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SELECTIVE INSULIN SIGNALING IN THE PANCREATIC β-CELL VIA THE TWO INSULIN RECEPTOR ISOFORMS

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Stockholm 2005

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Published and printed by Repro Print AB
Box 21085, SE-100 31 Stockholm, Sweden

ISBN 91-7140-258-6

‘Everyone is trying to accomplish something big,
not realizing that life is made up of little things.’

Frank A. Clark

To Claudia and Felipa

ABSTRACT

Insulin exhibits pleiotropic effects that are tissue- as well as development-dependent. However, the mechanisms by which insulin gains selective effects are poorly understood. Selectivity in insulin signaling is currently discussed as the result of the activation of specific signal transduction pathways. This may be gained by activating specific adapter proteins, such as IRS proteins and Shc, that 'channel' the insulin signal in a more defined way by specifically interacting with downstream located effector proteins. 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 pro-receptor transcript. IR-A lacks whereas IR-B contains the respective sequence coding for 12 amino acids in the C-terminus of the α-chain of the receptor. 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. However, these knockouts do not discriminate between the two IR isoforms. Besides their different affinity for insulin, differences in kinase activity as well as internalization and recycling for IR-A and IR-B have been described. These data implied differences in the function of either IR isoform. Although all cell types express both isoforms to a various degree, little is known about the mechanisms that underlie IR isoform-specific signaling and their biological importance remains obscure.

Besides the classical insulin target tissues liver, muscle and fat, recent research disclosed the pancreatic β-cell as an important target for pleiotropic insulin action, here involving signal transduction through IR and IGF-I receptors. The overall objective of the present thesis work was to test the hypothesis that the two IR isoforms contribute to selective insulin signaling. Specifically, we aimed to investigate the molecular mechanisms that allow simultaneous and selective transcriptional activation of three model genes encoding insulin, β-cell glucokinase (βGK) and c-fos by insulin signal transduction via the two IR isoforms in the pancreatic β-cell.

We show here that insulin activates the transcription of these three genes by different mechanisms. Insulin activates transcription of its own gene by signaling via IR-A and IRS/PI3K Ia/mTOR/p70s6k. In contrast, βGK and c-fos genes are activated by insulin signaling via IR-B but employing different signaling cascades. While insulin-stimulated βGK promoter up-regulation requires the integrity of the IR-B NPEY-motif and signaling via PI3K-C2α/PDK1/PKB, c-fos gene activation needs the intact YTHM-motif and signaling via PI3K Ia/p52-Shc/MEK1/ERK1/2. Studying the molecular mechanisms that underlie the selective signaling via IR-A versus IR-B, we found that both IR-A-mediated insulin and IR-B-mediated βGK promoter activation are not dependent on IR isoform-specific differences in internalization but on their spatial segregation in the plasma membrane. Our data demonstrate that localization and function of the two receptor types depend on the 12 amino acids encoded by exon 11. Moreover, our data suggest that selective activation of the insulin and βGK promoters occurs by signaling from non-caveolae plasma membrane micro-domains that are differently sensitive towards cholesterol depletion. Analyzing the mechanisms that allow activation of selective signaling cascades downstream of IR-B, we found that insulin activates the βGK promoter from membrane-standing IR-B, while c-fos promoter activation is dependent on clathrin-mediated IR-B endocytosis.

In conclusion, the results of the present thesis work clearly demonstrate that spatial segregation of selective signaling pathways originating from IR-A and IR-B allows the simultaneous activation of discrete signaling cascades that lead to specific insulin effects.

LIST OF PUBLICATIONS

I. Leibiger, B., Leibiger, I.B., Moede, T., **Kemper, S.***, Kulkarni, R.N., Kahn, C.N., Moitoso de Vargas, L., and Berggren, P.-O. (2001). Selective insulin signaling through A and B insulin receptors regulates transcription of insulin and glucokinase genes in pancreatic β cells. Mol. Cell *7*, 559-570.

II. **Uhles, S.**, Moede, T., Leibiger. B., Leibiger. I.B., and Berggren, P.-O. PI3K-C2α is involved in insulin receptor B-type mediated activation of glucokinase gene transcription in insulin-producing HIT-T15 cells. Manuscript.

III. **Uhles, S.**, Moede, T., Leibiger, B., Berggren, P.-O., and Leibiger, I.B. (2003). Isoform-specific insulin receptor signaling involves different plasma membrane domains. J. Cell. Biol. *163*, 1327-1337.

IV. **Uhles, S.,** Moede, T., Leibiger, B., Berggren, P.-O., and Leibiger, I.B. Spatial segregation of insulin receptor B-type signaling allows selective and simultaneous activation of glucokinase and c-fos gene transcription. Manuscript submitted.

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CONTENTS

LIST OF ABBREVIATIONS

APS	adaptor protein containing PH and SH2 domains
βGK	β-cell isoform of glucokinase
BSA	bovine serum albumin
CaMKII	Ca^{2+}/calmodulin dependent kinase II
CFP	cyan fluorescent protein
α-cyclodextrin	Schardinger α-dextrin; cyclohexaamylose
β-cyclodextrin	Schardinger β-dextrin; cycloheptaamylose
CMV	cytomegalovirus
DMEM	Dulbecco′s modification of Eagle′s medium
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
ERK1/2	extracellular signal-regulated kinase 1/2
FCS	fetal calf serum
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
HEPES	hydroxyl-ethyl-piperazine ethanesulfonic acid
IR	insulin receptor
IGF-I/-II	insulin like growth factor-I/-II
IGF-1R	insulin like growth factor-I receptor
IRS	insulin receptor substrate
JNK	c-jun-N-terminal kinase
MAPK	mitogen-activated protein kinase
MEK1	MAP and ERK kinase 1
p70s6k	ribosomal p70 s6 kinase
PBS	phosphate-buffered saline solution
PDK1	phosphatidylinositol-dependent kinase 1
PI3K	phosphatidylinositol 3-kinase
PI3K-C2α	phosphatidylinositol 3-kinase C2α
PKB/c-Akt	protein kinase B/c-Akt
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
PTEN	phosphatase and tensin homologue deleted on chromosome 10
RNAi	RNA mediated interference, siRNA; small interference RNA
RT-PCR	reverse transcriptase polymerase chain reaction
SDS-Page	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Shc	Src-homology 2 domain containing transforming protein 1
SH2	Src-homology 2
SHIP	Src-homology 2 (SH2)-containing phosphatase
SRE	serum response element
TBST	Tris-buffered SDS buffer with 0.1% Tween20
YFP	yellow fluorescent protein

1 INTRODUCTION

Insulin is an anabolic hormone with powerful effects on a wide range of physiological processes involving mitogenic and/or metabolic events [1]. The most examined is insulin's important role in the regulation of glucose homeostasis. Maintenance of normal glucose metabolism requires a tightly coordinated control of insulin secretion and action. In response to elevation in plasma glucose the secretion of insulin by pancreatic β-cells is increased. Elevated insulin levels stimulate glucose uptake by muscle and fat, increase glycogen synthesis and inhibit glycogenolysis and gluconeogenesis in the liver, thus maintaining normoglycemia. In addition to these well-established short-term actions, insulin exerts a number of long-term effects, many of which are mediated by regulating the expression of hundreds of genes [2] involved in amino acid uptake, lipid metabolism, cell growth, development and survival [3-8]. Malfunction of either release of insulin by the β-cell (i.e. β-cell dysfunction) or insulin action (i.e. peripheral insulin resistance) lead to the development of the most common metabolic disorder in man, type II diabetes mellitus.

Although the pleiotropic action of insulin is well appreciated, the molecular mechanisms involved in their selective regulation remain poorly understood. Selectivity in insulin signaling is currently discussed as the result of the activation of specific signal transduction pathways. This can be achieved by involving specific adapter proteins that transduce the insulin signal in a more defined way by selectively interacting with downstream located effector proteins [9,10]. The fact that insulin may transduce its signal through a variety of pathways has been discussed in extensive detail [1]. The two major pathways described to date, which employ insulin receptors as the primary target, include signaling via mitogen-activated protein kinases (MAPK) and via phosphoinositol 3-kinases (PI3K). 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 pro-receptor transcript [11]. The A-type (IR-A) [12] lacks whereas the B-type (IR-B) [13] contains the respective sequence coding for 12 amino acids in the C-terminus of the α–chain of the receptor. Although all cell types express both isoforms of the IR to a various degree, little is known about the mechanisms that underlie IR isoform-specific signaling and their biological importance remains obscure.

Data gathered over the last years clearly demonstrate that the insulin-producing β-cell is a target for pleiotropic insulin actions [14], here involving signal transduction through IRs and IGF-I receptors [15,16]. In the present work we aimed to understand how the two IR isoforms contribute to selective insulin effects in the pancreatic β-cell by using the insulin-stimulated transcription of three model genes, i.e. insulin, β-cell glucokinase (βGK) and c-fos, as functional read-outs.

2 BACKGROUND

2.1 THE IR STRUCTURE

In humans the IR gene is located on the short arm of chromosome 19 and contains 22 exons and 21 introns [11-13]. The mature IR is a heterotetrameric glycoprotein composed of two α-subunits and two membrane-spanning β-subunits. It is synthesized as a single high molecular weight pro-receptor (Mr ~180,000) that is proteolytically cleaved at a tetrabasic amino acid sequence (RLRR) located at the junction of the α- and β-subunits to yield α-β monomers bridged by disulfide bonds. Two α-β monomers are linked together by disulfide bonds resulting in the mature heterotetrameric α2β2 configuration (*Figure 2.1*).

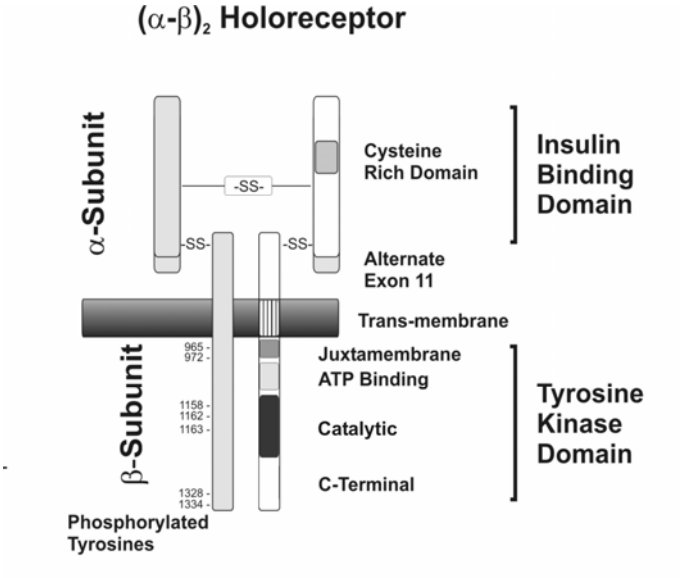

Figure 2.1. IR structure.

The α-subunit is entirely extracellular and is responsible for high affinity binding of insulin. The N-terminal (residues 1-155) and the cysteine-rich domains (residues 155-312) in combination with a C-terminal domain (residues 705-715) of the α-subunit are required for high affinity binding [17,18]. Furthermore, the presence of a 12 amino acid sequence encoded by exon 11 contributes to the modulation of insulin binding affinity as discussed in [19,20]. The unoccupied α-subunit may be viewed as a regulatory subunit of the catalytic intracellular β-subunit because it inhibits the intrinsic tyrosine kinase activity. Binding of insulin to the extracellular α-subunit results in a change within the quarternary structure of the receptor, which places the phosphorylation sites of one β-chain within reach of the active site of the other β–chain, allowing autophosphorylation and, as a consequence, increase in kinase activity [21]. Thus, in the absence of insulin, the IR α-subunit maintains a structural constraint on the constitutively active β-subunit kinase.

The β-subunit of the IR is composed of a short extracellular domain, the membrane-spanning domain, and the cytoplasmic domain, which possesses the intrinsic tyrosine kinase activity. Distinct functional regions have been defined in the cytoplasmic portion of the β-subunit: the ATP binding domain (GXGXXG), the juxtamembrane region (~30 residues C-terminal to the transmembrane helix, which includes the two autophosphorylation sites Y_{965} and Y_{972}), the tyrosine kinase (catalytic) domain (~300 residues including $Y_{1158}XXX\ Y_{1162}Y_{1163}$), and the C-terminal region (~70 residues including Y_{1328} and Y_{1334}) [22-26] (numbering of IR amino acids is according to [13]). Autophosphorylation of Y_{1158}, Y_{1162} and Y_{1163} leads to a major conformational change of the activation loop of the kinase, allowing unrestricted access of ATP, resulting in a 10-20-fold increase in the kinase activity and recruitment of protein substrates to the kinase active site [27-31].

Phosphorylation of Y_{972} in the juxtamembrane region creates a recognition motif (NPEY) for phosphotyrosine binding (PTB)-domain-containing proteins, e.g. the insulin receptor substrate (IRS) proteins (see 2.2.1.1). Mutation of this tyrosine completely inhibits subsequent

phosphorylation of IRS-1 and other IRSs and leads to a loss of most insulin-dependent biological responses [24,32]. Although the NPEY-motif resembles a classical recognition site for adapter proteins involved in receptor endocytosis, i.e. AP-2, mutation of this motif to APEA had only a modest effect on internalization of the IR [33]. In contrast, mutation of a similar sequence in the same region, $GPLY_{965}$ to APLA, resulted in a significant decrease in the endocytotic rate compared to wild type IR [33]. The double mutant APLA/APEA showed almost no internalization [33]. Recent studies demonstrated that Y_{1158} and Y_{1162} in the activation loop also serve as recruitment sites for downstream signaling proteins [34-36]. Like the C-terminal pY_{1334}THM-motif, they represent a preferred binding motif for Src homology 2 (SH2)-domain-containing proteins. However, while phosphorylated Y_{1158} and Y_{1162} are binding motifs for APS [34-36], the C-terminal YTHM-motif was reported to be involved in the binding and subsequent activation of the regulatory subunit p85 of PI3K class Ia [37,38]. Other studies suggested that the C-terminal region is involved in mediating the interaction between the IR and Shc [39]. This idea was supported by data that demonstrated a preferential association of the three isoforms of Shc with an IR C-terminal (amino acids 1257-1353) peptide [40].

2.2 INSULIN SIGNALING VIA THE IR

By interacting with adapter proteins, i.e. insulin receptor substrate proteins (see 2.2.1.1), and creating recognition sites for SH2-domain containing downstream adapter/effector proteins, the IR is able to initiate multiple signaling cascades. The two most studied propagate signal transduction through the IRS/PI3K pathway and through the Ras/MAPK pathway and are often referred to as the 'metabolic' and 'mitogenic' branches, respectively, in insulin signal transduction. Hence, it is believed that the acute metabolic effects of insulin require activation of the IRS/PI3K pathway, whereas the Ras/MAPK pathway may play a role in certain tissues to stimulate the actions of insulin on growth and proliferation.

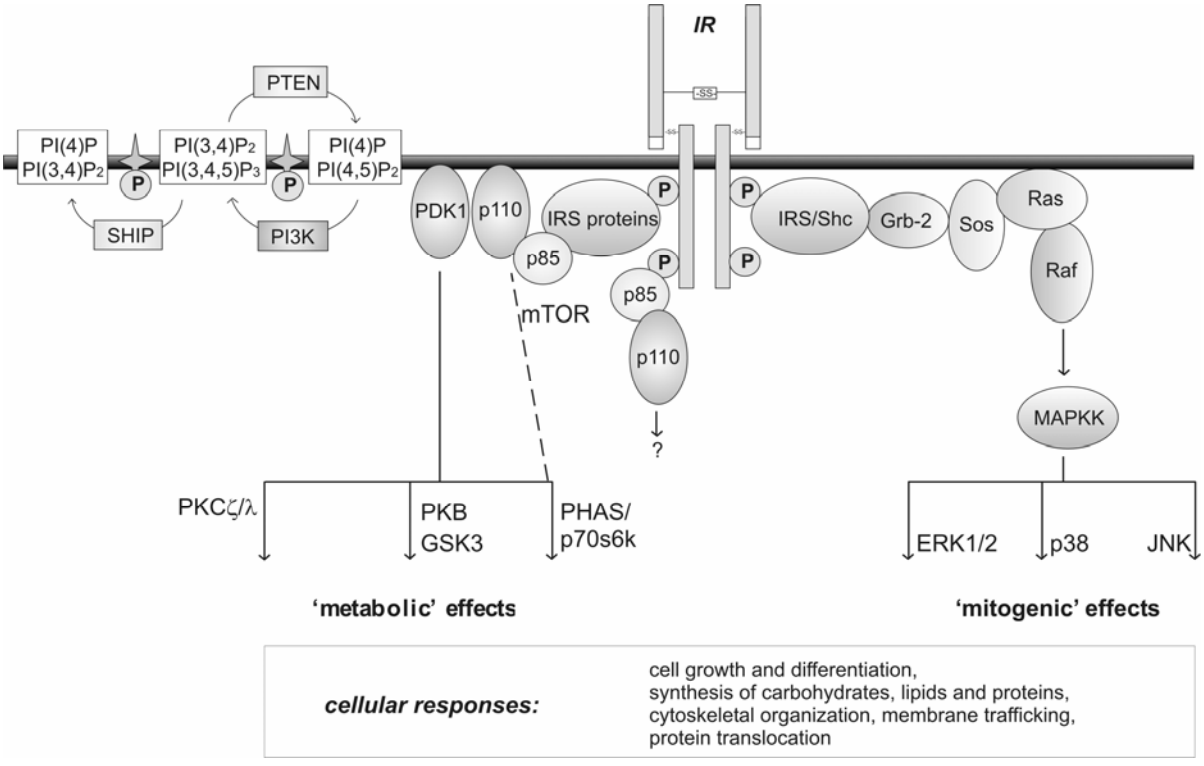

Figure 2.2. A model of the two main insulin signaling pathways.

