). Since mutation of both PDX-1 binding sites in the rat insulin-I promoter, i.e. A1 and A3/4, led to a disturbed glucose/insulin-stimulated insulin gene transcription, we focused on PDX-1 as a potential transcription factor involved in the short-term regulated insulin gene transcription by glucose/insulin. One of the possible mechanisms by which PDX-1 might activate insulin gene transcription is its glucose-dependent cytoplasmic-nuclear translocation (Macfarlane et al. 1999; Rafiq et al. 1998).

Nuclear translocation of proteins can be accomplished by a diversity of mechanisms (for review see Mattaj et al. 1998). In general the cargo is recognized in the cytoplasm by a receptor via a nuclear localization signal, NLS, presented by the cargo. Following binding to the receptor, often called importin or karyopherin, the cargo-receptor complex is recognized by GTP-bound Ran, translocated through the nuclear pore complex and the cargo is released inside the nucleus. In case the cargo itself does not possess a NLS, it can be imported by a piggyback mechanism after binding to an NLS-containing piggyback partner. Data from a recent report by Macfarlane et al. (Macfarlane et al. 1999) suggest that when PDX-1 is translocated to the nucleus it undergoes a modification that results in a 15-kDa shift in Western blot analysis and that translocated PDX-1 is phosphorylated. Both phosphorylation/dephosphorylation and the presence of a NLS, very often formed by a stretch of the positively charged amino acids arginine and lysine, have been shown to be involved in nuclear translocation of transcription factors (for review see Boulukas et al. 1994).

In order to identify amino acid residues in PDX-1 that are responsible for its nuclear translocation we performed site directed mutagenesis of putative phosphorylation sites as well as of stretches of positively charged amino acids in the GFP-tagged PDX-1 (paper III, fig. 1) and studied the nuclear translocation of the respective mutants by laser scanning confocal microscopy. In addition, this approach allowed us to monitor online stimulus-induced nuclear translocation of transcription factors.

Computer analysis of the PDX-1 amino acid sequence revealed a number of putative phosphorylation sites within the PDX-1 amino acid sequence. Each of these sites was mutated by site-directed mutagenesis to an alanine or arginine residue in C-terminally GFP-tagged PDX-1. Analysis of these constructs by laser scanning confocal microscopy, however, demonstrated that none of those single mutations was able to disturb the cytoplasmic-nuclear translocation of PDX-1~GFP (paper III, fig. 2).

Analysis of the PDX-1 amino acid sequence did not show the presence of a 'classical' NLS. A 'classical' NLS is formed by a hexapeptide that (a) consists of four or more positively charged amino acids (Arg or Lys), (b) contains no Asp and Glu residues, (c) contains no bulky amino acids such as Phe, Trp or Tyr, (d) is flanked by acidic residues proline and glycine that break α -helices and (e) contains no hydrophobic amino acids in the core NLS flanking region (Boulikas et al. 1994). Three amino acid sequences came close to an NLS and were studied for their impact on the localization of PDX-1~GFP and GFP~PDX-1 by site directed mutagenesis (paper III, fig. 3a-d). Only mutation of the sequence R¹⁹⁸RMKWKKEED²⁰⁷ (mutated: R199A+K203A) changed the distribution of PDX-1~GFP. The presence of Met and Trp within the string of the amino acid sequence makes this potential NLS rather untypical. We therefore tested whether this potential NLS is able to enforce nuclear localization of a heterologous protein by fusing the sequence PDX-1₁₉₄₋₂₀₈ to GFP. While this sequence clearly allowed a nuclear import, it did not confer an exclusive nuclear localization (paper III, fig. 3E,F). This was achieved by extending the sequence tag to PDX-1₁₈₅₋₂₀₉ (paper III, fig. 3g). Mutation of the additional positively charged amino acids in that sequence did not change the complete nuclear localization pattern obtained with GFP~PDX-1₁₈₅₋₂₀₉ (paper III, fig. 3 h-j). The nuclear localization sequence of PDX-1 is therefore RRMKWKK, with the additional requirement of the structurally intact 'helix 3' domain of the antennapedia-homeodomain of PDX-1. The identification of the 'helix-3' domain is in agreement with earlier findings by (Lu et al. 1996), demonstrating the necessity of the presence this domain for the *in vivo* activity of PDX-1, but not for the *in vitro* DNA-binding. Our data show that exclusive nuclear translocation requires the structural integrity of 'helix 3' of the homeodomain. Hessabi et al. (Hessabi et al. 1999) come to a similar conclusion in their analysis, however they identified additional amino acids as part of the NLS, namely R¹⁸⁹ and H¹⁹⁰. We mutated these two amino acids as well, but did not see an impact of this mutation on the nuclear localization of the GFP~PDX-1₁₈₅₋₂₀₉ construct (paper III, fig. 3h,i). This can be explained by the fact that their point mutations (R189I and

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H190L) disturbed the structural integrity of 'helix 3', thereby disturbing its ability to function as NLS, while ours (R189A and H190D) tried to retain the structural integrity of 'helix 3'.