2.2.1 The IRS/PI3K pathway

2.2.1.1 Members of the IRS-protein family

As described in 2.1, binding of insulin to its receptor activates the IR tyrosine kinase, resulting in autophosphorylation of tyrosine residues in the receptor β-subunit. This in turn leads to the recruitment and phosphorylation of several protein substrates, primarily the IRS proteins. These proteins have an important regulatory role, providing an interface between the IR and downstream effector molecules (reviewed in [10]). The IRS family is composed of four closely related members IRS-1 [41,42], IRS-2 [43], IRS-3 [44], and IRS-4 [45]. More distantly related members are Gab-1 [46], Shc [47], and $p62^{dok}$ [48]. Newly identified IR substrates are APS (adaptor protein containing PH and SH2 domains) [34,35], SIRPs (signal-regulatory proteins) [49], SH2-B [50,51], and Grb10 [52-54].
Most attention has focused on IRS-1 and IRS-2. Mice lacking IRS-1 are insulin resistant but do not develop overt diabetes [55,56]. By contrast, animals lacking IRS-2 develop diabetes as a result of peripheral insulin resitance and β-cell dysfunction [57]. Despite the similarity in structure and function, the apparent differences in phenotype between IRS-1 and IRS-2 knockout mice emphasize a signaling specificity that probably results from their tissue distribution, subcellular location, activation-inactivation kinetics and combinatorial interactions with downstream effectors [19]. Ablation of IRS-3 is devoid of a clear phenotype [58], whereas lack of IRS-4 expression is associated with modest growth retardation and insulin resistance [59]. Inactivation of Gab-1 has an embryonic lethal phenotype [60].
IRS proteins contain an N-terminal pleckstrin homology (PH) domain that is involved in their targeting to the membrane close to the IR. All IRS family members, except Gab-1, possess a PTB domain located adjacent to the PH domain. The PTB domain is critical for recognition of the NPEY sequence of the IR. During interaction with the IR, the IRS proteins become phosphorylated on several tyrosine residues, which allow them to interact with multiple SH2 domain-containing proteins [61]. All proteins in the IRS family bind to the autophosphorylated IR only transiently, dissociate and subsequently can be recognized by the SH2 domains of the adapter proteins. The IR substrate Shc binds to the NPEY-motif of activated IR via its PTB domain [62] or, alternatively, may bind via its SH2-domain to the C-terminal YTHM-motif of the IR, although the latter possibility has been controversially discussed [63]. Following association Shc becomes tyrosine phosphorylated [64], thus providing a binding site for the SH2-domain of Grb2/Sos. This is discussed to lead to the activation of the Ras/MAPK cascade and stimulation of the 'mitogenic' signaling pathway [47]. Gab-1 and $p62^{dok}$ are both phosphorylated by the IR [46,48], however their role in insulin signaling have not been determined.
The newly identified adapter proteins APS [34,35], SH2-B [50,51], and Grb10 [52-54] interact with phosphotyrosines in the activation loop of the autophosphorylated IR. However, while APS and SH2-B become themselves phosphorylated by the IR kinase and propagate signal transduction, Grb10 is not a substrate for the IR kinase and is discussed do negatively regulate IR function [65,66].

2.2.1.2 PI3K and downstream signaling proteins

Activation of the PI3K and its downstream effector proteins by insulin leads to a multitude of cellular responses. Blocking PI3K with, for example, the fungal inhibitor wortmannin is associated with inhibition of insulin-stimulated glucose uptake [67,68], glycogen [69,70], lipid [68], and protein [71,72] synthesis, modulation of gene expression [73,74], and cell survival

[75-77] in different cell systems. The enzyme PI3K catalyzes the addition of phosphate to the D3 position of the inositol ring of phosphoinositol and phosphoinositol phosphates leading to the generation of phosphatidylinositol 3-phosphate (PI(3)*P*), phosphatidylinositol 3,4-bisphosphate (PI(3,4)P_2) and phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P_3). 3-phosphorylated phosphoinositides act as intracellular messengers that allow the activation of PI-dependent kinases [78]. In addition, the enzyme has protein kinase activity, although there is no evidence yet for the involvement in insulin action [79].

To date three classes of mammalian PI3Ks have been identified based on their domain structures, differences in catalytic activity towards defined substrates, and modes of regulation (reviewed in [80]). Only class Ia and class II PI3Ks can be activated by insulin. Class Ia PI3Ks are heterodimers consisting of a catalytic subunit (p110) and a regulatory subunit (p85, p55, or p50). The catalytic subunit contains the kinase domain and an N-terminal regulatory subunit-binding domain, which is constitutively associated with the regulatory subunit. The regulatory subunit possesses two SH2 domains that bind specifically to phosphorylated tyrosine residues of either the IR or of adapter proteins, such as IRS1. The two SH2 domains are linked with the inter-SH2 domain, which allows the regulatory subunit to associate with the catalytic subunit p110. Activation of PI3K by phosphorylated IRS proteins binding to the NPEY-motif of the IR is well appreciated. A further way for PI3K class Ia to associate with the IR is the direct interaction via the SH2 domain of the regulatory subunit with the phosphorylated YTHM-motif in the C-terminal region of the IR [37,38], however, no functional consequence has yet been reported. Class II PI3Ks were originally identified by sequence homology with other PI3Ks [81] and so far, three mammalian isoforms, i.e. PI3K-C2α [82,83], PI3K-C2β [84,85], and PI3K-C2γ [86-88] have been described. They all contain a putative PTB motif and a C-terminal C2-domain. Class II PI3Ks prefer phosphatidylinositol (PI) and phosphatidylinositol 4-phosphate (PI(4)*P*) as substrates and therefore show a different substrate specificity than class Ia PI3Ks, which prefer phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) as a substrate [82,84,89].

The generation of increased levels in PI(3,4,5)P_3 and PI(3,4)P_2 in response to insulin stimulation activates PI-dependent serine/threonine kinases, e.g. PDK1 and -2 [90], which in turn activate protein kinase B (PKB) [91,92], p70s6k [93], salt- and glucocorticoid-induced kinases [94], and atypical protein kinase C (PKC) isoforms [95]. Among the PI-dependent kinases, PKB has received most attention. Upon insulin stimulation, PKB translocates to the plasma membrane, where it becomes phosphorylated by PDK1. All three PKB isoforms, i.e. PKBα, -β and -γ, are activated by phosphorylation on T_{308} and S_{473} [96,97]. It has been shown that PKB directly phosphorylates and thereby inactivates glycogen synthase kinase 3 (GSK3), thus leading to increased glycogen synthesis in the liver [98,99]. In addition, PKB phosphorylates proteins that regulate lipid synthesis [100], protein synthesis [101,102] and cell survival [103].

Another serine/threonine protein kinase which is activated in response to increased levels of PI(3,4,5)P_3 and PI(3,4)P_2, is p70 ribosomal S6 kinase (p70s6k). PKB is discussed in the activation process of p70s6k [99,104], however no direct phosphorylation of p70s6k by PKB has been demonstrated. Interestingly, PDK1 directly phosphorylates p70s6k at T_{252} [102] and T_{229} [105]. The phosphorylation of p70s6k is dependent upon PI3K activation, however, in contrast to PKB, p70s6k neither interacts with PI(3,4,5)P_3/PI(3,4)P_2 nor is the rate at which it is phosphorylated by PDK1 *in vitro* enhanced in the presence of PI(3,4,5)P_3/PI(3,4)P_2. Data from Alessi´s group demonstrated that a specific substrate recognition site on PDK1, called the PIF-binding pocket, plays a crucial role in enabling PDK1 to phosphorylate and activate p70s6k but

not PKB [106,107]. p70s6k activity has been discussed to be involved in protein synthesis, cell cycle control, cell migration and differentiation [93].
Members of the PKC family have been implicated in several of insulin´s actions. There are three groups of PKCs based on their domain structure: conventional (α, βI, βII, γ), novel (δ, ε, θ, η/L) and atypical (ζ, ι/λ) PKCs. The conventional ones are activated by Ca^{2+} binding, diacylglycerol and phosphatidylserine, whereas novel PKCs can be activated by diacylglycerol and phosphatidylserine, and atypical PKCs by phosphatidylserine [108-110]. Insulin-stimulated activation of atypical PKCs (ζ and ι/λ) through PI3K-dependent increases in phosphoinositides has been proposed to play a role in insulin-dependent glucose transport [50,111], protein synthesis [112] and gene expression [113].

2.2.2 The MAPK pathway

MAPK are serine/threonine protein kinases that can be activated by insulin and a variety of other external stimuli such as muscle contraction, cellular stress (osmotic stress and reactive oxygen species), growth factors, cytokines and ligands for G-protein coupled receptors [114]. When activated, they phosphorylate specific substrates such as phospholipase, transcription factors and cytoskeletal proteins. Thus, MAPK are involved in the regulation of cellular processes such as gene expression, proliferation, motility, metabolism and apoptosis.
The mammalian MAPKs can be subdivided into five families: the classical extracellular signal regulated kinases ERK1/2, c-jun N-terminal kinases JNK1/2/3, p38 (JNK and p38 are also referred to as the stress-activated protein kinases SAPK), ERK3/4 and ERK5. MAPKs are regulated by phosphorylation cascades. Two upstream protein kinases activated in series lead to activation of a MAPK, and additional kinases may also be required upstream of this three-kinase module. Signal amplification (each successive protein in the kinase cascade is more abundant than its regulator) [115,116] and dual non-processive phosphorylation (e.g. the tyrosine-phosphorylated ERK1/2 is not active but must accumulate before threonine phosphorylation and conversion to the active state) [117,118] are characteristic regulatory features of MAPKs.
In all currently known MAPK cascades, the kinase immediately upstream of the MAPK is a member of the MAP/ERK kinase (MEK or MKK) family. The substrate specificity of the known MEKs is very narrow: each MEK phosphorylates only one or a few of the MAPKs. MEKs are also activated by phosphorylation of two residues, either serine or threonine, in their activation loops [119,120]. The MEK kinases (MEKKs) that activate MEKs are many and diverse. Few generalizations can yet be made about regulation of these MEKKs themselves, except that they may be subject to multiple regulatory inputs. Most, if not all, of these MEKKs are not abundant, suggesting that the MEKK-MEK step amplifies the signal originating from a given MEKK.
Activation of the MAPK ERK1/2 pathway by insulin involves the interaction of tyrosine phosphorylated IRS family members with the adapter protein Grb2 via its SH2-domain [121]. Grb2 is constitutively complexed with Sos [122-125], the guanine nucleotide exchange factor for plasma membrane-bound Ras. Recruitment of the Grb2-Sos complex results in the membrane relocalization of Sos, an event considered sufficient to induce Ras activation [126]. Activated Ras then associates with and activates Raf-1, leading to the phosphorylation and activation of MEK1 and its downstream substrate ERK1/2. Aktivated ERK1/2 phosphorylates a variety of substrates, including the ternary complex factors (TCFs). A complex consisting of one phosphorylated TCF and two serum response factor proteins interacts with serum response

elements (SREs) localized, for example, in the promoter of the c-fos gene and allows up-regulation of transcription in response to insulin [127].

2.3 GENERAL PRINCIPLES TO GAIN SELECTIVITY IN INSULIN SIGNAL TRANSDUCTION

As mentioned above, selectivity in insulin signaling is currently discussed as the result of the activation of specific signal transduction pathways. This can be achieved by involving specific adapter proteins that transduce the insulin signal in a more defined way by selectively interacting with downstream located effector proteins [9,10]. Here, for example, the PH-doamins of IRS-1, -2, and -3 have been shown to exhibit different affinities for different membrane lipids [128]. Membranes are composed of a mosaic of different lipids, hence, exhibiting various membrane micro-domains and, thus, allow the micro-domain-selective recruitment of different lipid-anchored proteins [129]. Thus, targeting of the different IRS isoforms to different membrane micro-domains would support the concept of compartmentalization as a mechanistic basis for signal transduction from selective cellular membrane compartments, such as the plasma membrane, endosomes, endoplasmic reticulum or nuclear membranes, all discussed to be potentially involved in insulin signaling.

While the IRS proteins are early components of insulin signaling pathways, the subsequent specific recruitment of multiple downstream signaling proteins further contribute to the unique, selective insulin response in various cells and tissues, e.g. binding and subsequent activation of differnt isoforms of effector proteins via their SH2-domains. Here, the various members of the PI3K class Ia adapter protein family p85, i.e. p85, p55 and p50 together with the two different catalytic isoforms p110α and p110β (see *Table 4*) have been reported to be involved in gaining selective effects by insulin. For example, significant differences in the recruitment of the adapter protein splice variants (p50α, p85α, p55α) into insulin-induced signaling complexes [130-132], positive and negative roles of p85α and p85β in insulin signaling [133], or different kinetic properties of p110α and p110β [134] have been described. The latter raising the possibility of distinct subcellular localization/different compartmentalization of p110α and p110β in areas with low versus high substrate density such as lipid rafts [134-137].

The further involvement of multiple effector-protein isoforms downstream of PI3K, such as PKBα,β,γ (reviewed in [138]) or the atypical PKC isoforms PKCζ and PKCλ [113,139], or the GSK3 isoforms GSK3α and -β [98] and finally the isoforms p70s6k-1 and -2 [140] shall again, only exemplatory, illustrate the broad potential of possibilities to gain selective effects in insulin signaling by employing selective effector-protein isoforms.

Spatial resolution as a way to gain selectivity in insulin signaling involves the use of different subcellular compartments as sites for signal transduction, both regarding signal initiation at the plasma membrane as well as signal reception inside the cell [141-143]. Earlier studies have suggested that signal initiation may be functionally segregated into distinct domains of the plasma membrane. Caveolae [144-146], clathrin-coated pits [147,148], and glycolipid rafts [149,150] represent specialized regions of the plasma membrane that are crucial for specificity in signal transduction. While the plasma membrane is generally accepted as a site for IR-mediated signal transduction, the role of internalized IR complexes in insulin signaling is dicussed controversially. One obvious role for endocytosis in signaling is to provide temporal regulation, as the duration of signaling is an important parameter determining the biological output. The duration of the signaling process depends on the proportion of receptors undergoing degradation compared to those recycling to the plasma membrane [151,152]. Even between IR isoforms these parameters can differ (see 2.4). Dissociation and degradation of the ligand is

required to prevent continuous stimulation of autophosphorylation. Dephosphorylation of the IR leads to deactivation of the receptor kinase activity before the receptor is recycled to the cell surface [153]. Movement of IR signaling complexes via membrane vehicles, e.g. early endosomes, on the other hand can protect the active phosphorylated proteins against access of phosphatases [154]. Communication between endocytic organelles requires actin- and microtubule-dependent motility [155-157]. Insulin-induced IR internalization has been shown to require proper actin organization, while the organization of microtubules seems to be less critical for IR endocytosis [158]. Insulin-induced endocytosis of the IR differs from insulin-induced endocytosis of GLUT4 in that the latter depends upon intact microtubular network and the presence of micortubule motors [159].
Taken together, spatial segregation of signal initiation and propagation by using different plasma membrane compartments and intracellular compartments, in combination with the selective utilization of isoforms of adapter and effector proteins, can be seen as general principles to gain selective insulin effects. To what extend and how the two isoforms of the IR contribute to these principles to achieve selectivity in insulin signal transduction is poorly understood.

2.4 IR ISOFORMS IR-A AND IR-B

The IR exists in two isoforms which differ in the absence (IR-A or Ex11- [12]) or presence (IR-B or Ex11+ [13]) of 12 amino acids (residues 718-729) encoded by exon 11 at the C-terminus of the α-subunit (*Figure 2.1 and 2.3*). Two pro-receptor mRNA transcripts are generated as a consequence of alternative splicing of exon 11 [160]. Two sequences in intron 10 have been reported to modulate alternative splicing of exon 11: a 48 nucleotide purine-rich sequence at the 5′-end that functions as a splicing enhancer leading to an increase in exon 11 inclusion, and an inhibitory 43 nucleotide sequence at the 3′-end upstream from the branch point sequence favors skipping of exon 11 [161]. In addition, nucleotides within exon 11 itself appear to have both positive and negative regulatory effects on the alternative splicing [161]. The relative expression of the two isoforms is regulated both in a developmental and tissue-specific manner. All cell types express both isoforms to a various degree. IR-A is expressed predominantly in the central nervous system and hematopoietic cells, while IR-B is expressed predominantly in the liver [162-164]. Studies have suggested that hormonal and metabolic factors can regulate the alternative splicing of the IR mRNA [165,166]. Preferential expression of IR-A occurs in many cancers including those of the lung, colon [167], breast [167,168], ovaries [169], thyroid [170] and smooth and striated muscle [171]. Expression of IR-A in cancer correlates well with observations that IR-A is expressed in dedifferentiated cells [167,172].

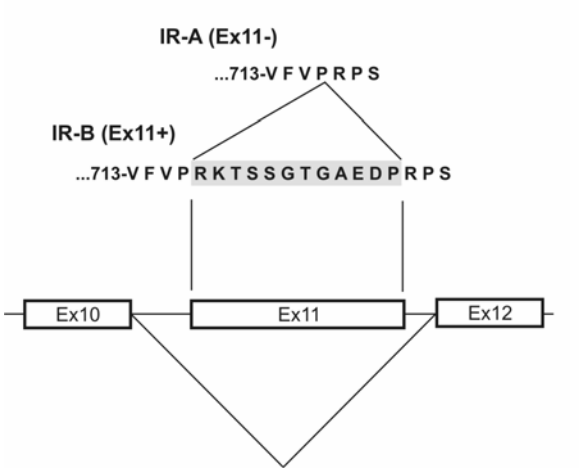

Figure 2.3. Amino acid sequence encoded by exon 11.

The tissue-specific expression of IR-A and IR-B led to several studies designed to detect functional differences between the receptor isoforms. These studies revealed an approximately two-fold higher affinity for insulin for IR-A compared to IR-B [163,173,174]. This difference could have significant physiological consequences: the presence of the lower affinity receptor

(IR-B) in liver may allow that organ to respond appropriately to portal insulin concentrations that are normally 2- to 3-fold higher than insulin concentrations in the periphery. The differences in affinities could also be important in pathological states of hyperinsulinemia, where the higher affinity receptor (IR-A) would be expected to be continuously occupied and, therefore, down-regulated to a greater degree than IR-B. Thus, subjects with hyperinsulinemia might have a higher proportion of lower affinity receptors present in insulin-responsive tissues. Along this line, expression of IR-B has been reported to be significantly increased in skeletal muscle and adipocytes of obese and type 2 diabetic subjects compared with controls, proposing a contribution to insulin resistance [19,175-177]. In contrast, no difference in the expression of IR splicing products between diabetic and healthy controls were reported by others [177-180].

The lower insulin affinity of IR-B is reflected by a lower insulin sensitivity and right-shifted dose-response curves for autophosphorylation, mitogenesis, and activation of glycogen synthase compared to IR-A [181]. The IR-A has been described to bind IGF-II with an affinity close to that of insulin and similar to the affinity of IGF-II-binding to the IGF-1R [167,182]. Studies in mouse fibroblasts lacking the IGF-1R demonstrated that activation of IR-A by insulin led primarily to metabolic effects, whereas activation of IR-A by IGF-II led primarily to mitogenic effects, thereby utilizing different intracellular signaling pathways [167]. IGF-II led to a significantly lower autophosphorylation of IR-B, probably because the 12 amino acids, that are encoded by exon 11 and influence insulin binding, hinder IGF-II binding to IR-B [167,182]. Generation of hybrid receptors of IGF-1R/IR-A and IGF-1R/IR-B revealed that IGF-1R/IR-A could be stimulated by IGF-I, IGF-II and insulin, while IGF-1R/IR-B was activated with high affinity by IGF-I, with low affinity by IGF-II, and insignificantly by insulin [183]. As a consequence, cell proliferation and migration in response to insulin and IGFs were more effectively stimulated in cells expressing IGF-1R/IR-A. [183]. IR-A and IR-B exhibit different kinetics in internalization and recycling, with IR-B exhibiting lower rates of internalization and, in contrast to IR-A, almost no recycling [173,184].

Despite the large numbers of studies, the differences that have been found between the two IR isoforms seem to be of small magnitude and to some degree controversial. Nonetheless, they suggest the possibility that IR-A and IR-B, despite their overall structural homology, have functionally distinct properties. Therefore, learning how signaling via IR-A differs from signal transduction through IR-B may prove useful not only in the context of insulin action/IR signaling but as a more general example for how signaling specificity can be achieved when one signal in the same cell at the same time is transduced via different receptor isoforms, activating selective signaling pathways and resulting in specific cellular responses.

For studies presented in this thesis we generated a series of expression constructs encoding tagged (with GFP, YFP, CFP, DsRed, or FLAG) or non-tagged wild type IR and IR mutants (A- and B-type). *Table 1* presents the variety of IR mutants used in paper III, where we successively deleted amino acids encoded by exon 11.

Table 1. Amino acid sequences of the α-chain C-termini of wild type IRs and deletion mutants

Receptor type	Amino acid sequence (C-terminus of α-chain)
B-type	…713-VFVPRKTSSGTGAEDPRPS
Δ1	…713-VFVPRKTSSGTGAEPRPS
Δ2	…713-VFVPRKTSSGTGAPRPS
Δ3	…713-VFVPRKTSSGTGPRPS
Δ4	…713-VFVPRKTSSGTPRPS
Δ5	…713-VFVPRKTSSGPRPS
Δ6	…713-VFVPRKTSSPRPS
Δ7	…713-VFVPRKTSPRPS
Δ8	…713-VFVPRKTPRPS
Δ9	…713-VFVPRKPRPS
Δ10	…713-VFVPRPRPS
A-type	…713-VFVPPRPS

IR variants tagged with a fluorescent protein or a FLAG-epitope were generated by either fusing the tag to the C-terminus of the β-subunit, to a C-terminus lacking the last 23 amino acids (and therefore the YTHM-motif) or to a 380 amino acid-truncated β-subunit (lacking the catalytic domain, the kinase domain and part of the juxtamembrane domain) (*Figure 2.4*).

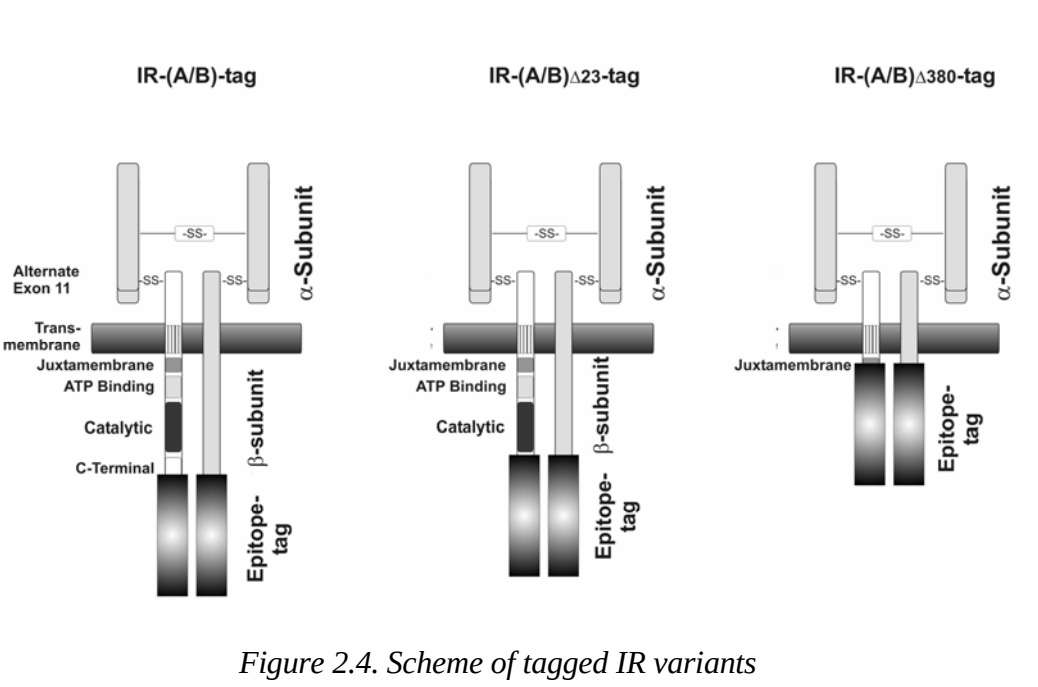

Figure 2.4. Scheme of tagged IR variants

2.5 THE PANCREATIC β-CELL AS AN INSULIN TARGET

The unique function of the pancreatic β-cell is to synthesize and release insulin in appropriate rates to keep blood glucose concentration within narrow physiological limits. To achieve this, strict regulation and fast acting mechanisms that guarantee efficient insulin secretion and biosynthesis are necessary.

It has been shown that glucose itself acts as the major nutrient regulator by triggering a cascade referred to as the glucose-stimulation/insulin-secretion coupling (*Figure 2.5*). In brief, glucose is taken up by the β-cell high-K_m/low affinity glucose transporter and is phosphorylated to glucose-6-phosphate by the β-cell isoform of glucokinase. The following metabolism of glucose-6-phosphate 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 the voltage-gated L-type Ca^{2+} channels leads to an increase in the cytoplasmic free Ca^{2+} concentration, which promotes insulin secretion [185,186].

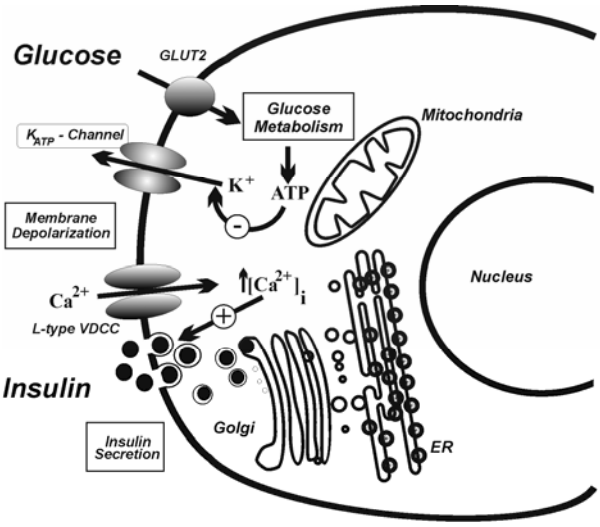

Figure 2.5. The glucose-stimulation/insulin-secretion coupling in the pancreatic β-cell.

Multiple signals of different origin guarantee appropriate β-cell function under both basal and glucose-stimulated conditions. These signals include humoral factors (hormones, vitamins, nutrients, etc), nerve stimulation, as well as factors of intraislet cell-cell communication. Whereas the paracrine effects on β-cells of glucagon, secreted from pancreatic α-cells and stimulating insulin release, and of somatostatin, secreted from δ-cells and inhibiting insulin release, are well accepted (reviewed in [187]), the autocrine effect of secreted insulin on β-cell function was and still is a matter of debate.

Although the idea of an autocrine feedback by insulin is not new and dates back to the 1940s [188], both conceptual disagreement and different results in respective experiments contribute to this still ongoing controversy. With regard to the conceptual disagreement, the major argument is that β-cells are exposed to so much insulin that the respective signal transduction pathways must be desensitized. Experimentally, with regard to the effect of insulin upon insulin secretion for example, all possible outcomes like negative feedback [189-194], positive feedback [195-197], and no effect at all [198-201] have been reported. One of the major points discussed as a source for controversial results and conceptual disagreement was the question whether the observed insulin effect upon β-cell function is a direct one or rather secondary, mediated by factors of non-β-cell origin. This mainly concerned experiments on whole animals and perfused pancreata, but also the 'artifical diffusion effect' in studies on cultured/perifused isolated islets.

Usually, the first step in the insulin cascade is binding of insulin to the IR [1]. However, because pancreatic β-cells are surely exposed to insulin concentrations that are higher than those in the periphery [202], IGF-I and IGF-II receptors, which have a lower affinity for insulin [203], cannot be excluded as receptors involved in insulin binding. That β-cells are targets for insulin was shown already in the 1980s in conventional radioligand binding assays [204] as well as by quantitative electron microscopic autoradiography [205]. The presence of IR and IGF receptors in insulin-producing cell lines was reported in [206] and [207,208], respectively. It was a major breakthrough when Rothenberg and Velloso [209,210] in 1995 demonstrated that

insulin, secreted upon glucose stimulation, activated the β-cell IR and the downstream located IRS and PI3K.

Analysis of kinases involved in insulin signal propagation in insulin-producing HIT-T15 cells demonstrate that signaling through both, the PI3K and the MAPK cascades are functionally active. Exogenous administered insulin results in elevated activation of ERK1/2 but not p38 and JNK, increased activity of p70s6 kinase and PKBα but no increase in PDK1 activity, and in an activation of PLCγ and GSK3 with slower dynamics (*Figure 2.6*).

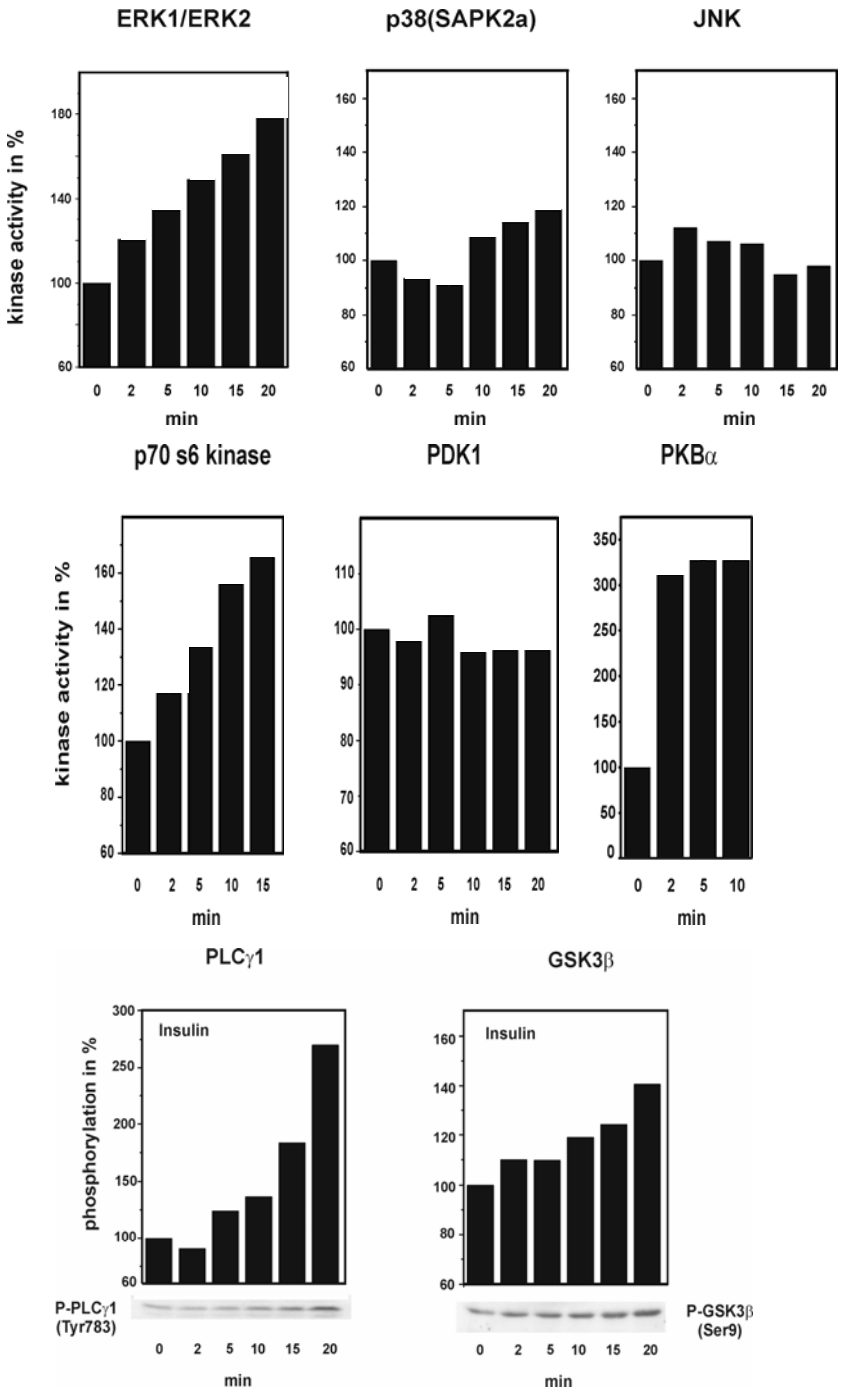

Figure 2.6. Effect of insulin stimulation on signaling pathways and kinases in the pancreatic β-cell.

All these data provided evidence for an autocrine feedback action of insulin at the molecular level but did not yet resolve whether insulin is a negative, positive or complex (negative and positive) signal in β-cell function.

Data gathered over the last six years clearly give evidence for a positive role of insulin in several cellular processes in β-cell function, namely regulation of gene transcription [16,211-217, paper I], translation [212,218-220], glucokinase activity [221,222], glucose metabolism (oxidation and utilization) [223], PHAS-I phosphorylation [224], Ca^{2+} flux from the endoplasmic reticulum [195,196,225,226] and of insulin exocytosis [195,196,227].

Substantial evidence for the requirement of IRs in insulin synthesis and release, as well as postnatal β-cell proliferation and survival, provided the development of the β-cell restricted knockout of IRs in mice (βIRKO) [227]. These mice exhibit a selective impairment in the first phase of glucose-stimulated insulin secretion and a reduction in pancreatic insulin content, which led consequently to the development of a type 2 diabetes-like phenotype. In addition, βIRKO mice show an age-dependent decrease in β-cell mass and glucose intolerance. Along this line, knockdown of IR expression in pancreatic β-cells by RNA silencing showed a marked impairment in the ability of glucose to activate the expression of target genes, i.e. PDX-1, insulin, and βGK [16].

Earlier studies from our group had shown that glucose-induced exocytosis of insulin activates insulin gene transcription by signaling via the A-type IR/PI3K and p70s6k [211]. Because the promoters of both the insulin and the βGK genes contain many similar cis-elements, we sought to analyze whether transcription of the βGK gene is regulated by a similar mechanism as that of the insulin gene. However, preliminary data at that time already indicated differences in signal transduction. Since over-expression of IR-A failed to further activate the βGK promoter in response to insulin stimulation, we hypothesized the B-type IR isoform may be involved in insulin-dependent up-regulation of βGK gene transcription.

3 AIMS

The overall objective of the present thesis work was to test the hypothesis that the two IR isoforms can contribute to selective insulin signaling, and if so, what the underlying molecular mechanisms are. Moreover, we aimed to analyze whether and how selective signaling pathways can be operative simultaneously in the same cell.

The specific aims of this study were to investigate the molecular mechanisms that allow selective and simultaneous transcriptional activation of the three model genes, i.e. insulin, βGK and c-fos, by insulin signaling via the two IR isoforms in the pancreatic β-cell:

1. To investigate the molecular mechanisms underlying the differences between glucose-stimulated βGK gene transcription and glucose-stimulated insulin gene transcription postulating here the involvement of different IR isoforms.

2. To analyze the role of the 12 amino acids encoded by exon 11 of the IR gene in different functions of IR-A and IR-B.

3. To study the molecular mechanisms that underlie insulin-stimulated c-fos gene transcription via the 'mitogenic' branch of insulin signal transduction.

4. To identify the molecular mechanisms that allow the simultaneous but selective activation of insulin, βGK and c-fos genes in the same cell, i.e. to understand how selectivity in insulin signaling is gained i) when utilizing the two IR isoforms (A- and B-type) and ii) when utilizing the same receptor isoform.

4 MATERIALS AND METHODS

4.1 MATERIALS

Materials used in the experiments reported in this work are described in more detail in the respective paper (I-IV).

4.2 CELL CULTURE

4.2.1 Pancreatic islets and primary β-cells

Pancreatic islets of male Wistar rats or normoglycemic ob/ob mice were isolated by collagenase digestion as described by Lacy [228]. Isolated islets and cells of disaggregated islets were cultured at 5% CO_2 and 37°C in RPMI 1640 culture medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 10% FCS and incubated for 2 hours at 3 mM glucose in fully supplemented RPMI 1640 culture medium before starting experiments.

4.2.2 β-cell lines

4.2.2.1 HIT-T15

HIT-T15 cells were obtained from American Type Collection (Manassas, VA) and cultured in RPMI 1640 culture medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 10% FCS at 5% CO_2 and 37°C. Because HIT-T15 cells were reported to show glucose responsiveness at sub-physiological concentrations, i.e. between 0.1 mM and 2 mM glucose [229], they were cultured overnight in RPMI 1640 culture medium, supplemented as above, but containing 0.1 mM glucose overnight before starting experiments.