Recent reports addressed the question of glucose-dependent cytoplasmic-nuclear translocation of PDX-1 with different results (Macfarlane et al. 1999, Rafiq et al. 1998). We therefore aimed to address this question by tracking GFP-tagged PDX-1 in response to glucose stimulation by laser scanning confocal microscopy, an approach that has been proven to allow monitoring of stimulus-induced translocation of a glucocorticoid receptor~GFP fusion protein (paper III, fig. 4 a-d).

Because we could not demonstrate that GFP-tagged PDX-1 was localized outside the nucleus at non-stimulatory glucose concentrations (it was always localized within the nucleus, (paper III, fig. 4e-h), we were unable to confirm the data by Macfarlane et al. (Macfarlane et al. 1999).

In conclusion, we identified the nuclear localization signal of PDX-1 being RRMKWKK, which represents a novel type of NLS, in the context of the integrity of the 'helix 3' domain of the PDX-1 homeodomain. However, we did not find evidence supporting a glucose-dependent cytoplasmic-nuclear shuttling of PDX-1.

5.4 Short-term regulation of β -cell glucokinase (BGK) gene transcription by glucose

To test whether β -cell glucokinase gene (BGK) transcription is controlled by similar mechanisms as the insulin gene, we studied the impact of glucose on steady-state BGK mRNA levels. Stimulation of islets or HIT-T15 cells with 16.7 mM glucose led to an increase of BGK mRNA levels, similar in dynamics to the increase in insulin mRNA levels (paper V, fig. 1a).

To define the dynamics of BGK mRNA, we analyzed the half-life time, stability and transcription initiation rate of the BGK mRNA pool. The half-life time of BGK mRNA was about 60 min and did not change in response to glucose stimulation (paper V, fig. 1b). Transcription initiation as measured by nuclear run-off assay was increased as early as 15 min after start of stimulation and reached its maximum at 30 min after stimulation. This effect was observed in both HIT-T15 cells and pancreatic islets (paper V, fig 1c,d).

To further corroborate these data, we established a reporter gene assay using the BGK promoter coupled to GFP (prBGK.GFP). We used the rat BGK promoter fragment up to nucleotide -278 since this has been shown to contain all *cis*-elements responsible for both glucose-dependent and cell-type-specific transcriptional control (Jetton et al. 1994, et al. 1998). Stimulation with 16.7 mM glucose led to an increase in BGK promoter-driven GFP fluorescence in HIT cells, isolated primary pancreatic β -cells as well as in intact pancreatic islets (paper V, Fig. 1e). In agreement with the nuclear run-off assay, the dynamics of the activation of BGK promoter-

driven GFP expression were similar, if not identical, to those of the glucose-stimulated insulin gene promoter (paper I, paper II).

In conclusion, BTK gene transcription is also controlled by short-term glucose-stimulation.

5.5 The mechanisms involved in glucose-stimulated BGK gene transcription

To determine whether glucose metabolism *per se* or secreted insulin is a requirement for the up-regulation of BGK transcription, we investigated the effect of insulin secretagogues on BGK mRNA steady-state levels and BGK promoter-driven GFP expression. Stimulation with either 50 mM KCl or 1 μ M glibenclamide for 5 min, at sub-stimulatory glucose concentrations, led to an increase in β GK mRNA steady-state levels (paper V, fig. 2a) and to an elevation in BGK promoter-driven GFP expression (paper V, fig. 2c). Alternatively, preventing stimulus-induced insulin secretion by blocking L-type Ca^{2+} channels using nifedipine, abolished up-regulation of BGK mRNA levels (paper V, fig. 2b). Addition of only 50 μ U insulin per ml to fully supplemented culture medium was sufficient to evoke BGK mRNA levels in pancreatic islets (paper V, fig. 2d). Interestingly, a more detailed comparison of the necessary amounts of exogenous insulin to trigger promoter activities, revealed that instead of 5-10 μ U insulin per ml, as is the case with the insulin gene, the addition of 20 μ U per ml was required to gain an effect on BGK promoter activation (paper V, fig. 2e). Stimulation with 5 mU insulin per ml culture medium for 5 min led to an BGK promoter-driven increase in GFP fluorescence in isolated primary pancreatic β -cells (paper V, fig. 2c), HIT cells as well as in intact pancreatic islets. Thus, our data support the view that the insulin gene and the BGK gene are both stimulated by insulin secreted in response to glucose. Interestingly, a higher concentration of insulin is needed to activate BGK transcription when compared with the insulin gene.

As we have demonstrated before, glucose/insulin-stimulated insulin gene transcription involves PI3K, p70s6k and CaM kinases. The characterization of the 5'-flanking region of the β -cell transcription unit of the rat glucokinase gene by others (Shelton et al. 1992; Watada et al. 1996) and us (Leibiger et al. 1994a, 1994b, 1994c) revealed *cis*-elements similar to those within the insulin gene promoters, suggesting common mechanisms in transcriptional regulation. Therefore, we postulated that both insulin- and BGK-genes might be regulated by the same signaling pathways. To test if the same protein kinase pathways are involved in the insulin-stimulated transcription of the two genes, we employed various protein kinase inhibitors in combination with a stimulus of 5mU/ml insulin on both, BGK promoter-driven GFP expression and insulin promoter-driven GFP expression. As shown in (paper V, fig. 3a) insulin promoter-driven GFP expression was blocked, as expected, by inhibitors of the IR tyrosine kinase (HNMPA-(AM)₃), p70s6k (rapamycin), PI3K (LY 294002) and CaM kinase II (autocamtide-2

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related inhibitory peptide), while blockers of PKA (Rp-isomer of cAMPS), PKC (bisindolylmaleimide I), MAP kinases Erk1/2 (PD 98059) or p38/RK/SAPK2a+SAPK1/JNK (PD 169316) had no effect (paper V, fig. 3a). Interestingly, BGK promoter-driven GFP expression, was only sensitive to inhibition of the insulin receptor tyrosine kinase by HNMPA-(AM)₃ (paper V, fig. 3a). These findings were confirmed by measuring steady-state BGK mRNA levels by RT-PCR (paper V, fig. 3b).

In conclusion, BGK gene transcription is short-term regulated through a mechanism involving secreted insulin, but which is different from the mechanism controlling insulin-stimulated insulin gene transcription.

5.6 Differences in the pathways involved in the insulin stimulated insulin and glucokinase gene expression

To confirm the involvement of different protein kinases in insulin-stimulated insulin and BGK gene transcription, we applied the inhibitors for p70s6k, PI3K, CaM kinase II and insulin receptor tyrosine kinase in cells that were co-transfected with prIns1.DsRed and pBGK.GFP. By combining insulin stimulation with the pharmacological inhibitors we were able to show that activation of the insulin promoter is abolished (no increase in DsRed fluorescence). On the other hand, no effect on insulin-stimulated BGK promoter activity (increase in GFP fluorescence) was observed in the same cell (paper V, fig. 3c).

Insulin has been shown to activate not only the MAP kinases and the PI3K/mTOR/p70s6k pathways, but also PKB(c-Akt) (Coffer et al. et al. 1998). To test whether stimulation with either glucose or insulin leads to the activation of PKB in pancreatic β cells, we studied PKB activity following stimulation with either 16.7 mM glucose or 5 mU insulin/ml. Indeed, PKB activation was observed 5 min following stimulation with 16.7 mM glucose (paper V, fig. 4a) and 2 min following stimulation with 5 mU insulin/ml, at sub-stimulatory glucose concentrations (paper V, fig. 4b).

Because of the lack of a pharmacological inhibitor of PKB(c-Akt), we tested the involvement of this protein kinase in insulin-stimulated transcription by over-expressing PKB α . While this approach did not affect insulin-stimulated insulin promoter-driven DsRed expression, BGK promoter-driven GFP expression was pronounced (paper V, fig. 4c).

According to the current model, activation of PKB involves the phosphorylation of PKB by phosphoinositol-dependent kinase 1, PDK1 (Vanhaesebroeck et al. 2000). Transient over-expression of PDK1 in β -cells did enhance the effect of insulin on BGK promoter activity, while over-expression of a PDK1-antisense construct abolished the response of BGK promoter-driven GFP expression to insulin (paper V, fig. 4c). Neither PDK1 nor PDK1-antisense expression had an effect on insulin-stimulated insulin gene transcription (paper V, fig. 4c).

Interestingly, the activation of PKB has so far been shown to be dependent on the activity of PI3K (Vanhaesebroeck et al. 2000) and therefore to be sensitive to the independent pharmacological inhibitors wortmannin and LY 294002. However, while treatment with 25 μ M LY 294002 abolished the stimulatory effect of insulin on insulin promoter activity, it did not block insulin-stimulated BGK promoter activity (paper V, fig. 3a-c). When investigating the effect of LY 294002 on both BGK and insulin promoter activity in a dose-dependent manner, we observed that LY 294002 inhibits insulin-stimulated activation of the two promoters at different concentrations. While 25 μ M LY 294002 was sufficient to block the stimulatory effect of insulin on insulin promoter activity, 100 μ M LY 294002 were required to completely abolish BGK promoter up-regulation (paper V, fig. 4d). A similar dose-dependency was found for wortmannin. Whereas 50 nM wortmannin was sufficient to block the increase in insulin promoter-driven DsRed expression upon insulin stimulation, 150 nM were needed to abolish the up-regulation of BGK promoter-driven GFP expression.

In conclusion, these data indicate that insulin-stimulated BGK gene transcription occurs by signaling via PDK1/PKB, whereas insulin-stimulated insulin gene transcription is mediated via PI3K/p70s6k and CaM kinase II. The activation of PKB/PDK1 is dependent on a PI3 kinase activity.

5.7 The involvement of insulin receptors in the differential regulation of insulin and glucokinase gene transcription by insulin

Pancreatic β -cells express insulin receptors (IR) (Harbeck et al. 1996, Velloso et al. 1995), insulin receptor related-receptor (Hirayama et al. 1999), IGF-II and IGF-I receptors (Katz et al. 1997), all potential targets for the feedback action by insulin. The loss of the insulin-effect when treating cells with HNMPA-(AM)₃, an inhibitor of the IR tyrosine kinase (Saperstein et al. 1989), supported the idea that signaling via IR is crucial. Consequently, we examined whether the expression of IR *per se* is an absolute requirement for insulin-stimulated insulin- and BGK-gene expression.