4.2.2.2 INS1

INS1 cells were obtained from Dr. C.B. Wollheim (Centre Médical Universitaire, Geneva, Switzerland) and cultured in RPMI 1640 culture medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 10% FCS, 1 mM pyruvate, 10 mM HEPES, and 50 µM β-Mercaptoethanol at 5% CO_2 and 37°C. INS1 cells were incubated for 6 hours in RPMI 1640 medium, supplemented as above, but containing 2.0 mM glucose before starting experiments.

4.2.2.3 MIN6

MIN6 cells [230] were obtained from Dr. J. Miyazaki (Osaka University School of Medicine, Osaka, Japan) and were adapted to culture at 11.1 mM glucose in DMEM culture medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FCS at 5% CO_2 and 37°C.

4.2.2.4 Non-insulin-producing cells (HEK293 and COS7)

HEK293 and COS7 cells were grown in DMEM culture medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 10% FCS, and 5.5 mM glucose at 5% CO_2 and 37°C.

4.2.3 Stimulation of cells and application of pharmacological inhibitors and antibodies for online monitoring experiments

HIT-T15 cells, INS1 cells or cultured islets and cells of disaggregated islets were stimulated with either 16.7 mM glucose or 100 ng/ml PMA for 15 min, 50 mM KCl, 1 µM glibenclamide, or 5 µU/ml to 5 mU/ml insulin for 5 min. Following stimulation, the cells were washed with PBS and further incubated in RPMI 1640 culture medium containing sub-stimulatory glucose concentrations.
Pharmacological inhibitors and antibodies were given to the fully supplemented culture medium at sub-stimulatory glucose concentrations at the indicated concentrations 30 min before and throughout stimulation.

Table 2. Name, reference, target and concentration of administered inhibitors:

name	reference	target	concentration
actinomycin D	Sigma	DNA transcription	5 µg/ml
autocamtide-2 related inhibitory peptide	Calbiochem	CaMKII	400 nM
bisindolylmalemide I	Calbiochem	PKC	150 nM
HNMPA-$(AM)_3$	Calbiochem	IR tyrosine kinase	100 µM
LY294002	Calbiochem	PI3K	25 µM to 100 µM
nifedipine	Calbiochem	L-type Ca^{2+} channel	10 µM
PD169316	Alexis Biochemicals	p38/RK/SAPK2a + JNK/SAPK1	10 µM
PD98059	Calbiochem	ERK kinase MEK1	20 µM
rapamycin	Calbiochem	mTOR	10 nM
SB203580	Calbiochem	p38/RK/SAPK2a	20 µM
SP600125	A.G. Scientific Inc.	JNK/SAPK1	25 µM
wortmannin	Calbiochem	PI3K	20 nM to 150 nM

Blocking antibodies against IR-B (Rabbit anti-Insulin Receptor B (αIR(B)), Biodesign), against both isoforms of IR (Rabbit anti-Insulin Receptor α (αIR(AB)), Biodesign) and against IGF-1R (Mouse monoclonal IGF-IRα (αIGF1R), Pharmingen) were applied to the cells in a concentration of 0.67 µg/ml.
Cholesterol-depletion studies using α- or β-cyclodextrin were performed in RPMI 1640 culture medium at substimulatory glucose containing 0.5% BSA instead of 10% FCS. Indicated concentrations of α- or β-cyclodextrin were applied to the cells 10 min prior to and throughout stimulation.

4.3 PLASMIDS AND ADENOVIRUSES

4.3.1 RT-PCR cloning

Total RNA was isolated from islets or β-cells employing the RNeasy kit (QIAGEN). The RNA was reverse-transcribed using the RT-PCR kit from Stratagene. Aliquots of the obtained cDNA were subjected to PCR with primers described in the respective paper/manuscript. PCR products were separated on a 2% agarose gel, and the DNA was eluted and cloned into pCRII using the TA cloning kit (Invitrogen AB). All subcloned DNA fragments were analyzed by DNA sequencing.

4.3.2 Expression constructs

4.3.2.1 Plasmids

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

4.3.2.2 Site-directed mutagenesis

All mutations were performed by using the QuikChange Mutagenesis kit (Stratagene), and respective oligonucleotides were purchased from Proligo (Paris, France).

4.3.2.3 Adenovirus constructs

The adenovirus construct Ad.rβGK.GFP was constructed by subcloning the rβGK.GFP cassette into the pAC.CMV.pLpA and performing homologous recombination by Dr. Moitoso de Vargas (School of Medicine, Boston University, Boston, Massachusetts).

4.4 TRANSFECTION AND TRANSDUCTION

4.4.1 Lipofectamine transfection method

Lipofectamine transfection was carried out 48-72 hours prior to online monitoring or confocal microscopy experiments. Cells were seeded on 24 mm glass coverslips in 35 mm dishes 24 hours prior to transfection. The transfection was performed using 2 to 3 µg plasmid-DNA and 6-9 µl lipofectamine (Invitrogen) in unsupplemented RPMI 1640 per 35 mm dish for 12-14 hours.

4.4.2 Calcium phosphate/co-precipitation method

Cells were grown in 10 cm dishes (3.5-5 x 10^6 cells/dish) and transiently transfected with the respective expression constructs according to the calcium phosphate/co-precipitation technique as described in [231] and cultured for additional 36 hours.

4.4.3 Transduction of islets with adenovirus constructs

Transduction of whole pancreatic islets with the adenovirus constructs were performed as described by Dr. Moitoso de Vargas [232].

4.5 MOLECULAR BIOLOGICAL AND PROTEIN BIOCHEMICAL METHODS

4.5.1 Quantification of mRNA amounts

4.5.1.1 Quantification by RNase-protection assay

For RNase-protection analysis radiolabeled cRNA was generated on the respective linearized cDNA-containing plasmids by employing the SP6/T7 *in vitro* transcription kit (Boehringer Mannheim) and [α-^{32}P]CTP (Amersham Biosciences). After purification by polyacrylamide gel electrophoresis, equal cpm of the labeled cRNA probes (8 x 10^4 cpm/µl, 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 employing the RPA II kit (Ambion). Quantification of protected complexes was performed by phosphorimaging and values obtained for βGK mRNA were normalized to β-actin mRNA values.

4.5.1.2 Quantification by comparative RT-PCR

Levels of insulin, βGK, c-fos and β-actin mRNA were analyzed by comparative RT-PCR as described in [233]. The employed PCR-primers are described in detail in the respective paper (primers for insulin [233], βGK mRNA in paper I, primers for c-fos mRNA in paper IV, primers for β-actin mRNA in paper I and IV). In brief, total RNA was reverse-transcribed by using the RT-PCR Kit (Stratagene). Aliquots of the generated cDNA and [α-^{32}P]dCTP were used for PCR-mediated amplification. PCR conditions were chosen to guarantee the amplification of insulin, βGK, c-fos and β-actin fragments in the linear range. PCR was performed in an AutogeneII thermocycler (Grant) or GeneAmp® PCR System 9700 (Applied Biosystems) using a linked program. ^{32}P-labeled PCR products were separated on a 6% polyacrylamide sequencing gel and analyzed by phosphorimaging. Quantification was performed with TINA-software 2.07d (Raytest), using co-amplified RT-PCR products for β-actin as the internal standard.

4.5.2 Nuclear run-off analysis

5 x 10^7 HIT cells were pre-incubated overnight at sub-stimulatory glucose concentrations (0.1 mM) in fully supplemented RPMI 1640 culture medium. After stimulation with 16.7 mM glucose for 15 min, cells were washed and incubated in sub-stimulatory RPMI 1640 medium for the indicated time (15, 30, 45, and 60 min). Nuclei were isolated and run-off reactions were performed as described in [234]. For nuclear run-off on islets, nuclei from 2000 islets per experiment were used. The labeled RNA was hybridized to 2.5 µg βGK, β-actin and control (pBluescript) DNA, immobilized on nitrocellulose filters. Hybridization was performed with equal amounts of cpm (2.5 x 10^6 cpm) for each sample from all experimental conditions. After hybridization, unhybridized RNA was digested by RNase A. Filters were dried and analyzed by phosphorimaging. Values obtained for βGK mRNA were normalized to β-actin mRNA values.

4.5.3 Membrane preparation of islets and rat tissue

Lysates for membrane preparation were obtained from normoglycemic ob/ob mouse islets or rat tissue (muscle, brain, liver, fat, and kidney). Islets and tissue were washed three times with HB buffer (12 mM HEPES, 300 mM mannitol (pH 7.6), 1 mM PMSF, 0.5 µg/ml pepstatin, 0.5 µg/ml aprotinin, and 0.5 µg/ml antipain), centrifuged for 1 min at 20,000 *g*, resuspended in HB buffer, and homogenized for 1 min in a glass-glass homogenizer followed by passing the homogenate five times through an insulin syringe needle (0.33 x 13 mm/ 29G x 1/2). The homogenate was centrifuged for 5 min at 600 *g*. The supernatant was collected and kept on ice while the pellet was homogenized and centrifuged again for 5 min at 600 *g*. The supernatants were combined and centrifuged for 20 min at 20,000 *g*. The resulting supernatants were collected and centrifuged for 30 min at 60,000 *g*. The pellets containing the crude membrane were resuspended in 200 µl HB buffer. After adding 200 µl of percoll (Sigma-Aldrich) and 800 µl HB buffer, the samples were homogenized and centrifuged for 30 min at 70,000 *g*. The fraction between the aqueous and the percoll phase was collected, and the amount of protein was measured by the Bradford method. All working steps were performed either at 4°C or on ice.

4.5.4 Western blot analysis

Western blot analysis was performed either to determine the profile of protein expression and/or phosphorylation or, in combination with immunoprecipitation, to detect protein-protein interaction with co-immunoprecipitated proteins. If no membrane preparation was needed, lysates of cell monolayers were obtained by washing the (treated or untreated) cells two times with ice-cold PBS and harvesting them in 400 µl lysis buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 4 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 20 mM Tris (pH 8.0), 1 µg/ml aprotinin, 1 mM PMSF and 10 mM NaF). The lysed cells were homogenized by passing them ten times through an insulin syringe needle (0.33 x 13 mm/ 29G x 1/2), centrifuged for 10 min at 600 *g* and the supernatants collected. The amount of protein was measured in the supernatants by the Bradford method. All working steps were performed either at 4°C or on ice.

4.5.4.1 Quantification of expressed proteins by Western blotting

Equal amounts of protein (50-250 µg) per sample were mixed with 4 x SDS sample buffer [235], boiled for 5 min at 95°C and separated over a normal or gradient SDS-polyacrylamide gel (buffering system according to [235]). After electrophoresis, proteins were transferred to polyvinylidenedifluoride (PVDF) membranes (Millipore, Bedford, MA). The membrane was blocked in TBST (10 mM Tris (pH 7.6), 100 mM NaCl, 0.1% Tween20) containing 3-5% nonfat dried milk or 1% BSA (anti-phosphotyrosine-specific antibodies) for ≥ 1 hour at RT, washed with TBST and incubated with the respective primery antibody according to the manufacturer's instructions. The membrane was washed five times with TBST and incubated for 1 hour at RT with the appropriate horseradish peroxidase-conjugated secondary antibody (BioRad). Thereafter, membranes were washed with TBST for 30-40 min at RT and immunoreactivity was detected by using the ECL system (Amersham Biosciences) and quantified by densitometry and TINA-software 2.07d.

4.5.4.2 Immunoprecipitation analysis

Equal amouts of protein (1-1.5 mg per sample) were incubated with 4-5 µg of a primary polyclonal antibody on a rotator for 16 hours at 4°C. 50 µl of pre-equilibrated Protein-G Plus Agarose were added and incubated for additional 3 hours. Immunoprecipitates were washed twice with lysis buffer (described in 4.5.4), twice with buffer A (137 mM NaCl, 100 mM Tris (pH 8.0)), once with buffer B (150 mM NaCl, 10 mM Tris (pH 7.6), 1 mM EDTA) and once with buffer C (20 mM HEPES (pH 7.6), 1 mM DTT, 5 mM $MgCl_2$). All buffers contained 4 mM Na_3VO_4, 10 mM NaF, 1 mM PMSF and 1 µg/ml aprotinin. Each working step was performed at 4°C or on ice. The beads were then resuspended in 75 µl buffer C, mixed with 4 x SDS sample buffer, boiled for 5 min at 95°C and separated over a normal or gradient SDS-polyacrylamide gel. Western blot analysis was performed as described in 4.5.4.1.

4.5.5 Kinase activity analysis

4.5.5.1 PI3K activity analysis

Cell lysates containing 1-1.5 mg of protein were subjected to immunoprecipitation with the respective primary antibody and washed twice with lysis buffer, twice with buffer A, once with buffer B, once with buffer C (all buffers described in 4.5.4.2), and once with kinase assay buffer (10 mM sodium glycerophosphate, 5 mM sodium pyrophosphate, 30 mM NaCl, 1 mM DTT). The beads were resuspended in 20 µl kinase assay buffer and preincubated for 10 min with 50 µg L-α-phosphatidylinositol (Avanti Polar Lipids) in 10 µl 1% (w/v) sodium cholate at 37°C. In

wortmannin inhibition studies the samples were incubated 30 min prior to and throughout the initiated reaction with the respective wortmannin concentrations (1-100 nM). The reaction was started by the addition of 10 µCi of [γ-^{32}ATP] in 20 µl of reaction mix (3 µM Na_2ATP, 7.5 mM $MgCl_2$) and incubated for 15 min at 37°C. The reactions were terminated by the addition of 50 µl 1M HCl and 160 µl $CHCl_3$:CH_3Cl (1:1 v/v), the phosphatidylinolphosphate (PI) was extracted from the watery phase by extensive vortexing and centrifugation (5 min at 14000 *g*). 40 µl of the reaction product were separated by thin layer chromatography (run in a pre-equilibrated tank containing methanol:chloroform:ammonia:water, 75:54:20:10) and quantified with phosphorimaging and TINA-software 2.07d.

4.5.5.2 PKB activity analysis

Analysis of PKB activity was performed employing the Akt1/PKBα Immunoprecipitation Kinase Assay Kit (Upstate Biotech.) according to the manufacturer's instructions.

4.5.5.3 p70s6k activity analysis

p70s6 kinase was immunoprecipitated from cell lysates using a p70s6k antibody (Upstate Biotech.). Analysis of p70s6k activity was performed employing the PhosphoPlus p70S6 Kinase Antibody Kit (Upstate Biotechnology) according to the manufacturer's instructions.

4.5.5.4 ERK1/2 kinase activity

For determination of ERK1/2 kinase activity Western blott analysis using the MAPK Immunoprecipitation Kinase Assay Kit (Upstate Biotechnology) was performed. Phosphorylation was quantified by TINA software.

Table 3. Name, origin and properties of primary antibodies

Name	origin	WB	IP	reference
Caveolin-1	mouse	1:1000		Transduction Laboratories
Caveolin-2	mouse	1:1000		Transduction Laboratories
Caveolin-3	mouse	1:1000		Transduction Laboratories
anti-Dynamin (Hudy 1)	mouse	1:1000		Upstate Biotechnology
anti-ERK	rabbit		4 µg	Upstate Biotechnology
anti-FLAG (M2)	mouse	1:1000		Sigma
anti-FLAG	rabbit		4 µg	Sigma
anti-GFP (JL-8)	mouse	1:1000		Clontech Laboratories, Inc.
anti-GFP (A11122)	rabbit		5 µg	Molecular Probes
Insulin Rβ (C-19)	rabbit	1:1000		Santa Cruz Biotechnology, Inc.
anti-Myc tag (clone 9E10)	mouse	1:1000		Upstate Biotechnology
anti-p70s6k	rabbit	1:1000	4 µg	Upstate Biotechnology
phospho-Tyr (pY99)	mouse	1:1000		Santa Cruz Biotechnology, Inc.
anti-PI3 Kinase p85	rabbit	1:5000	4 µg	Upstate Biotechnology
anti-PI3 Kinase C2α	rabbit	1:2000	4 µg	kindly provided by Dr. J.Domin (Faculty of Medicine, Imperal College, London)
anti-Akt1/PKBα	sheep		1.2 µg	Biolabs
SHC	mouse	1:1000		Transduction Laboratories
anti-Shc	rabbit		4 µg	Upstate Biotechnology

WB: Western blot; IP: immunoprecipitation

4.6 DETECTION OF FLUORESCENCE BY DIGITAL IMAGING FLUORESCENCE MICROSCOPY

Expression of DsRed and GFP was detected using digital imaging fluorescence microscopy as described in [211,233,236]. In brief, transfected cells (described in 4.4.1) were grown on 24 mm glass coverslips. After stimulation and further incubation up to 60 min after start of stimulation in RPMI 1640 culture medium with sub-stimulatory glucose concentrations at 5% CO_2 and 37°C, the coverslips were placed in a temperature controlled perifusion chamber and mounted on an inverted microscope (Zeiss Axiovert 133TV; Carl Zeiss MicroImaging, Inc.) equipped with a Zeiss plan NEOFLUAR x25/0.8 Imm Korr lens (Carl Zeiss MicroImaging, Inc.). During experiments, the cells were kept at 37°C and were perifused with fully supplemented RPMI 1640 culture medium at sub-stimulatory glucose concentrations. Excitation light was obtained from SPEX fluorolog-2 MM1T11I spectrofluorometer (Spex Industries). The following settings were used: for GFP detection, excitation at 485 nm, a 505-nm dicroic mirror, and a 505-535-nm band-pass emission filter; for DsRed excitation at 558 nm, a 565-nm dicroic mirror and a 580-620-nm band pass emission filter. Cells were imaged using a cooled charged-coupled device (CCD) camera (CH250 with KAF 1400; Photometrics) connected to an imaging system (Inovision). Online monitoring was initiated 60 min after start of stimulation. Cells to be monitored were chosen randomly in 6-12 fields of view containing at least 9 transfected cells. Cells were exposed to the excitation light for 1 min every 20 min during the experiments. The correlation of fluorescence with cells was verified by overlaying the fluorescence and the phase contrast image. For calculation, the fluorescence intensity of an individual cell at the beginning of the experiment (t = 60 min after start of stimulation) was set as 1. The fluorescence intensity of each monitored cell was followed over time up to t = 240 min and calculated relative to its intensity at t = 60 min by using the Isee software for UNIX (Inovision).

4.7 CONFOCAL MICROSCOPY AND CO-LOCALIZATION ANALYSIS

4.7.1 Detection of fluorescence by laser scanning confocal microscopy

Laser scanning confocal microscopy was performed using the Leica TCS SP2 confocal microscope equipped with a Leica HCX PI Apo x63/1.20/0.17 UV objective lens. The following settings were used: for GFP and DsRed fluorescence, excitation at 488 nm (Ar laser) and 543 nm (HeNe laser), a 488/543-nm double dicroic mirror, and detection at 505-525 nm for GFP and 605-670 nm for DsRed; for CFP and YFP detection, excitation at 458 nm for CFP and 514 nm for YFP (Ar laser), a 458/514-nm double dicroic mirror, and detection at 465-495 nm (CFP) and 535-600 nm (YFP). To eliminate fluorophore cross contamination, detection of CFP and YFP was performed using the "between lines" sequential scan mode of the confocal software.

Co-localization of GFP/DsRed and CPF/YFP fluorescence within the plasma membrane was quantified using the 2D scatterplot analysis function of Leica confocal software version 2.5. To exclude signals originating from the cytoplasm or non-cellular sources, the analysis was limited to the plasma membrane by using the "region of interest" feature.

Fluorescence in Ad.βGK-GFP transduced islets was monitored with the following settings: Leica x10 objective lens, excitation at 488 nm (Ar laser), a 488/543-nm double dicroic mirror, and detection at 505-525 nm. After stimulation, the transduced islets were placed into the perifusion chamber and fixed with metallic mesh to stabilize its position during the experiment.

For experiments described in paper III, where confocal microscopy and online monitoring techniques were used to analyze IR-A and IR-B distribution in the β-cell plasma membrane, we tagged the receptor isoforms with monomeric forms of CFP, YFP, GFP (indicated in paper III as mCFP, mYFP, mGFP) and the improved version of DsRed, i.e. DsRed2. These fluorescent protein variants display a reduced aggregate formation compared to their wild type forms [237] and consequently exclude the possibility of artifacts in receptor distribution studies. We continued to employ these variants of fluorescent proteins in paper IV without emphasizing it. For simplicity, the monomeric nature of the fluorescent proteins in the particular chapters of results and discussion (section 5), was not indicated and fluorescent proteins were just termed CFP, YFP, GFP or DsRed.

4.7.2 Fluorescence resonance energy transfer (FRET) analysis

FRET analysis was performed by digital imaging fluorescence microscopy as described in 4.6. The following filter settings were used: for detection of CFP fluorescence, excitation at 435 nm, a 455-nm dicroic mirror, and a 460-500-nm band pass filter; for YFP detection, excitation at 495 nm, a 505-nm dicroic mirror, and a 520-550-nm band pass filter; for detection of the FRET signal, excitation at 435 nm, a 455-nm dicroic mirror, and 520-550-nm band pass filter. The FRET image was generated by linear unmixing as described by [238] using the FRET, CFP, and YFP signals as raw data.

5 RESULTS AND DISCUSSION

5.1 SELECTIVE INSULIN SIGNALING VIA IR-A AND IR-B REGULATES TRANSCRIPTION OF INSULIN AND βGK GENES IN PANCREATIC β-CELLS

5.1.1 Short-term regulation of βGK gene transcription is activated by insulin, secreted upon glucose stimulation

Earlier studies from our group demonstrated that insulin, secreted in response to glucose stimulation, is a major player in the short-term activation of insulin gene transcription. This positive feedback mechanism involves signaling through the IR/PI3K/p70s6k and CaM kinase pathways [211]. Because the insulin promoter and the βGK promoter contain similar *cis* elements [231,239-241], we sought to analyze whether transcription of the βGK gene is regulated by similar or even the same mechanism(s). We found that stimulation with 16.7 mM glucose for 15 min led to an increase in βGK mRNA levels in both islets and insulin-producing HIT-T15 cells (Paper I, Figure 1A), similar compared to the effect of glucose on insulin mRNA levels [211,233]. The half-life time of βGK mRNA was determined as ~60 min and did not change in response to glucose stimulation (Paper I, Figure 1B). Glucose-dependent βGK transcription initiation, measured by an nuclear run-off assay in HIT-T15 cells, was elevated as early as 15 min after start of stimulation and reached its maximum at 30 min (Paper I, Figure 1C). The elevation in βGK transcription initiation was confirmed in stimulated rat islets (Paper I, Figure 1E).

To analyze the molecular mechanisms involved in glucose-dependent transcriptional activation of the βGK gene, we established a reporter gene assay employing the green fluorescent protein (GFP) gene under control of the rat βGK promoter (prβGK.GFP). Here we used the promoter fragment up to nucleotide -278, because this fragment has been shown to contain all *cis* elements responsible for both glucose-dependent and cell type-specific transcriptional control [242]. This enabled us to monitor the increase in fluorescence of GFP as a read-out for βGK promoter activity in HIT-T15 cells, isolated primary β-cells, and intact pancreatic islets after glucose stimulation (Paper I, Figure 1E). In agreement with the nuclear run-off assay, the dynamics of the activation of βGK promoter-driven GFP expression were very similar to those reported for the glucose-stimulated insulin gene promoter [211,233].

To investigate whether glucose metabolism *per se* or secreted insulin is responsible for the up-regulation of βGK gene transcription, the latter being the case for short-term insulin gene transcription [211], we analyzed the effect of insulin secretagogues and a Ca^{2+} channel blocker on βGK mRNA levels in cultured islets. Indeed, stimulation with either 50 mM KCl or 1 μM glibenclamide for 5 min at sub-stimulatory glucose concentrations led to an increase in βGK mRNA levels (Paper I, Figure 2A), while preventing stimulus-induced insulin secretion by blocking L-type Ca^{2+} channels with 10 μM nifedipine abolished up-regulation of βGK mRNA levels (Paper I, Figure 2B). In addition, administration of insulin secretagogues led to an increase in βGK promoter-driven GFP expression in transiently transduced islets (Paper I, Figure 2C). These data indicated that insulin secreted in response to glucose metabolism is a major player in short-term βGK gene transcription.

Addition of 50 μU insulin per ml to fully supplemented culture medium was sufficient to elevate βGK mRNA levels in pancreatic islets (Paper I, Figure 2D). Interestingly, a more detailed analysis of the necessary amounts of exogenous insulin to trigger promoter activity revealed distinct concentrations required for up-regulation of insulin compared to βGK

promoter activation. While 5-10 μU insulin per ml were sufficient to up-regulate insulin transcription, 20 μU per ml were needed to up-regulate βGK transcription (Paper I, Figure 2E).

5.1.2 The signal transduction pathway involved in insulin-stimulated βGK gene transcription differs from that leading to insulin-stimulated insulin gene transcription

Previous studies by our group and others suggested that both the insulin and the βGK promoter can bind the same transcription factors [231,239-240]. This together with the observation that both genes respond positively to the same stimuli (glucose, insulin and secretagogues), led us to postulate that βGK and insulin genes may be regulated by the same signaling pathway. To analyze whether the same protein kinases are involved in the insulin-stimulated up-regulation of the two genes, we combined insulin stimulation with treatment with various pharmacological protein kinase inhibitors on insulin- and βGK promoter-driven GPF expression in islet cells and HIT-T15 cells. Insulin-stimulated insulin promoter activation was sensitive to inhibition of IR tyrosine kinase activity (HNMPA-$(AM)_3$), p70s6k (rapamycin), PI3K (LY294002), and CaMKII (autocamtide-2 related inhibitory peptide), while blocking of PKC (bisindolylmaleimide I), MEK1 (PD98059) or p38/RK/SAPK2a + JNK/SAPK1 (PD169316) had no effect (Paper I, Figure 3A). In contrast, insulin-stimulated βGK promoter activation was only sensitive to inhibition of IR tyrosine kinase activity (Paper I, Figure 3A). These data were confirmed by measuring βGK mRNA levels after treatment with inhibitors of IR tyrosine kinase, CaM kinase II and PI3K (Paper I, Figure 3B). In order to analyze insulin-stimulated up-regulation of both genes in the same cell at the same time, we co-transfected islet cells and HIT-T15 cells with an expression vector encoding insulin promoter-driven DsRed together with an expression vector encoding βGK promoter-driven GFP. Because of the different excitation and emission profiles of DsRed and GFP, this technique allowed us to monitor simultaneously the effect of pharmacological inhibitors on both insulin and βGK promoter activities. Insulin stimulation led to an increase in both insulin promoter-driven DsRed and βGK promoter-driven GFP expression, while treatment of cells with the IR tyrosine kinase inhibitor HNMPA-$(AM)_3$ abolished this effect. βGK promoter activity was not affected by inhibitors of mTOR/p70s6k, CaMKII and PI3K while, in contrast, up-regulation of insulin gene transcription was totally suppressed (Paper I, Figure 3C).
Thus, activation of βGK gene transcription by secreted insulin is regulated via a signaling pathway that is different from that involved in the activation of insulin gene transcription.

Insulin has been shown to activate not only MAPKs and the PI3K/mTOR/p70s6k pathways, but also PKB/c-Akt [243]. 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/ml insulin. Indeed, PKB activation was observed as early as 5 min following glucose stimulation (Paper I, Figure 4A) and 2 min following insulin stimulation (Paper I, Figure 4B). Moreover, transient co-expression of PKBα/c-Akt1 had no effect on insulin-stimulated insulin promoter-driven DsRed expression, while it led to a significant increase in βGK promoter-driven GFP expression (Paper I, Figure 4C). Finally, expression of a dominant-negative acting form of PKB (PKB-CAAX) [276] almost completely abolished insulin-stimulated βGK promoter activity *(Figure 5.1).*

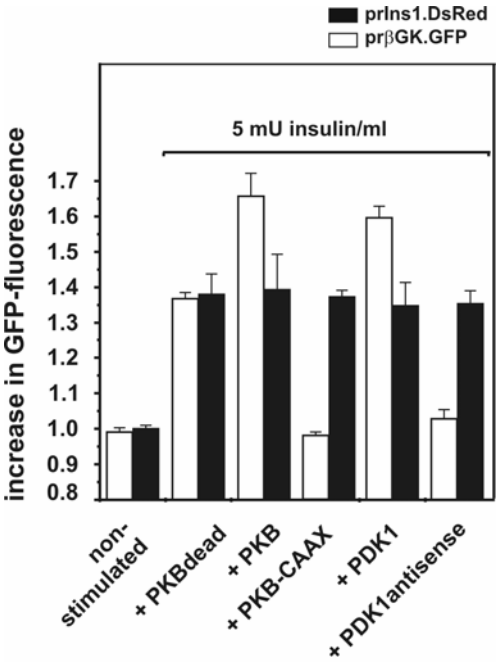

Figure 5.1. Role of PKB and PDK1 in insulin-stimulated insulin and βGK gene transcription.

Insulin-stimulated PKB activity is depending on phosphorylation by phosphatidylinositol-dependent kinase 1 (PDK1) [138]. Transient co-expression of PDK1 led to a pronounced elevation of βGK promoter activity, whereas co-transfection of a PDK1 antisense RNA abolished the stimulatory effect of insulin. In contrast, neither PDK1 nor PDK1 antisense had any effect on insulin-stimulated insulin promoter activity (Paper I, Figure 4C and *Figure 5.1*). Activation of PKB by PDK1 has been shown to be dependent on PI3K activity [138] and is therefore sensitive to the pharmacological PI3K inhibitors LY294002 and wortmannin. Surprisingly, 25 μM LY294002 did not block βGK promoter-driven GFP expression in transfected islet cells, while it completely inhibited insulin promoter-driven DsRed expression (Paper I, Figure 3A). When we analyzed the effect of LY294002 and wortmannin on transfected HIT-T15 cells in a dose-dependent manner, we found that 25 μM LY294002 abolished insulin promoter-driven DsRed expression, while 100 μM LY294002 were needed to inhibit βGK promoter-driven GFP expression (Paper I, Figure 4D). Similarily, 50 nM wortmannin led to a marked decrease in insulin promoter activity, while on the other hand, remarkably higher amounts (150 nM) were necessary to block βGK promoter activity (Paper I, Figure 4E).

We concluded from these data that up-regulation of insulin and βGK gene transcription in the pancreatic β-cell occurs via different insulin-dependent signaling pathways. While the signaling pathway leading to insulin gene transcription is sensitive to PI3K inhibitors at lower concentrations and involves mTOR/p70s6k and CaMKII, signaling leading to the activation of the βGK promoter is less sensitive to PI3K inhibitors and is mediated via PDK1 and PKB.

5.1.3 Insulin signaling via IR-A activates insulin gene transcription whereas signaling via IR-B activates βGK gene transcription

To further investigate the nature of selective signaling leading to insulin-stimulated up-regulation of insulin and βGK gene transcription, we wanted to know whether selectivity might start already at the receptor level, where insulin is bound and the initiating signal is transduced to further downstream intracellular kinases. Pancreatic β-cells express IRs [210,244], insulin receptor related receptor [245], IGF-1R and IGF-IIR [208], all potential targets for the feedback action of insulin. Our experiments, using the IR kinase inhibitor HNMPA-$(AM)_3$ ([211] and Paper I, Figure 3A), suggested that signaling via IR is crucial for insulin-stimulated insulin gene transcription. Consequently, we examined whether the expression of IR *per se* is an absolute requirement for insulin-stimulated insulin and βGK gene transcription.

We analyzed insulin and βGK mRNA levels in isolated pancreatic islets from βIRKO mice in response to stimulation with 16.7 mM glucose or 5 mU/ml insulin. βIRKO mice, which do not

express IR specifically in pancreatic β-cells, have an impaired first phase of glucose-stimulated insulin secretion and a decreased β-cell insulin content, a phenotype similar to that seen in patients with type 2 diabetes [227]. This defect ultimately leads to age-dependent glucose intolerance and, in some mice, to overt diabetes. Interestingly, stimulation with either glucose or insulin led to a remarkable increase in both insulin and βGK mRNA levels in islets from wild type mice, whereas no elevation in insulin and βGK gene transcription was observed in islets prepared from βIRKO mice (Paper I, Figure 5A).
These findings, together with the observation that stimulation of cells with IGF-I did not lead to either insulin or βGK promoter activation, clearly demonstrated that expression of IR in pancreatic β-cells is an absolute requirement for the stimulatory effect of insulin on insulin and βGK gene expression and that signaling via the IGF-1R is unlikely to be involved.
Our group had previously shown that both IR isoforms, IR-A and IR-B, are expressed in insulin-producing cells in an approximately 1:1 ratio [211]. Moreover, we could demonstrate that over-expression of IR-A, but not IR-B, leads to enhanced insulin-stimulated insulin gene transcription [211]. When we transiently co-transfected cells with prIns1.DsRed and prβGK.GFP in combination with either pRcCMV.HIRA or pRcCMV.HIRB, we discovered that βGK promoter activity was only up-regulated in cells over-expressing IR-B (Paper I, Figure 5B). This was supported by the observation that co-transfection of cells with expression constructs encoding the inactive mutants of IR-A and IR-B, i.e. IR-Am or IR-Bm, described as M1153I [246], led to diminished up-regulation of insulin promoter activity in cells expressing IR-Am, while activation of the βGK promoter was abolished in cells expressing IR-Bm (Paper I, Figure 5B). Finally and most convincingly, application of receptor-specific blocking antibodies that inhibit signal transduction via these endogenous receptors, i.e. either both IR isoforms (αIR(AB)) or selectively via IR-B (αIR(B)), confirmed that indeed, activation of insulin gene transcription involves signaling via IR-A, while signaling via IR-B is required to activate βGK gene transcription. As we expected, incubation of cells with an antibody blocking signaling via IGF-1R, did not influence either insulin promoter or βGK promoter activity (Paper I, Figure 5C). Noteworthy, the selective signaling pathways activating insulin gene transcription via IR-A/IRS/PI3K Ia/mTOR/p70s6k and βGK gene transcription via IR-B/PI3K class II/PDK1/PKB are also operative in primary human β-cells [247].

5.2 MOLECULAR MECHANISMS UNDERLYING THE SELECTIVE SIGNALING VIA IR-A AND IR-B

So far our data demonstrated that in pancreatic β-cells insulin activates the transcription of its own gene by signaling via IR-A, IRS, a PI3K activity, mTOR/p70s6k and CaMKII, while activation of the βGK gene transcription involves signaling via the B-type IR, a PI3K activity that is less sensitive to LY294002 and wortmannin, PDK1 and PKB.
We now aimed to understand the different sensitivities towards PI3K inhibitors that we observed in insulin-stimulated insulin versus βGK promoter activation (Paper I, Figure 3A, and 4D,E).

5.2.1 Different classes of PI3K contribute to selectivity in insulin signal transduction via IR-A and IR-B

One interpretation of the different sensitivity towards PI3K inhibitors *in vivo* is the different accessibility of the inhibitor for the same type of PI3K as a result of different localization/compartmentalization of IR-A/PI3K and IR-B/PI3K complexes in the β-cell.

Another interpretation is the involvement of different classes of PI3K, exhibiting different sensitivities for LY294002 and wortmannin, as described for PI3K classes I and III versus PI3K class II (reviewed in [248]).
In order to analyze whether insulin signaling in β-cells that leads to selective activation of insulin and βGK gene transcription involves activation of a PI3K class Ia, we transiently co-transfected cells with prIns1.GFP or prβGK.GFP and an expression construct encoding the dominant-negative form of the adapter protein p85, i.e. Δp85 [249]. Whereas transient expression of Δp85 abolished insulin-stimulated insulin promoter activity, this approach had no effect on insulin-stimulated βGK promoter activity (Paper I, Figure 6C). Thus, these data suggest the involvement of PI3K class Ia in the IR-A-mediated activation of the insulin promoter, while a PI3K activity different to class Ia is required for IR-B-mediated up-regulation of the βGK promoter.