We therefore analyzed insulin and BGK mRNA steady-state levels in response to stimulation with either glucose or insulin in islets from normal and β IRKO mice, the latter a knockout model that lacks expression of IRs specifically in the β -cell (Kulkarni et al. 1999). Stimulation with either 5mU/ml insulin or 16.7mM glucose led to an increase in insulin- and BGK mRNA levels in islets of wild type animals, whereas no increase in insulin- and BGK-mRNA levels was observed in islets prepared from β IRKO mice (paper V, fig. 5a). These data suggest that the expression of the IR in pancreatic β -cells is an absolute requirement to gain the stimulatory

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effect by insulin on both insulin- and BGK-gene transcription and that signaling via IGF-I receptors is unlikely to be involved.

β -Cells express both isoforms of the IR, i.e. IR-A and IR-B. As shown in paper II, over-expression of IR-A, but not of IR-B, leads to a pronounced effect in insulin-stimulated insulin gene transcription (paper II, fig. 3a). To test a similar effect for insulin-stimulated BGK transcription we co-transfected islet cells and HIT cells with prBGK.GFP and prIns1.DsRed in combination with either IR-A or IR-B. To our surprise we found that over-expression of IR-B led to a pronounced activation of the BGK promoter while over-expression of IR-A had no effect. Moreover, over-expression of the respective inactive mutants of IR-A and IR-B did not allow a further up-regulation of the respective promoter activities by insulin (paper V, fig. 5b).

To test the involvement of IR-B in insulin-stimulated BGK promoter activation in more detail we treated islet cells and HIT cells, prior to glucose/insulin stimulation, with an anti-IR-B antibody. This antibody selectively binds to the α -subunit of IR-B and thereby selectively blocks insulin binding to IR-B and thereby signaling via IR-B. Employing co-expression of prBGK.GFP and prIns1.DsRed in the same cell, we observed that treatment with the B type receptor-specific antibody (α IR-B) abolished insulin-stimulated prBGK.GFP expression, whereas it did not affect insulin-stimulated prIns1.DsRed expression (paper V, fig. 5c). As expected, treatment of transfected cells with an antibody that blocks insulin signaling via both receptor isoforms (α IR-AB) suppressed insulin-stimulated activation of BGK and insulin promoters (paper V, fig. 5c). Accordingly, treatment of insulin producing cells with this antibody also abolished insulin-stimulated elevation of BGK mRNA steady state levels (data not shown). By contrast, treatment of transfected cells with an antibody that blocks signaling via IGF-I receptors (α IGF-1R) did not affect insulin-stimulated activation of BGK- and insulin promoter-driven reporter gene expression (paper V, fig. 5c). Furthermore, stimulation of β -cells with 2.6 nM IGF-1 failed to up-regulate either insulin- or BGK-transcription.

These data indicate that insulin stimulates the insulin gene promoter through IR-A whereas it stimulates the BGK gene promoter via IR-B.

5.8 The molecular mechanisms underlying the selective signaling through insulin receptor A and B

To start to understand the molecular mechanisms that underlie the selectivity in insulin signaling via the two IR isoforms, we aimed to explain the different sensitivity for PI3K inhibitors we observed between insulin-stimulated insulin- and BGK-promoter activation.

One possible interpretation would be that the same PI3K is involved in the transcription of both genes but that a lower PI3K activity is sufficient to trigger the cascade that activates BGK gene transcription via PKB. If this is the case, inhibition of PI3K-mediated BGK transcription should

require higher concentrations of wortmannin or LY294002 to be fully effectuated. This should also be reflected upon by an inverse relationship between required inhibitor concentration and sensitivity of the respective promoter activity to insulin. If the same PI3K is sufficient to lead to the activation of both insulin- and BGK-promoters, then the promoter activity, which is least sensitive to the inhibitors, i.e. BGK, would be expected to require less insulin to become stimulated. As demonstrated in paper V figure 2e this is not the case. On the contrary, more insulin is needed to stimulate BGK promoter-driven GFP expression.

Another interpretation is that the different sensitivity *in vivo* could be due to a different accessibility of the inhibitor for the same type of PI3K as a result of a different distribution/localization of the two IR isoforms or it could be due to the involvement of different classes of PI3K, exhibiting a different sensitivity to wortmannin and LY294002 as described for PI3K classes I and III versus class II (reviewed in Fruman et al. 1998). To test whether IR-A and IR-B exhibit a distinct distribution *in vivo*, we tagged both receptor isoforms with GFP and DsRed at the C-terminus of the β -subunit. Tagging both IR isoforms did not interfere with their physiological function, e.g. over-expression of the tagged IR isoform led to a pronounced insulin-effect on the respective promoter activity to the same extent as the untagged IR. Whereas transient co-expression of the same, but differently tagged (IR-A~DsRed/IR-A~GFP and IR-B~GFP/IR-B~DsRed), IR isoform led to a complete co-localization (data not shown), co-expression of the differently tagged IR-A and IR-B in either combination (IR-A~DsRed/IR-B~GFP and IR-A~GFP/IR-B~DsRed) clearly showed IR isoforms that are not co-localized (paper V, fig. 6a). To test whether the two IR isoforms do utilize different classes of PI3K, we over-expressed either IR-A~GFP or IR-B~GFP in HIT cells and studied the sensitivity of PI3K activity to wortmannin *in vitro* following immunoprecipitation with GFP-antibodies. Whereas the PI3K activity in the IR-A immunoprecipitate was inhibited by wortmannin in the low nanomolar range, as typical for PI3K class I and III, the PI3K activity in the IR-B immunoprecipitate was only inhibited at higher concentrations (paper V, fig. 6b), as described for PI3K class II (see Fruman et al. 1998). To test whether insulin-stimulated insulin gene transcription involves IR-A-mediated insulin signaling via PI3K class Ia, we combined insulin-stimulation with the transient over-expression of the dominant negative form of the PI3K class Ia adapter protein p85, i.e. Δ p85 (Dhand et al. 1994). Whereas transient over-expression of Δ p85 totally abolished insulin-stimulated insulin promoter activity this approach had no effect on insulin-stimulated BGK promoter activation (paper V, fig. 6c).

When separately over-expressing IR isoforms in HIT cells, we observed a more pronounced activation of p70s6k in cells over-expressing IR-A in response to insulin stimulation, while cells over-expressing IR-B showed a trend towards a higher PKB activity (paper V, fig. 6d).

Taken together these data suggest a selectivity in insulin signaling in insulin-producing cells via IR-A through PI3K class Ia and p70s6k on the one hand, and via IR-B through a different PI3K activity, very similar to that of class II, and PKB on the other.

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This led us to propose the following model of glucose-stimulated insulin and BGK gene transcription (fig. 8):

Following uptake via GLUT2, glucose is metabolized by glycolysis and oxidative phosphorylation (Krebs cycle) and leads to the release of insulin by triggering the cascade of events, that are described in 2.4 as the stimulus-secretion-coupling. Secreted insulin then binds

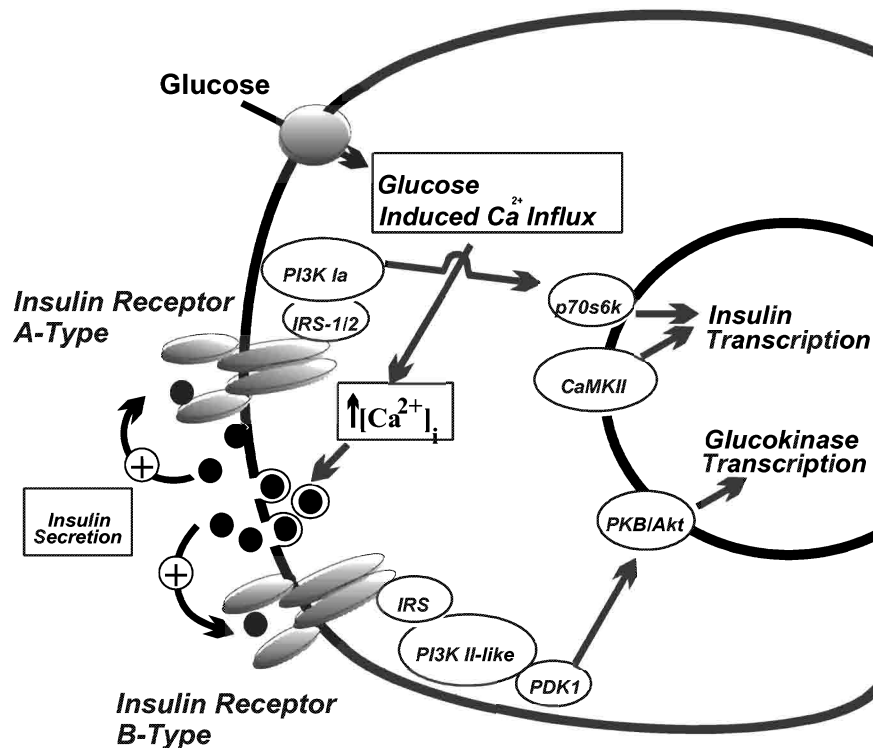


Figure 8 **Insulin-secretion/insulin-gene-transcription coupling.** The scheme illustrates the coupling between the insulin exocytosis and insulin gene transcription. K_{ATP}-channel - ATP-sensitive K⁺ channel; L-type VDCC - L-type voltage-dependent Ca²⁺ channel; Glc – glucose (adopted from Leibiger et al. 2001; paper V, fig. 7)

β-cell IR, both IR-A and IR-B. Whereas signaling via IR-A/PI3K class Ia/p70sk and CaM kinase II activates the transcription of the insulin gene, signaling via IR-B/PI3K class II-like activity/PDK1/PKB(Akt) leads to the up-regulation of BGK gene transcription.