5.2.2 Signaling via IR-A involves a class Ia PI3K leading to up-regulation of the insulin promoter while signaling via IR-B and the PI3K class II member PI3K-C2α up-regulates the βGK promoter

Our data so far demonstrated 1) that IR-B is involved in insulin-stimulated βGK gene transcription, 2) that βGK gene transcription is not sensitive to PI3K inhibitor concentrations that abolish the PI3K class Ia-dependent signal transduction, 3) that expression of Δp85 had no effect on βGK promoter activity, and 4) that βGK promoter activity was increased when PDK1 or PKB were overexpressed and vice versa was decreased when dominant-negative PKB-CAAX or PDK1 antisense were expressed.
To date two classes of mammalian PI3Ks have been identified that can be activated by insulin, i.e. class Ia and class II PI3Ks. The class II PI3K member PI3K-C2α differs from class Ia PI3Ks in its domain structure, substrate specificity and sensitivity towards pharmacological inhibitors [80] illustrated in *Table 4*.

Table 4. Classes of PI3K

	isoforms (subunits)		insulin	substrates	inhibitors
class	catalytic	regulatory	signaling	(*in vitro*)	(*in vitro*)
Class Ia	p110α	p85α, p55α, p50α	+	$PI(4,5)P_2$	
	p110β	p85β	+	PI(4)P	
	p110γ	p50γ	?	PI	
				PI(5)P	
Class Ib	p110γ	p101	-	$PI(4,5)P_2$	Wortmannin
				PI(4)P	(1-10 nM)
				PI	LY294002
Class II	PI3K-C2β		+	PI	(1 μM)
	PI3K-C2γ		?	PI(4)P	
				($PI(4,5)P_2$)	
Class III	Vps34p	p150	?	PI	
					Wortmannin
Class II	PI3K-C2α		+	PI	(50-450 nM)
				PI(4)P	LY294002
					(>20 μM)

Since 150 nM wortmannin and 100 μM LY 294002 were needed to abolish insulin-stimulated βGK promoter activation (Paper I, Figure 4D,E), PI3K-C2α could be involved in this specific signaling pathway. Western blot analysis demonstrated the expression of PI3K-C2α in insulin-producing cells (Paper II, Figure 1). We next sought to examine whether the PI3K activity associated with IR-B exhibited the same or similar sensitivity towards wortmannin as PI3K-C2α. We investigated the wortmannin sensitivity profile of PI3K activities associated with GFP-tagged IR isoforms and compared those with the wortmannin sensitivity profiles of endogenous PI3K-C2α and of PI3K class Ia. We found that the wortmannin sensitivity profile of the PI3K associated with IR-A, i.e. showing inhibition in the lower nanomolar range, was very similar to that obtained in immunoprecipitates of p85, the adapter protein of PI3K class Ia (Paper II, Figure 2A,B). In contrast, the PI3K activity associated with IR-B was inhibited only at higher wortmannin concentrations, exhibiting a similar wortmannin sensitivity profile as that obtained in PI3K-C2α immunoprecipitates (Paper II, Figure 2C,D).

Class Ia and class II PI3K also differ in their preference for lipid substrates in response to insulin. PI3Ks class Ia mainly use PI(4,5)P_2 as their substrate to generate PI(3,4,5)P_3, while class II PI3Ks prefer PI(4)P to produce PI(3,4)P_2 [89]. Identification of the lipid product that is involved in the up-regulation of βGK gene transcription might be a second helpful indicator for the involvement of PI3K-C2α. To identify the lipid kinase product required in this signaling pathway, we transiently transfected HIT-T15 cells with the PI-phosphatases PTEN and SHIP. To ensure the expression of PTEN or SHIP in the same cells that were monitored for βGK and insulin promoter activities, we transfected cells with expression constructs that contained the combination of βGK promoter-driven GFP (or insulin promoter-driven GFP) and the expression cassette for either PTEN or SHIP. PTEN is a 3´-phosphatase that converts PI(3,4,5)P_3 into PI(4,5)P_2 [250], while SHIP is a 5´-phosphatase that dephosphorylates PI(3,4,5)P_3 to PI(3,4)P_2 [251,252]. In agreement with earlier observations by da Silva Xavier et al. [213], over-expression of either PTEN or SHIP led to a drastic reduction in PI3K class Ia-mediated PI(3,4,5)P_3-dependent insulin promoter activity (Paper II, Figure 3). In case of IR-B/PI3K-C2α/PDK1/PKB-mediated activation of βGK gene transcription, we expected over-expression of PTEN to be inhibitory while over-expression of SHIP could have a stimulatory effect. The latter expectation was based on the observation that the SHIP product PI(3,4)P_2 is the preferred lipid for PKB recruitment to the plasma membrane via its PH-domain [253,254]. Indeed, over-expression of PTEN significantly decreased insulin-stimulated βGK promoter activity, while over-expression of SHIP, to our surprise, did not lead to an additional increase (Paper II, Figure 3). We concluded from this data, that the PI3K-C2α product PI(3,4)P_2, although involved in insulin-stimulated up-regulation of βGK gene transcription via IR-B/PI3K-C2α/PDK1/PKB, may not be the rate-limiting factor in this signaling cascade. Another possible explanation is that the additional production of PI(3,4)P_2 by SHIP takes place in a membrane micro-domain that does not contribute to the specific signaling pathway leading to βGK promoter activation.

Finally, to verify the involvement of PI3K-C2α, we examined whether selective down-regulation of PI3K-C2α interferes with insulin-stimulated βGK promoter activation via IR-B. Cells were co-transfected with an expression vector encoding both, insulin promoter-driven DsRed and βGK promoter-driven GFP, together with a plasmid containing an antisense construct for PI3K-C2α. While insulin-stimulated insulin promoter activity was not affected, here serving as a control, βGK promoter activity was clearly down-regulated (Paper II, Figure 4). This approach convincingly demonstrated that in the pancreatic β-cell insulin-stimulated βGK gene transcription requires activity of PI3K-C2α.

5.2.3 Up-regulation of the βGK promoter via IR-B involves the NPEY-motif in the juxtamembrane region of the receptor

While our previous studies suggested the involvement of IRS-2 in insulin-stimulated insulin gene transcription [211], thus implicating the NPEY-motif in the cytoplasmic juxtamembrane region of IR-A, the domain of IR-B responsible for activating PI3K-C2α remained unclear. The NPEY-motif is described to recruit adapter proteins possessing a PTB domain, such as IRS proteins [255-258], which bind and phosphorylate members of the regulatory subunit of class Ia PI3K and stimulate PI3K activity. On the other hand, the YTHM-motif at the intracellular C-terminus has been shown to directly interact and activate class Ia PI3Ks [37,38,259] (see 2.1). Interestingly, expression of FLAG- or GFP-tagged IR-B variants, lacking the last C-terminal 23 amino acids and therefore the YTHM-motif, led to the same additional increase in βGK promoter activity as obtained with full-length IR-B (Paper I, Figure 5B; Paper II, Figure 5A, Paper III, Figure 3A,B). These data suggest the involvement of the juxtamembrane NPEY-motif, rather than the C-terminal YTHM-motif, in this specific signaling cascade. This is further corroborated by the observation that expression of a full-length IR-B variant bearing a mutation in the YTHM-motif allowed the same additional increase in βGK promoter activation as wild type IR-B (Paper IV, Figure 5A). To test whether the NPEY-motif is required for the recruitment of PI3K-C2α, we studied the increase in βGK promoter-driven GFP expression in β-cells co-transfected with FLAG-tagged IR-B wild type or a FLAG-tagged IR-B variant bearing a mutation in the NPEY-motif, i.e. NPEF. Expression of FLAG-tagged wild type IR-B led to a pronounced increase of βGK promoter activity while, in contrast, expression of the NPEF-mutant did not (Paper II, Figure 5B, Paper IV, Figure 5A). These results identified the NPEY-motif of IR-B being crucial in insulin-stimulated βGK gene transcription. Finally, we examined whether PI3K-C2α is directly associated with IR-B and performed a Western blot analysis with an anti-PI3K-C2α antibody in immunoprecipitates of over-expressed FLAG-tagged IR-A or IR-B. We detected a weak PI3K-C2α-signal in the IR-B- but no signal in the IR-A-immunoprecipitate (Paper II, Figure 6).

In conclusion, our data suggest that insulin-stimulated activation of the βGK gene transcription requires signaling via the B-type IR, here involving the juxtamembrane NPEY-motif, association of PI3K-C2α and recruitment of PDK1 and PKB *(Figure 5.2)*. Thus, we demonstrated that different classes of PI3K contribute to selectivity in insulin signaling and, hence, insulin action.

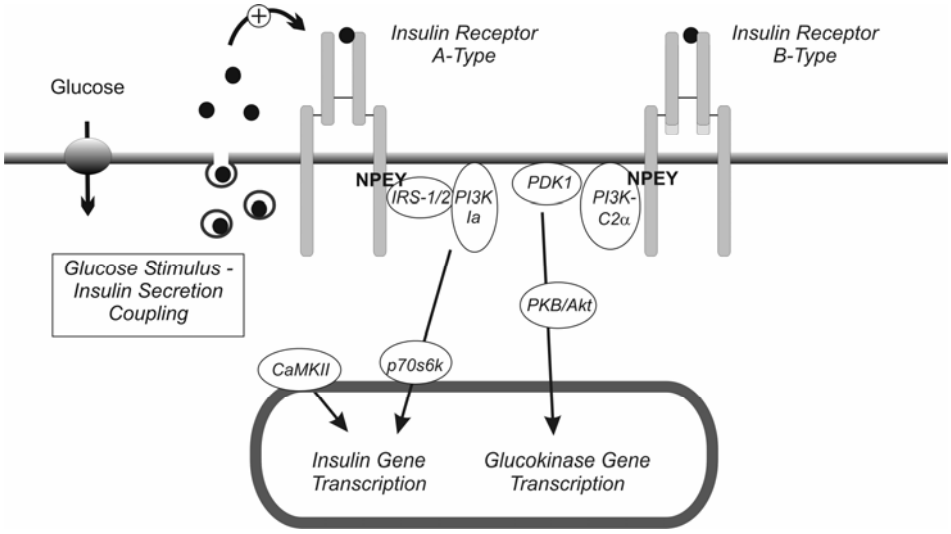

Figure 5.2. Selective activation of insulin and βGK gene transcription by selective signaling via IR-A and IR-B.

5.3 ISOFORM-SPECIFIC IR SIGNALING INVOLVES DIFFERENT PLASMA MEMBRANE DOMAINS

5.3.1 Insulin-stimulated up-regulation of insulin promoter via IR-A and βGK promoter via IR-B is not dependent on IR internalization

In order to analyze whether activation of different signaling cascades downstream of IR-A and IR-B is dependent on different internalization and recycling pattern of the two IR isoforms [173,184], we examined whether endocytosis of the two receptor isoforms is required. To test whether internalization of IR-A or IR-B is specifically necessary for the activation of insulin and βGK gene transcription, respectively, we studied insulin-dependent promoter activation in β-cells that were transiently transfected with the dominant-negative mutant of dynamin, i.e. the K44A-mutant that prevents clathrin-dependent endocytosis [260,261]. We found that neither the activation of the insulin promoter nor the activation of the βGK promoter was affected by the expression of the interfering dynamin mutant (Paper III, Figure 1A), indicating that the signals responsible for the insulin-dependent up-regulation of the two genes originate from membrane-standing IRs rather than from internalized receptor complexes.

Next we sought to analyze whether a different localization of the two insulin receptor isoforms within the β-cell plasma membrane, and consequently the access to different adapter and effector proteins, might explain the difference in IR isoform-specific activation of the insulin and the βGK genes. We tagged both receptor isoforms with GFP and DsRed at the C-terminus of the β-subunit (lacking the last 23 amino acids) and examined their plasma membrane distribution in co-expression studies. Tagging both isoforms did not interfere with their physiological function, e.g. over-expression of tagged IR isoforms led to a pronounced insulin effect on the respective promoter activity to the same extent as the untagged IR (Paper II, Figure 5A), and allowed co-immunoprecipitation of associated PI3Ks (Paper I, Figure 6B; Paper II, Figure 2). Whereas transient co-expression of the same, but differently tagged (IR-A~DsRed/IR-A~GFP and IR-B~DsRed/IR-B~GFP), receptor isoform led to a high degree of co-localization, co-expression of 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 complexes that are not co-localized. We observed this pattern of distinct distribution in insulin-producing HIT-T15 cells (Paper I, Figure 6A), INS1 and MIN6 cells, as well as in non-insulin-producing HEK293 and COS7 cells.

These data were confirmed by studies where both isoforms were tagged with monomeric forms of CFP and YFP, which exclude potential artifacts in receptor distribution resulting from aggregate formation. Co-expression of differently tagged receptors of the same isoform led to a high degree (>70%) of co-localization (Paper III, Figure 2Aa,b), while co-expression of differently tagged IR-A and IR-B showed areas in the plasma membrane where the two isoforms did not co-localize (~40% co-localization in total) (Paper III, Figure 2Ac).

5.3.2 Different function and localization of the IR isoforms depend on the 12 amino acids encoded by exon 11

The two isoforms of the IR are the result of alternative RNA splicing [11]. They either lack (IR-A) or contain (IR-B) the 12 amino acids encoded by exon 11, which are located at the C-terminus of the extracellular α-chain of the receptor (see 2.1 and 2.4). To test whether these 12 amino acids are responsible for the differences in localization and function of the IR isoforms, we generated a series of deletion mutants of IR-B by successive shortening the 12-amino acid

string from the C-terminal side. Because the α-chain of both isoforms ends with the identical 4-amino acid string PRPS, which directly flanks the proteolytical processing site of the pro-receptor, we decided to successively eliminate 10 amino acids in front of these 4 amino acids, thus generating deletion mutants Δ1-Δ10 (*Table 1* in 2.4). Over-expression of IR-A, IR-B and the intermediates Δ1-Δ10 in cells co-expressing insulin promoter-driven DsRed/βGK promoter-driven GFP revealed that IR-B, Δ1 and Δ2 led to an enhanced activity of the βGK promoter but not of the insulin promoter in response to insulin stimulation, whereas Δ7-Δ10 and IR-A exhibited a pronounced activation of the insulin promoter but no further activation of the βGK promoter (Paper III, Figure 3A). Interestingly, expression of receptor mutants Δ3-Δ6 interfered with insulin-dependent up-regulation of both the insulin and βGK promoter in a dominant-negative way, decreasing promoter activities below the level of mock-transfected cells (Paper III, Figure 3A).
To test whether function of the receptor can be related to its localization, we tagged mutants Δ2, Δ3 and Δ6, Δ7 with GFP and co-expressed them with their closest wild type counterpart, i.e. IR-B-DsRed with Δ2-GFP and Δ3-GFP and IR-A-DsRed with Δ6-GFP and Δ7-GFP. While the biologically active mutants Δ2 and Δ7 co-localized with IR-B and IR-A, respectively, to the same degree as full-length receptors (Paper III, Figure 4Aa,d), the non-active mutants Δ3 and Δ6 showed a much lower degree of co-localization with their respective wild type receptor counterparts (Paper III, Figure 4Ab,c).
Taken together these data show that the 12 amino acids encoded by exon 11 are responsible for both different localization and function of the two IR isoforms.

5.3.3 Insulin-stimulated up-regulation of insulin and βGK gene transcription involves signaling through IR isoforms located in different membrane micro-domains but is not dependent on caveolin

One possible explanation for the different localization of the two IR isoforms in the β-cell plasma membrane and, hence, their different accessibility for subsequent adapter and effector proteins linking the insulin signal with specific biological functions might be their residence in specific micro-domains within the plasma membrane. IR-mediated signaling has been discussed in the context of lipid rafts, i.e. cholesterol-enriched membrane micro-domains (reviewed in [262]), however, without discriminating the IR isoform. To elucidate whether the two IR isoforms are located within cholesterol-enriched plasma membrane domains, we transfected β-cells with expression vectors encoding either IR-A-YFP or IR-B-YFP together with Myr-Palm-CFP. Myr-Palm-CFP is a monomeric CFP variant fused with an amino acid string that allows lipid modification by myristoylation/palmitoylation and results in the localization of Myr-Palm-CFP in cholesterol-enriched membrane domains [237]. Expression of both combinations, i.e., IR-A-YFP/Myr-Palm-CFP and IR-B-YFP/Myr-Palm-CFP, resulted in a high degree of co-localization, suggesting that both, IR-A and IR-B, are mostly located within cholesterol-enriched membrane domains (Paper III, Figure 6).
We next examined whether IR isoform specific function is differently sensitive towards cholesterol depletion. Cholesterol depletion has been shown to inhibit lipid raft-dependent signaling in general [263] and IR-mediated signaling in particular [264-266]. When we treated β-cells, transiently transfected with prIns1.DsRed/prβGK.GFP, with different amounts of β-cyclodextrin as a tool to gradually deplete cholesterol from the plasma membrane, we observed that the two promoters are to a different extent sensitive towards cholesterol-depletion (Paper III, Figure 7A). This led us to hypothesize that the two plasma membrane-standing IR

isoforms that activate these specific signal transduction pathways reside in membrane micro-domains with distinct cholesterol composition.
Caveolae represent a subgroup of lipid raft micro-domains that are, besides rich in glycosphingolipids and cholesterol, enriched in caveolin-1, -2 and/or –3 [263,267,268]. Studies, mostly performed in adipocytes/preadipocytes, contradictorily describe caveolins to be directly, indirectly or not involved in IR-dependent signaling (reviewed in [262]).
To examine whether caveolins are involved in selective signaling via the two IR isoforms in the pancreatic β-cell, we first investigated the expression of caveolins in rat and mouse islets. RT-PCR analysis as well as Western blot analysis revealed that caveolin-1α, caveolin-1β and caveolin-2, but not caveolin-3, are expressed in mouse and rat pancreatic islets (Paper III, Figure 8A,B). However, co-transfection of β-cells with expression vectors encoding wild type caveolin-1α, -1β or -2, the combinations caveolin-1α + caveolin-2 or caveolin-1β + caveolin-2, respectively, and both insulin promoter-driven DsRed and βGK promoter-driven GFP had no effect on either promoter (Paper III, Figure 9A). These data suggest that, although present in the β-cell, caveolins do not seem to be involved in either IR-A-dependent up-regulation of the insulin gene or in IR-B-dependent βGK promoter activation. This, on the other hand, does not exclude the localization of IRs in caveolin-containing lipid domains, but makes a direct/indirect action of caveolins themselves in these specific signaling pathways unlikely. Moreover, caveolae only represent a subset of lipid rafts. The involvement of non-caveolin-containing lipid rafts in IR-mediated signaling has been shown in human HuH7 hepatoma cells that express IRs but lack caveolae [266]. Further studies in pancreatic β-cells should be performed to clarify the effect of these lipid rafts on selective IR isoform-dependent biological responses.
In summery, our data showed that IR-A-dependent up-regulation of the insulin gene and IR-B-dependent up-regulation of the βGK gene results from IRs located in distinct plasma membrane micro-domains, which might mechanistically explain the access to different adapter and effector proteins (e.g. different classes of PI3Ks) and the subsequent activation of selective signaling pathways leading to specific downstream responses.