5.9 Regulation of insulin stimulated insulin and glucokinase gene transcription in human β -cells

The above described results for glucose/insulin-stimulated up-regulation of insulin and BGK gene transcription were obtained in primary β -cells from mouse and rat and in insulinoma cell lines HIT-T15 (hamster), INS-1 (rat) and MIN6 (mouse). In order to test whether the described mechanisms involved in the stimulus-dependent up-regulation of the two genes are the same or different in human β -cells, we performed the following experiments on human islet cells.

First, we demonstrated that stimulation with 5mU/ml insulin for 5 min at sub-stimulatory glucose concentrations results in the up-regulation of BGK promoter-driven GFP and insulin promoter-driven DsRed expression.

To test, whether also here signaling via IR is required for the up-regulation of insulin and BGK promoter activity, we treated transfected human islet cells with blocking antibodies that either prevent signaling through both IR-A and IR-B (α IR-AB) or that block selectively signaling through IR-B

only (α IR-B). Treatment with α IR-B abolished the up-regulation of both insulin- and BGK-promoter-driven expression of DsRed and GFP, respectively. However, selective inhibition of signaling via IR-B abolished up-regulation of BGK promoter-driven GFP expression but did not

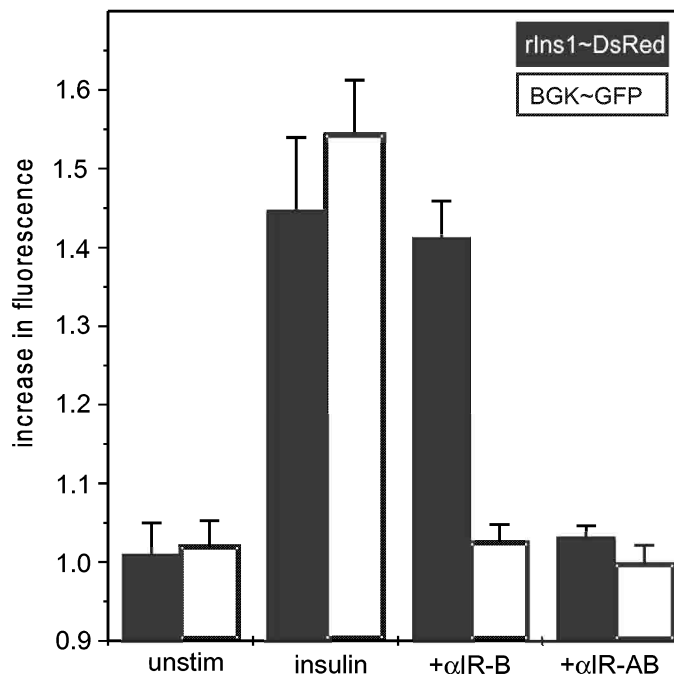


Figure 9 The role of insulin receptors in gene transcription in human β -cells Effect of antibodies that block signaling through insulin receptor A and B (α IR-AB) and through insulin receptor B (α IR-B) on insulin stimulated rat BGK promoter-driven GFP expression (open bars) and rat insulin-I promoter-driven DsRed expression (closed bars) in co-transfected human islet cells. Cells were incubated with 0.67 μ g/ml of the respective antibodies 30 min prior to and throughout stimulation with 5 mU/ml insulin. As controls cells were either not stimulated (unstim) or stimulated with 5 mU/ml insulin. Data are presented as the ratio of fluorescence obtained at minutes 240 and 60 and represent mean values \pm S.E. (n = 11)

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affect the up-regulation of insulin promoter-driven DsRed expression in response to insulin stimulation (Figure 9)

To test, whether in human β -cells the same or different protein kinases as compared to the rodent cell systems contribute to the up-regulation of insulin and BGK gene expression, we employed inhibitors for CaM kinase II (autocamtide-2 related inhibitory peptide [AC]), p70s6k (rapamycin) and IR tyrosine kinase (HNMPA-(AM)₃). Furthermore, we studied the impact of two different concentrations of the PI3K inhibitors LY294002 and wortmannin, respectively, in order to test whether also in human β -cells different classes of PI3K are involved in the up-regulation of the insulin and BGK promoters by insulin. The experiments showed (fig. 10), that insulin-stimulated insulin gene expression is sensitive to inhibition of CaM kinase II, p70s6k and inhibited by low concentrations of wortmannin or LY 294002, indicating the involvement

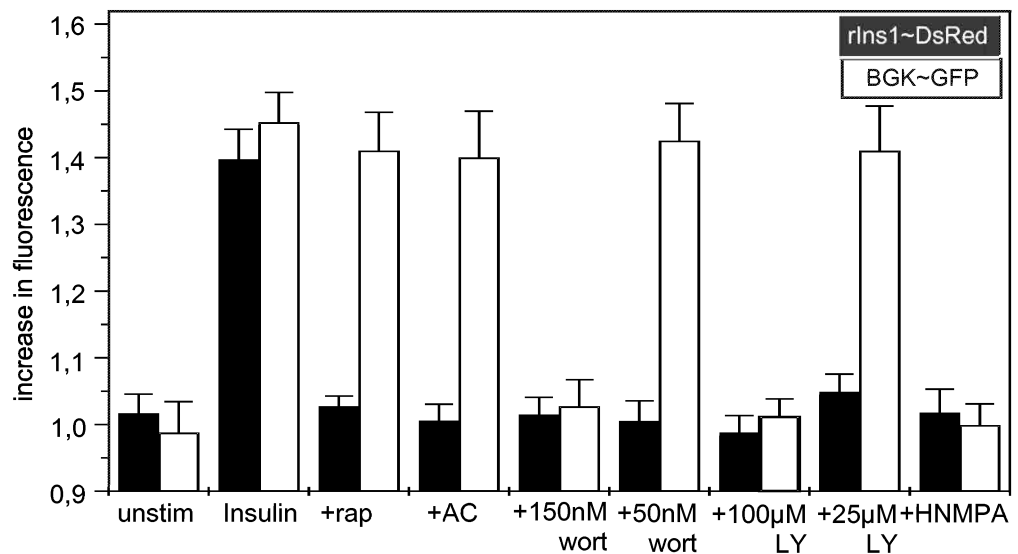


Figure 10 Influence of various protein kinase inhibitors on gene transcription in human β -cells Effect of various protein kinase inhibitors on insulin stimulated BGK-promoter driven GFP expression (open bars) and insulin-promoter driven DsRed expression (closed bars) in co-transfected human islet cells. Cells were incubated with 10 nM Rapamycin (p70s6kinase, rap), 400 nM autocamtide-2 related inhibitory peptide [AC] (Ca²⁺/calmodulin dependent kinase II; AC), 50 nM or 150 nM Wortmannin (PI3 kinases; 50nM or 150nM wort), 25 μ M or 100 μ M LY 294002 (PI3 kinases; 25 μ M LY or 100 μ M LY) or 100 μ M HNMPA-(AM)₃ (IR tyrosine kinase; HNMPA) 30 min prior to and during stimulation with 5 mU/ml insulin. As controls cells were either not stimulated (unstim) or stimulated with 5 mU/ml insulin. Data are presented as the ratio of fluorescence obtained at minutes 240 and 60 and represent mean values \pm S.E. (n = 11)

of class Ia PI3K. This, together with the data employing the IR antibodies, confirmed, that insulin stimulated gene transcription is regulated through a pathway involving IR A/PI3K class Ia/p70s6k and CaM K II.

Only the inhibitor for the IR tyrosine kinase and higher concentrations of wortmannin or LY 294002 had an inhibitory effect on insulin-stimulated glucokinase transcription. Together with the effect of the IR-B-antibody as described above, these data suggest, that the up-regulation of BGK promoter activity by insulin involves a pathway that includes the IR B and a PI3K class II in human β -cells. Although the involvement of PDK-1/PKB in this mechanism is likely, considering the congruency of pathways in mouse β -cells/HIT T15 cells and human β -cells so far, it still must be proven experimentally in an approach similar to that used in 5.6.

In conclusion, we were able to demonstrate, that the regulation of insulin and glucokinase gene transcription follows the same pathways in human β -cells as in rat, mouse β -cells or HIT T15 cells, showing also the distinction between signaling through IR-A and IR-B. This proves that the positive feedback of secreted insulin on insulin and BGK gene transcription is not limited to rodent β -cells or cell lines but is also active in human β -cells.

6 GENERAL REMARKS

The results of the present thesis work clearly show that short-term stimulation (15 min) of pancreatic β -cells with stimulatory glucose concentrations is sufficient to up-regulate the transcription of both the insulin gene and the β -cell transcription unit of the glucokinase gene. The time frame of this stimulatory effect of glucose is in agreement with the dynamics of post-prandial blood glucose elevation.

Studying the mechanism underlying the short-term glucose response of insulin- and glucokinase gene transcription we found that neither glucose or its metabolites, nor elevation in free cytoplasmic Ca^{2+} , but that insulin, secreted in response to glucose stimulation, is the key factor in up-regulating the transcription of the two genes. The major consequence of this finding is that it actually designates the β -cell as an insulin target tissue. The idea of an autocrine feedback of insulin on pancreatic β -cell function is not new, already in 1941 Best and Haist (Best et al. 1941) suggested a negative feedback of insulin on insulin secretion by the pancreatic beta cell (see Koranyi et al. 92), this feedback was generally discussed in the context of being inhibitory to β -cell function (Iversen et al. 1971; Draznin et al. 1986). Our data (paper II) together with data from Macdaniel's laboratory (Xu et al. 1998a) and Rothenberg's group (Xu et al. 1998b) were in 1998 the first to report a positive effect of secreted insulin on β -cell function. Since then growing evidence has been provided showing that insulin is a key player in β -cell physiology, including processes such as the regulation of gene transcription (Leibiger et al. 1998b, Xu et al. 1998b, Leibiger et al. 2001, da Silva Xavier et al. 2000, Larsson et al. 2000, Wu et al. 1999, Leibiger et al. 2000), translation (Xu and Macdaniel et al. 1998, Leibiger et al. 2000), ion flux (Aspinwall et al. 2000, Xu et al. 2000), beta cell proliferation (Kulkarni et al. 1999b, Welsh et al. 2000, Withers et al. 1998), cell survival (Kwon et al. 1999) and finally insulin secretion (Aspinwall et al. 1999, Aspinwall et al. 2000, Khan et al. 2001, Persaud et al. 2002). These findings led to the concept of ' β -cell insulin resistance' as a mechanism involved in development of type 2 diabetes mellitus.