5.4 SPATIAL SEGREGATION OF IR-B-TYPE SIGNALING ALLOWS SELECTIVE AND SIMULTANEOUS ACTIVATION OF βGK AND C-FOS GENES IN THE PANCREATIC β-CELL

Activation of the insulin gene and the βGK gene transcription units upon insulin stimulation exemplify β-cell-specific responses within the ‘metabolic’ branch of insulin signal transduction. To explore in more detail how insulin activates the ‘mitogenic’ branch in insulin signaling, i.e. signal transduction via the MAPK cascade, we employed the transcriptional regulation of the proto-oncogene c-fos as the functional read-out. c-fos has been shown to be activated by insulin in a variety of tissues and cell types [2]. Here, the involvement of MAPKs ERK1/2, p38/SAPK2a and JNK/SAPK1, has been discussed. Although the later steps in insulin-dependent c-fos gene activation have been studied in great detail, the molecular mechanisms of the early events, starting with the nature of the IR involved, remain poorly understood.
Our data so far have shown that one possibility to obtain selectivity in insulin action in the pancreatic β-cell is the utilization of signal transduction via the two IR isoforms, the A- and the B-type, situated in plasma membrane domains differently sensitive to cholesterol depletion. While signaling through IR-A and IRS/PI3K Ia/p70s6k activates transcription of the insulin gene, signaling through IR-B and PI3K-C2α//PDK1/PKB is required to activate the βGK gene

(Paper I,II,III). Therefore, compartmentalization of receptor isoforms with the subsequent access to a defined pool of adaptor/effector proteins may represent one mechanistic basis for selective signaling. Activation of the c-fos gene by insulin has to be initiated by signaling via either IR-A or IR-B. In studying how insulin-dependent c-fos promoter activation differs from either insulin (via IR-A) or βGK (via IR-B) gene up-regulation therefore allowed us to address the exciting and general question how selectivity in signal transduction is achieved when signaling is started utilizing the same receptor isoform, most probably situated in the same plasma membrane micro-domain.

5.4.1 Insulin-stimulated activation of c-fos gene transcription in pancreatic β-cells involves signaling through MEK1/ERK1/2 and the SRE of the c-fos promoter

In order to verify that c-fos gene transcription can be activated in pancreatic β-cells by insulin, we examined both endogenous c-fos mRNA levels as well as c-fos promoter activity. Semi-quantitative RT-PCR analysis of β-cells showed a more than 2-fold increase in c-fos mRNA levels 30 min after start of insulin stimulation (Paper IV, Figure 1A). To examine the activation of the c-fos promoter in INS1 cells and primary β-cells, we transiently transfected cells with an expression vector containing the expression cassette of the human c-fos promoter fused to GFP. In studies on transfected islet cells, β-cells were identified by insulin promoter-driven DsRed expression before monitoring c-fos promoter-driven GFP expression. Because insulin led to an up-regulation of c-fos promoter activity in both INS1 cells and in primary β-cells, this excluded the effect to be a 'cell line phenomenon'.

To investigate which of the MAP kinases is involved in insulin-stimulated c-fos promoter activation in β-cells, we studied the effect of pharmacological inhibitors of ERK1/2 kinase MEK1 (PD98059), p38 (SB203580) and JNK (SP600125). INS1 cells were transfected with a vector encoding both c-fos promoter-driven DsRed and βGK promoter-driven GFP (c-fos.DsRed/βGK.GFP). This allowed us to directly compare the influence of the inhibitors on IR-mediated activation of the two promoters in the same cell at the same time. In agreement with our earlier data (Paper I, Figure 3A), βGK promoter activation was not sensitive to inhibition of MEK1, p38 and JNK. However, insulin-stimulated c-fos promoter-driven DsRed expression was almost completely abrogated in cells treated with the inhibitor of MEK1, but was not affected by inhibitors of p38 and JNK (Paper IV, Figure 2A). Similarly, c-fos promoter activation in primary mouse β-cells was abolished by treatment with the MEK1-inhibitor, suggesting the direct involvement of ERK1/2 in insulin-stimulated c-fos transcription in pancreatic β-cells (Paper IV, Figure 2B).

The serum response element (SRE) of the c-fos promoter is responsible for serum and growth factor dependent transcriptional activation of c-fos via the MAPK cascade [269]. ERK1/2 activates the ternary transcription factor complex bound to the SRE [270]. Mutation of the SRE-motif inhibits formation of the transcription factor complex and, hence, serum- and growth factor-induced transcription of the c-fos gene [269,271]. To test whether activation of the c-fos promoter by insulin signaling via ERK1/2 requires the integrity of the SRE, we transiently transfected INS1 cells with either c-fos promoter-driven DsRed (wild type) or SRE-mutated (SRE-KO) c-fos promoter-driven DsRed and βGK promoter-driven GFP. SRE-KO abolished the insulin-dependent up-regulation of the mutant c-fos promoter compared to the wild type promoter (Paper IV, Figure 2C). βGK promoter-driven GFP expression was not affected by SRE-KO, and operated as the internal control (data not shown).

We conclude from these data that insulin stimulates c-fos gene transcription in the pancreatic β-cell by signaling via MEK1/ERK1/2 and activation of transcription through the SRE of the c-fos promoter.

5.4.2 Insulin stimulated activation of c-fos gene transcription involves IR-B

Mitogenic signaling via MAPK is described to involve mainly IGF-I receptors (IGF-1R) or, in case of insulin receptors, IR-A rather than the B-isoform.
We therefore sought to analyze whether insulin-stimulated c-fos gene transcription involves, as anticipated, the IR-A isoform and/or IGF-1R, or signal transduction via the IR-B isoform. We assessed the roles of IR isoforms and IGF-1R in INS1 cells, transiently transfected with c-fos promoter-driven GFP, by utilizing receptor-specific antibodies, blocking signal transduction through either IGF-1R (αIGF1R), both IR-A and IR-B (αIR(AB)) or through only IR-B (αIR(B)).
Treatment with αIGF1R did not affect insulin-stimulated c-fos promoter-driven GFP expression while blocking of signal transduction through both IR isoforms abolished c-fos promoter activation. Most interestingly and unexpectedly, application of the B-type receptor-specific antibody completely inhibited c-fos promoter activation (Paper IV, Figure 3A), suggesting activation of the c-fos promoter by signaling through IR-B. These results were confirmed in experiments performed on mouse islet cells, thus demonstrating the involvement of IR-B in primary β-cells (Paper IV, Figure 3B). To further corroborate these data, we investigated the effect of transiently overexpressed IR-A and IR-B on c-fos promoter activation. While over-expression of IR-B led to a pronounced insulin effect on c-fos promoter-driven GFP expression, over-expression of IR-A had no effect (Paper IV, Figure 3C). In all experiments either insulin promoter-driven (via IR-A) or βGK promoter-driven (via IR-B) reporter gene expression served as an internal control, verifying responsiveness of the cells/islets to insulin.
Taken together these data demonstrate that in pancreatic β-cells insulin-stimulated activation of the proto-oncogene c-fos is regulated by signaling via the IR-B isoform and the MEK1/ERK1/2 cascade.

5.4.3 Activation of c-fos gene transcription through IR-B involves p52-Shc and the C-terminal YTHM-motif of IR-B

As mentioned above (see 2.1 and 5.2.3), autophosphorylation of the tyrosine residue in the juxtamembrane NPEY-motif generates a recognition sequence for PTB-containing proteins such as IRS-proteins and Shc. In the C-terminus, autophosphorylation of the YTHM-motif creates a recognition site, which has been shown to recruit SH2 domain-containing proteins, e.g. the p85 regulatory subunit of PI3K. The adapter protein Shc, and especially the p52-isoform, has been described to be one of the major players involved in the activation of the MAPK cascade in response to insulin [272]. Once it associates with the IR, p52-Shc is phosphorylated on three tyrosine residues (Y_{239}, Y_{240} and Y_{313}) thus creating docking sites for the Grb2/Sos complex and, hence, further signal transduction via Ras/Raf and the MAPK cascade (see 2.2.2). Western blot analysis demonstrated the expression of the ubiquitously expressed isoforms p46, p52 and p66 [272] in insulin-producing cell lines as well as in islet cells of normoglycemic ob/ob mice (Paper IV, Figure 4A). In order to test whether signal transduction through p52-Shc is required for up-regulation of c-fos promoter activity in pancreatic β-cells, we analyzed the effect of transiently co-expressed wild type p52-Shc (WT-Shc) or a dominant-interfering variant of p52-Shc (DN-Shc). Over-expression of WT-Shc led to a further increase in c-fos promoter

activity in response to insulin compared to mock-transfected cells, while expression of DN-Shc almost completely inhibited c-fos promoter-driven GFP expression (Paper IV, Figure 4A).
We next wanted to examine whether the NPEY-motif of IR-B is involved in the p52-Shc/MEK1/ERK-pathway leading to up-regulation of c-fos gene transcription. Therefore, we co-transfected INS1 cells with c-fos.DsRed/βGK.GFP and with an expression vector encoding either wild type IR-B or an IR-B variant bearing a mutation in the NPEY-motif, i.e. IR-B-NPEF. Interestingly, expression of the NPEF-mutant had no inhibitory effect on insulin-stimulated c-fos promoter activation while, as expected, it inhibited further activation of the βGK promoter (Paper II, Figure 5B). This makes the involvement of the NPEY-motif for direct or indirect (in complex with IRS-1/Grb2/Sos [273]) recruitment of p52-Shc in this particular pathway very unlikely.
An alternative way for p52-Shc-binding to the IR is the direct association of p52-Shc via its C-terminal SH2 domain to the YTHM-motif, although this possibility is controversially discussed [40,63]. Indeed, when we expressed an IR-variant with a mutated YTHM-motif, i.e. the FTHM-mutant, we observed a significant decrease in c-fos promoter activation, while the pronounced activation of the βGK promoter was not affected (Paper IV, Figure 5A). We next performed immunoprecipitation studies to determine a possible direct interaction of p52-Shc with the YTHM-motif. Western blot analysis of the IR-immunoprecipitates with an anti-Shc antibody showed an increase of p52-Shc-binding to wild type IR-B in response to insulin stimulation, whereas no binding of p52-Shc to the FTHM-mutant could be detected (Paper IV, Figure 5B).
These data suggest the involvement of p52-Shc in insulin-stimulated c-fos gene transcription via the IR-B isoform, and more specifically, the recruitment of p52-Shc by the C-terminal YTHM-motif of IR-B.

5.4.4 Activation of c-fos gene transcription via IR-B/p52-Shc requires a PI3K class Ia activity

Recent data by Ugi et al. [274] show that insulin-stimulated tyrosine phosphorylation of p52-Shc in 3T3-L1 adipocytes is sensitive to the PI3K inhibitor wortmannin or to over-expression of a dominant-negative PI3K mutant. Therefore we had to consider that impaired co-immunoprecipitation of p52-Shc with the FTHM-mutant of IR-B may be secondary to a potentially abrogated PI3K activation via the YTHM-motif [37,38]. Because the PTB domain of p52-Shc may also function as a PH domain and, thus, can interact with PI(3,4,5)P_3 [275], a PI3K activity might be necessary to recruit p52-Shc to the plasma membrane in close proximity to the IR where it then becomes tyrosine-phosphorylated by the IR. In order to analyze whether the p52-Shc-mediated activation of c-fos gene transcription requires a PI3K activity, we transiently transfected β-cells with the vector containing the expression cassettes for c-fos promoter-driven DsRed and βGK promoter-driven GFP (c-fos.DsRed/βGK.GFP) and treated the cells prior to and throughout stimulation with the PI3K inhibitor wortmannin. Application of 100 nM wortmannin totally abolished insulin-stimulated c-fos promoter activity both in INS1 cells (Paper IV, Figure 6A) as well as in mouse islets cells (Paper IV, Figure 6B). Inhibition at a concentration of 100 nM wortmannin indicated the involvement of a class Ia-PI3K in this particular pathway while, as expected, this concentration was not sufficient to inhibit insulin-stimulated βGK promoter activity in the same cell (see 5.2.2).
To test whether the involved PI3K activity originates from a PI3K Ia directly bound to the YTHM-motif in the C-terminal region of IR-B, we performed Western blot analysis of associated p85 in FLAG-immunoprecipitates of wild type IR-B, the FTHM-mutant and the

NPEF-mutant of IR-B. Western blotting with an anti-p85 antibody clearly showed a strong band migrating at ~85 kDa as early as 1 min following stimulation, indicating a co-immunoprecipitation of the adapter protein p85, rather than p55 or p50, with wild type IR-B (Paper IV, Figure 6C). This band was much less intense in co-immunoprecipitates of IR-B-FTHM and IR-B-YTHM *(Figure 5.3).*

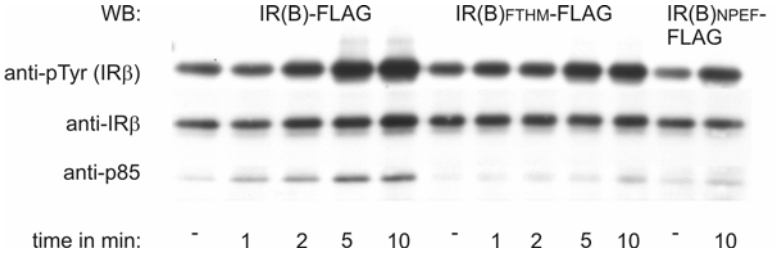

Figure 5.3. Western blot analysis of p85 co-immuno-precipitated with either wild type IR-B, the FTHM-mutant or the NPEF-mutant of IR-B.

Expression of the dominant-negative form of PI3K class Ia adapter protein p85 (Δp85) completely abolished insulin-stimulated c-fos promoter-driven DsRed expression, while up-regulation of βGK gene transcription in the same cell was not affected (Paper IV, Figure 6D). Finally, expression of the Shc-S154P PTB/PH-mutant did not allow the pronounced elevation in c-fos promoter activity seen in cells overexpressing p52-Shc but, most surprisingly, reduced c-fos promoter activity in a dominant-negative way to almost non-stimulated levels (Paper IV, Figure 6E). A reasonable explanation for the observed effect is that Shc-S154P still binds via its SH2 domain to the IR but, because of the mutation of its PTB/PH-domain and the therefore lacking interaction with PI(3,4,5)P_3 in the plasma membrane, does not become activated by the IR and, hence, interferes with signaling by competing for IR interaction with endogenous Shc molecules. If this is to be true, then mutation of the SH2 domain, i.e. R397L [256], in Shc-S154P should abolish its interaction with the IR and thus eliminate its dominant-negative effect. Indeed, expression of the double mutant Shc-S154P,R397L, where both the PTB/PH- and the SH2 domain were disrupted, resulted in a c-fos promoter activation similar to that observed in mock-transfected cells (Paper IV, Figure 6E).
Taken together, these data suggest that a PI3K Ia activity is needed to recruit p52-Shc to IR-B and to allow further signal transduction to activate the c-fos promoter. This is consistent with the two-step mechanism proposed in [274], where the generation of plasma membrane PI(3,4,5)P_3 by activated PI3K leads to the recruitment of p52-Shc to the plasma membrane via its PTB/PH domain and subsequent tyrosine-phosphorylation by the IR. More specifically, in case of IR-B/p52-Shc-mediated activation of c-fos, we suggest that the PI3K Ia activity allows recruitment of p52-Shc to the membrane in close proximity to IR-B. Both, binding of p52-Shc to PI(3,4,5)P_3 via its PTB/PH domain and to the YTHM-motif of IR-B via its SH2-domain result in a conformation of p52-Shc that allows insulin-dependent activation of c-fos transcription via IR-B/p52-Shc/MEK1/ERK1/2.

5.4.5 Insulin-stimulated c-fos gene transcription requires endocytosis of IR-B while βGK gene transcription is activated by signaling via the membrane-standing IR-B

In chapter 5.3 we described that in the pancreatic β-cell selectivity in insulin signaling can be gained by signal transduction through the two isoforms of the IR which are situated in different plasma membrane micro-domains. While signaling through IR-A and IRS/PI3K Ia/p70s6k activates transcription of the insulin gene, signaling through IR-B and PI3K-C2α/PDK1/PKB is

required to activate the βGK gene. Therefore, compartmentalization of receptor isoforms with the subsequent access to a defined pool of adaptor/effector proteins may represent one mechanistic basis for selective signaling. But how can selectivity be achieved when signal transduction involves the same isoform of the receptor, situated in the same plasma membrane micro-domain? To investigate whether the signaling pathways leading to up-regulation of the βGK and the c-fos promoters via IR-B originate from receptor complexes located in the same or different membrane compartments, we referred to β-cyclodextrin as a tool to differentially deplete cholesterol from plasma membrane domains (as described in 5.3.3) and thereby interfere with IR-B signaling. However, we observed no differences in cholesterol dependency with regard to IR-B mediated activation of βGK respective c-fos promoters in response to insulin (not shown). An alternative to explain the activation of different signaling cascades by IR-B is that these cascades are activated in different cellular compartments, i.e. plasma membrane-standing versus internalized receptor complexes. Ceresa and co-worker demonstrated that the dominant-interfering mutant of dynamin, i.e. dynamin-K44A, abolishes clathrin-dependent endocytosis of IRs, which leads to a reduction in insulin-meditated Shc tyrosine-phosphorylation in HII4E cells [260]. Although we have shown in 5.3.1 that transient over-expression of dynamin-2K44A had no inhibitory effect on either insulin-stimulated insulin promoter activity (via IR-A) or insulin-stimulated βGK promoter activity (via IR-B), we intended to analyze whether internalization of IR-B is required for insulin-dependent activation of c-fos gene transcription. We found that expression of dynamin-2K44A abolished insulin-stimulated c-fos promoter activation, while it did not affect up-regulation of the βGK promoter by insulin in the same cell (Paper IV, Figure 7A). To corroborate these data more specifically, we expressed an IR mutant where the endocytosis-responsible motif GPLY in the juxtamembrane region of IR-B was mutated to APLA ($IR(B)_{APLA}$-FLAG). This mutant is biologically active but shows a drastically reduced endocytosis [33]. Expression of this mutant abolished further up-regulation of the c-fos promoter by insulin while it allowed full activation of the βGK promoter (Paper IV, Figure 7B). This demonstrates, that in contrast to the βGK promoter, insulin-stimulated c-fos promoter activation is dependent on clathrin-mediated endocytosis. In conclusion, our data demonstrate that selectivity in insulin signaling via the same receptor isoform (IR-B) can be gained by signal transduction from different cellular compartments, i.e. plasma membrane-standing versus internalized receptors *(Figure 5.4).* Thus, our data allow a mechanistic understanding of how selective signaling in the same cell via the same receptor isoform simultaneously activates different signaling pathways in response to the same stimulus, here exemplified by insulin.

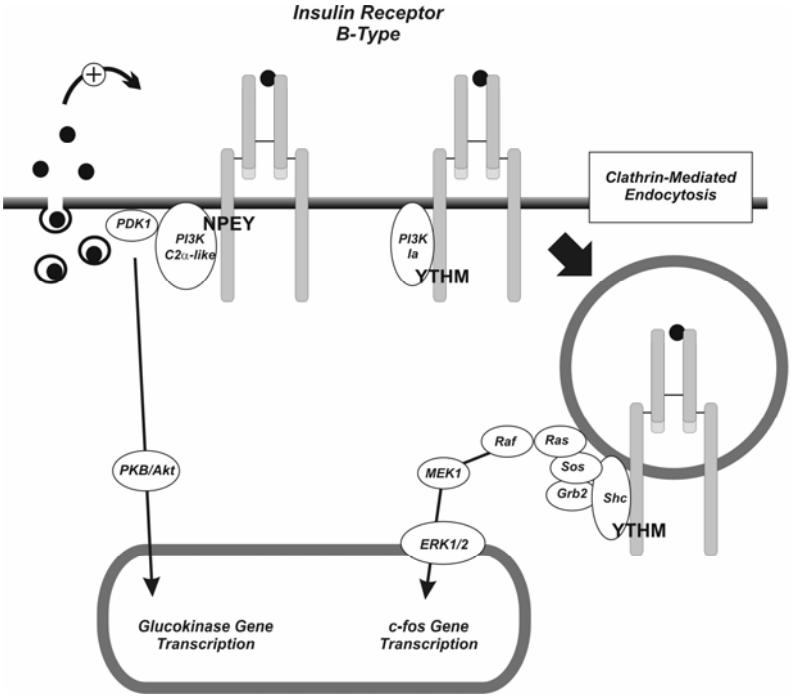

Figure 5.4. Spatial segregation of IR-B in different cellular compartments allows selective activation of βGK and c-fos gene transcription.

6 GENERAL REMARKS AND FUTURE PERSPECTIVES

The data of this thesis work cearly show that the IR isoforms IR-A and IR-B contribute to the variety of mechanisms that allow simultaneous and selective insulin effects in the same cell, here exemplified by the insulin-producing pancreatic β-cell.

In addition to the findings presented and discussed in the previous section (section 5), the following observations are interesting and therefore worthwile to address in future work.

One of the exciting findings was the localization of IR-A and IR-B in different plasma membrane micro-domains leading to different function (Papers I and III), which is dependent on the 12 amino acid string encoded by exon 11. Interestingly, as reported in Paper I, this difference in IR isoform distribution was not restricted to insulin-producing cells (HIT-T15, MIN6, INS1) but was also observed in non-insulin-producing cells such as HEK293 and COS7. To test whether the simple presence of 12 amino acids, acting as a 'spacer', results in the different, isoform-specific localization/function, we mutated amino acids S_{721} and D_{727} within the 12 amino acid string of IR-B to valine, thus generating IR-Bm. Expression of IR-Bm did not allow the pronounced βGK promoter activation as seen with wild type IR-B (Paper III, Figure 3B). Moreover, localization analysis of expressed GFP-tagged IR-A and IR-B mutants with a below the juxtamembrane region truncated β-subunit, thus missing the last C-terminal 380 amino acids (i.e. IR-$A_{\Delta 380}$-GFP and IR-$B_{\Delta 380}$-GFP), revealed a high degree of co-localization when co-expressed with their DsRed-tagged wild type counterpart. In contrast, co-expression of IR-$A_{\Delta 380}$-GFP together with IR-$B_{\Delta 380}$-DsRed resulted in a low degree of co-localization (Paper III, Figure 5). The observation that mutation of two amino acids within the 12 amino acid string results in both loss of function and shift in localization in the plasma membrane (Paper III, Figures 3B and 4A) does not only support the view that the function of the IR is linked to localization, but also suggests that these 12 amino acids do not simply function as a physical 'spacer' but may represent a specific protein motif. This, together with the data obtained with the C-terminally truncated IR isoforms IR-$A_{\Delta 380}$ and IR-$B_{\Delta 380}$, implies that the 12 amino acids encoded by exon 11 represent a signal involved in the IR isoform-specific sorting of the receptor to different plasma membrane micro-domains.

The second finding, which may have broader implications, concerns the question of hybrid receptors. As demonstrated in Paper III (Figure 2), co-expression of CFP/YFP-tagged receptor variants allowed the evaluation of hybrid formation of IR isoforms (i.e. IR-A/IR-A, IR-B/IR-B, IR-A/IR-B) by fluorescence resonance energy transfer (FRET). Interestingly, FRET was only observed in cells where differently tagged receptors of the same isoform had been expressed (Paper III, Figure 2Ad,e). In contrast, cells co-expressing differently tagged IR-A and IR-B did not lead to FRET (Paper III, Figure 2Af). Since control experiments outlined in Paper III indicated that the observed FRET results from intra-receptor fluorophore interaction, the absence of FRET in IR-A/IR-B hybrid receptors versus the presence of FRET in both IR-A and IR-B homo-receptors strongly suggests structural differences in the hybrid receptor. Interestingly, the analysis of interaction between APS with the IR activation loop indicated the requirement of symmetry between the two IR β-subunits [36]. Since our data may indicate a non-symmetric orientation of the β-subunits in IR-A/IR-B hybrid receptors, it will be interesting to test the hypothesis whether this would exclude IR hybrid receptors from APS-dependent

signaling and thus provide a mechanistic explanation for selective signaling via IR hybrid receptors in general.

The third interesting observation concerns insulin signaling from internalized IR complexes, as reported in Paper IV. Since internalization of IR complexes and their targeting to different subcellular compartments will provide a further way of gaining selective insulin effects, a more detailed analysis of the mechanisms underlying the IR-B mediated activation of c-fos gene transcription will be of importance. Here, the next steps will be the identification of the intracellular compartment from where IR-B-mediated signals activate the PI3K Ia/p52-Shc/MEK1/Erk1/2 cascade. Initial experiments using dominant-negative dynamin as a tool suggest endosomes as the compartment. Here, the use of dominant-interfering variants of Rab5 versus Rab7 may help to identify the early or late endosome pool as the origin of signal transduction in this particular pathway.

Data presented here show the involvement of the two IR isoforms in selective signaling in the pancreatic β-cell. Future work including the development of i) general as well as tissue-specific knockouts of IR isoform expression, ii) tissue-specific rescue by IR isoform expression in IR-null mice, and iii) RNAi-based knockdown of each IR isoform in various cell types will help to more globally understand the biological relevance of the two IR isoforms in insulin signal transduction.

7 CONCLUSIONS

1. Glucose-stimulated βGK gene transcription involves positive insulin feedback action similar to that observed for glucose-stimulated insulin gene transcription. However, while insulin activates transcription of its own gene by signaling via IR-A, it requires signal transduction through the IR-B isoform to up-regulate βGK gene transcription.

2. Secreted insulin activates insulin and βGK gene transcription by utilizing different signaling cascades. IR-A-mediated activation of the insulin promoter requires signaling via IRS/PI3K Ia/mTOR and p70s6k, while simultaneous activation of the βGK promoter, via IR-B involves signal transduction through PI3K-C2α/PDK1 and PKB.

3. IR-A mediated activation of the insulin promoter and IR-B-mediated activation of the βGK promoter are not dependent on IR isoform-specific differences in internalization but on the different localization of the receptor types in the plasma membrane. Here, localization and function of the two IR isoforms depend on the 12 amino acid string encoded by exon 11. Moreover, our data suggest that selective activation of the insulin and βGK promoters occurs by signaling from non-caveolae membrane micro-domains that are differently sensitive towards cholesterol depletion.

4. Insulin stimulates c-fos gene transcription in the pancreatic β-cell, like βGK, via the IR-B isoform. Analysis of both promoters revealed that insulin activates the βGK promoter from membrane-standing IR-B, while c-fos promoter activation is dependent on clathrin-mediated IR-B endocytosis. Moreover, βGK promoter up-regulation requires the integrity of the IR-B NPEY-motif and signaling via PI3K-C2α/PDK1/PKB, while c-fos gene activation requires the intact YTHM-motif and signaling via PI3K Ia/Shc/MEK1/ERK1/2.

In conclusion, the results of the present thesis work clearly demonstrate that simultaneous and selective signal transduction through the two isoforms of the IR, i.e. IR-A and IR-B, allow the simultaneous activation of discrete signaling cascades that, as exemplified here by using the transcriptional up-regulation of three model genes insulin, βGK and c-fos as functional read-outs, leading to specific insulin effects in the insulin-producing pancreatic β-cell (*Figure 7.1*).

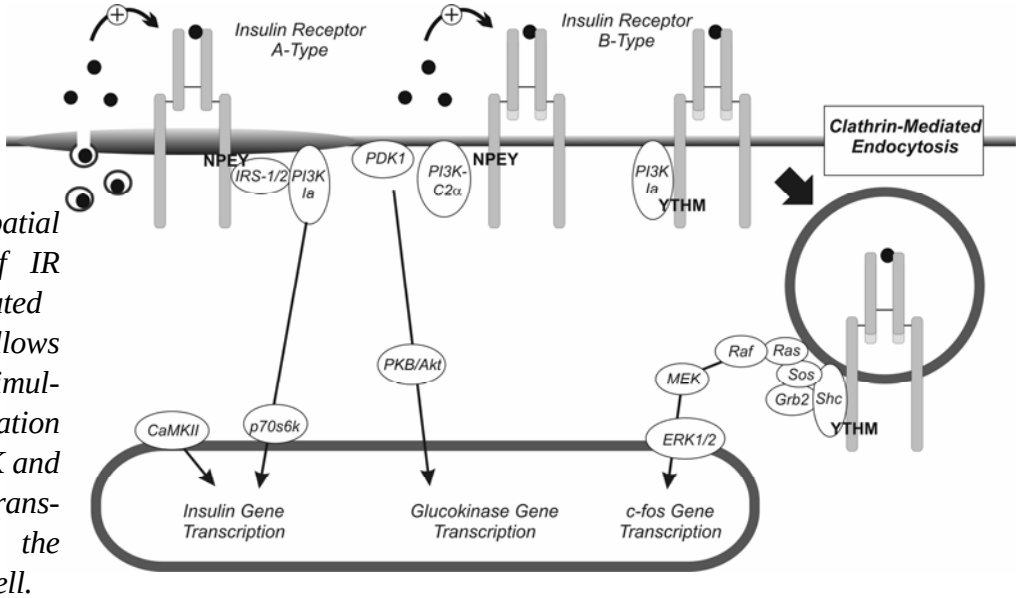

Figure 7.1. Spatial segregation of IR iso-form-mediated signaling allows selective and simultaneous activation of insulin, βGK and c-fos gene transcription in the pancreatic β-cell.

8 ACKNOWLEDGEMENTS

This thesis is the result of an exiting journey, which includes extremely valuable experiences and memories in both scientific and personal life. I would like to express my gratitude to the following persons, who have guided, contributed, supported, helped or cheered me up throughout this journey.

My supervisor Docent Ingo Leibiger, for your superiour guidance and endless optimism, for your shooting of provocative ideas and questions and for also teaching me to do so. For all the time you took to discuss with me thousands of topics concerning science – and life and for your valuable opinion and advice. It was a privilege for me to have you as my mentor. Your depth of knowledge and your dedication to science has inspired me throughout these years.

My co-supervisor Professor Per-Olof Berggren, co-chairman of the Department of Molecular Medicine, for your enthusiasm and insatiable curiosity for β-cell and diabetes research, for your ingenious financial management and for providing such a fruitful environment. Thank you for inviting me to the Karolinska Institute and giving me the opportunity to perform my PhD in such an excellent group.

My co-supervisor Docent Barbara Leibiger, for sharing your remarkable knowledge about all kind of techniques, teaching me confocal microscopy and the importance of the 'happiness of cells'. Thank you for taking care of the every-day 'administrative' matters of our group, for reading my manuscripts and your useful critism.

Professor Kerstin Brismar, chairman of the Department of Molecular Medicine, for always taking good care of our department, for your positive and calm attitude and your interest in personal needs of everyone.

Professor Rolf Luft for creating the Rolf Luft Center for Diabetes Research

Tilo Moede for being a great co-worker, your knowledge and help with computers and for teaching me digital imaging fluorescence microscopy.

My former supervisor Professor Dirk Mueller-Wieland at the Innere Klinik II, Universitätsklinik Köln in Germany, where I performed my diploma thesis, for introducing me to diabetes research, for his generous support and encouraging me to take the opportunity to work at the Karolinska Institute.

Svante Norgren, for introducing me to this great and outstanding group.

Katarina Breitholtz, Britt-Marie Witasp, Helene Zachrison, Christina Bremer and Annika Lindgren for their kindness and their invaluable assistance in the 1001 practical and administrative things necessary in the lab.

My co-authors Rohit N. Kulkarni, Ronald C. Kahn and Lina Moitoso de Vargas, for sharing their knowledge and allowing successful collaborations.

Lennart Helleday, our system administrator, for your kind help and support with computers whenever my laptop broke down again.

Thanks to all the present and former collegues at the Rolf Luft Center in no particular order: Mei Yu, Fan Zhang, Per Moberg, Mikael Turunen, Zongpei Chen, Alejandro Bertorello, Jenny Johansson, Lena Lilja, Slavena Mandic, Wei Zhang, Juliette Janson, Christina Bark, Jia Yu, Christopher Illies, Christopher Barker, Dominic-Luc Webb, Nancy Dekki, Rebekka Nilsson, Essam Refai, Lisa Juntti-Berggren, Hannelore Rotter, Daniel Nyqvist, Martin Köhler, Luosheng Li, Hua Qui, Lina Yu, Shao-Nian Yong, Qimin Zhang, Olga Kotova, Vladimir Sharoyko, Sergei Zaitsev, Irina Zaitseva, Andreas Fernström, Olof Larsson for creating a pleasant scientific atmosphere in the lab.

In addition I would like to thank former and present people at the Karolinska Institute whom I had the pleasure to meet and to spend joyful time outside the lab: Alexander Efanov and Raf Lemmens for sporty times at the gym and outside inline skating. Rafael Krmar for his patience trying to teach me Spanish and for all the talks about life and everything. Stefan Glund and Karim Bouzakri for the good time we shared in Nice and during coffee breaks with Lubna. It was a delight to meet you all!

Particularly I would like to thank my dear friends at the Karolinska Institute: Stefania Cotta-Doné, Gabriela Imreh, Lubna Al-Khalili, Tatjana Steiler, and Guillermo Yudowski, for knowing you, for your valuable friendship, for all the happy moments when we (too seldom) managed to spend time together. Thank you for charging me with positive energy and sharing hard and sweet times. I hope that these friendships will continue for life time, even when we finally will move to distant places.

My close friends in Germany, who always remind me where I come from, for all your care, your open ears and truly friendship. In particular, my dearest friend, Susi, for standing by my side since almost 25 years, knowing me to the bottom of my heart and always believing in me and my ideals. You, Rüdi and now little Lennart will always be part of my family. Babs, Moni and Ellen, for the great merry and brainstorming ☺ evenings during my visits to Köln, loading my battery. Nickelchen, Christian and Torsten, for the cheerful times we had during your visits in Stockholm and wherever we met somewhere in Germany during these years, for your support, your humor and true concern. My dear Mattes for always having a good advice when needed most, for help and for being such a reliable friend.

My brother Olaf Kemper for your love and care, for being such a great ‘big’ brother always at my side sharing hard and sweet times and for having patience with your sometimes hard-headed sister.

My dear grandmother-in-law, Adele Gutsch, for your never ending humor and wisdom to take life how it comes. For your tremendous support during the last years, for your care and endless love.

My grandmother, Hetwig Kemper, in loving memory. A remarkable woman with a never ending interest for life, nature and humans. For her joyful heart, her spirit and love.

To the memory of my late parents, Siegfried and Rita Kemper, who died much too early 13 years ago. Raising me up in a sheltered and happy childhood provided me with a solid fundament of strength to confront difficult situations in life. Especially I wish to thank my precious mother, for her endless love, for being a wonderful Mom and always believing in me. You would have been so proud. I cannot tell how much I miss you.

My soul mate Claudia. There are no words to express my gratitude to you. You and Felipa flood my heart with joy, light my day and fulfill my life. Thank you.

This work was supported by funds from Karolinska Institutet and by grants from the Swedish Diabetes Association, Swedish Research Council, The Novo Nordisk Foundation, Berth von Kantzow's Foundation, Juvenile Diabetes Research Foundation (JDRF), National Institutes of Health (DK58508), the Family Stefan Persson Foundation.

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