The second surprising, unexpected major finding of this thesis work is that insulin affects transcription of the insulin and BGK genes by signaling through insulin receptors.

As mentioned above, insulin feedback action on β -cell function has been discussed as a negative feedback loop. Nonetheless, the question whether insulin could affect β -cell function at all was the subject of debate over the last decades. The major argument against any feedback action was that insulin-secreting pancreatic beta cells face so much insulin that expression of insulin receptors would be down-regulated and/or insulin signaling had to be de-sensitized. If at

all, IGF-I receptors rather than insulin receptors, were seen as target receptors in a potential feedback action. However, data by Velloso et al. (Velloso et al. 1995) and Harbeck et al. (Harbeck et al. 1996) convincingly showed that pancreatic β -cells beside IGF-I-, IGF-II- (Katz et al. 1997), and insulin receptor related-receptors (Hirayama et al. 1999) do express insulin receptors. Moreover, data revealed that insulin, secreted upon glucose stimulation, activates insulin receptors and the downstream located PI3K and insulin receptor substrate (IRS) proteins (Velloso et al. 1995, Rothenberg et al. 1995). These studies provided evidence at the molecular level for an autocrine feedback action of insulin but did not resolve yet whether insulin is a negative, positive or complex (negative and positive) signal for β -cell function. Our data, when using the pharmacological inhibitor HNMPA-(AM)₃ or IR-specific blocking antibodies gave some indication that signaling via IR is important in the up-regulation of insulin and BGK gene transcription by insulin. However, the most convincing proof for the involvement of insulin receptors and not IGF-I receptors in our system, were the data obtained from experiments on islets prepared from β IRKO mice. These mice have a β -cell restricted knockout of IR expression but do express all other types of receptors in β -cells, including IGF-I receptors. Nonetheless, stimulation of β IRKO mouse islets with either glucose or insulin failed to activate transcription of insulin and BGK genes. It is noteworthy, that β IRKO mice at 6 to 8 month of age express a phenotype very similar to type 2 diabetes (Kulkarni et al. 1999)

The third surprising, unexpected outcome of this thesis work was the observation that selective insulin signaling can be gained by signal transduction through the two isoforms of the IR, i.e. IR-A and IR-B. While insulin gene transcription is activated by signaling through IR-A/ PI3K class Ia/p70s6k and CaM kinase II, the stimulation of glucokinase gene transcription is dependent on signaling through IR-B/PI3K class II-like/PKB.

Selectivity in insulin signaling is currently discussed as the result of the activation of specific signal transduction pathways. This selectivity may be gained by activating specific adapter proteins, i.e. IRS and Shc proteins, that 'channel' the insulin signal in a more defined way by specifically interacting with downstream located effector proteins (Myers et al. 1996; Virkamäki et al. 1999). Whereas the importance of IRS proteins in achieving insulin effects in different tissues is currently under extensive investigation, the possibility of selective insulin signaling via the two isoforms of the IR has been neglected. Studies on general and tissue-specific IR knockout models have demonstrated that a defect IR-mediated insulin signaling leads to a type 2 diabetes like phenotype (reviewed in Taylor et al. 1999). However, these knockouts do not discriminate between the two IR isoforms. This is of importance, since earlier studies clearly established differences in tissue-specific IR isoform expression as well as in their activation profile. To our knowledge our data provide the first functional 'read-out' for discriminating selective signaling via the two IR isoforms.

Taken together, the data of this thesis work do not only show that short-term stimulation with glucose up-regulates the transcription of two important β -cell genes, insulin and glucokinase

General Remarks

genes as a result of a positive feedback by secreted insulin, but also clearly demonstrate that selectivity of insulin signaling can be gained by signaling through the two IR isoforms.

7 CONCLUSIONS

1. Insulin gene transcription is up-regulated by short-term incubation with stimulatory glucose concentrations.
2. Insulin, secreted upon glucose stimulation, up-regulates insulin gene transcription by a pathway involving insulin receptor A/PI3K class Ia/p70s6k and CaM kinase II.
3. The nuclear translocation of PDX-1 depends on the NLS motif RRMKWKK and on the structural integrity of 'helix 3' of the homeodomain.
4. Glucokinase gene transcription in pancreatic β -cells is up-regulated by short-term incubation with stimulatory glucose concentrations.
5. Insulin, secreted upon glucose stimulation, up-regulates glucokinase gene transcription by a pathway involving insulin receptor B/PI3K class II like activity/PKB(c-Akt).
6. Selective up-regulation of insulin- and glucokinase gene transcription in response to secreted insulin is gained by the selective signaling through the two isoforms of the insulin receptor A type and B type, respectively. The regulation of insulin and glucokinase transcription by insulin in human β -cells seems to be identical that in rat and mouse β -cells and HIT T15 cells.

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