REGULATION OF INSULIN AND CHOP GENE EXPRESSION

IN PANCREATIC BETA CELLS

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Dedicated to My Family, My Husband,

and My Lovely Daughter.

REGULATION OF INSULIN AND CHOP GENE EXPRESSION IN PANCREATIC BETA CELLS

by

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Publication No._____

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Abstract: Insulin is a major hormone in maintaining glucose homeostasis. It is essential to understand the mechanisms by which insulin gene expression is regulated in pancreatic beta cells. In addition to examining histone modifications on the insulin gene promoter, I focused on the effect of MafA modification on insulin expression. MafA is a transcriptional activator of the insulin gene via binding to the RIPE3b/C1 (rat insulin promoter element 3b) element. Mutagenesis showed that MafA was post-translationally modified by SUMO-1/2 (small ubiquitin-like modifier) mainly at lysine 32. Low glucose

starvation or hydrogen peroxide stimulation increased sumoylation of MafA. Forced sumoylation of MafA reduced its transcriptional activity towards the insulin gene promoter and increased its suppression of the CHOP (C/EBP homologous protein) gene promoter. However, sumoylation of MafA did not alter its nuclear localization, protein stability, or apparently its DNA binding to the insulin promoter in beta cells. These studies suggest that MafA sumoylation modulates gene transcription in beta cells**.** In type I diabetes, beta-cell apoptosis is the major reason for immune-mediated pancreatic beta-cell death. IL-1β (interleukin 1β), a proinflammatory cytokine, induces ER (endoplasmic reticulum) stress and activates proapoptotic networks in beta cells, such as NF-κB (nuclear factor-kappaB) and JNK (c-Jun N-terminal kinase) signaling pathways. The second project focused on the mechanisms by which JNK and NF-κB regulate the expression of CHOP, a mediator of ER stress-induced apoptosis, upon IL-1β stimulation. Exposure of beta cells to IL-1β markedly increased CHOP messenger RNA and protein. Electrophoretic mobility shift assays showed that IL-1β-activated NF-κB bound to the CHOP promoter. Furthermore, immunoblot data indicated that expression of c-Jun was strongly increased, and that multiple residues on c-Jun were phosphorylated after IL-1β treatment. IL-1β also increased c-Fos expression in beta cells. These data suggest that IL-1β-induced activation of NF-κB and JNK controls CHOP gene expression in pancreatic beta cells, and that IL-1β influences beta-cell function through a variety of signaling pathways.

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LIST OF ABBREVIATIONS

bHLH basic helix-loop-helix

CHOP C/EBP homologous protein

CPH carboxypeptidase H

CRE cAMP-response element

CREB CRE binding protein

ER endoplasmic reticulum

ERK extracellular signal-regulated protein kinase

FoxO1 forkhead box O1

GlcNAc N-acetylglucosamine

GLP-1 glucagon-like peptide-1

GLUT glucose transporter

GSIS glucose-stimulated insulin secretion

GSK3β glycogen synthase kinase 3 beta

HAT histone acetyltransferase

HDAC histone deacetylase

HNF1-β hepatocyte nuclear factor-1 beta

IAPP islet amyloid polypeptide

IFNγ interferon gamma

IL-1β interleukin 1 beta

IP-10 IFNγ inducible protein-10

IRS1 insulin receptor substrate 1

JNK c-Jun N-terminal kinase

MafA v-maf musculoaponeurotic fibrosarcoma oncogene homolog A

MAP kinase mitogen-activated protein kinase

MARE Maf response element

MAZ myc-associated zinc finger protein

MCP-1 monocyte chemotactic protein-1

MODY maturity onset diabetes of the young

NFAT nuclear factor of activated T cells

NF-κB nuclear factor-kappa B

NRE negative regulatory element

PC1 prohormone convertase 1

Pdx-1 pancreatic duodenal homeobox-1

PEA-15 phosphoprotein enriched in astrocytes

PI3K phosphatidylinositol 3-kinase

RIPE3b rat insulin response element 3b

ROS reactive oxygen species

PPARγ peroxisome proliferator activated receptor γ

SENP SUMO-1/sentrin specific protease

SRP signal recognition particle

SUMO small ubiquitin-like modifier

TNF-α tumor necrosis factor alpha

Ubc-9 Ubiquitin-like conjugating enzyme 9

UCP-2 uncoupling protein 2

ZDF Zucker Diabetic Fatty rat

Chapter One: General Overview

I. Pancreatic beta cells and diabetes

A. Physiology of the beta cell

Pancreatic beta cells are located in the islets of Langerhans, where pancreatic endocrine cells are clustered. The islets of Langerhans constitute approximately 1-2% of the pancreatic volume and are distributed abundantly in the tail of the human pancreas (Brissova et al. 2005) (Figure 1-1A). Islets are vascularized by an extensive capillary network and innervated by various nerve fibers. A healthy adult human pancreas comprises about 1,000,000 islets, each containing approximately 2000 beta cells (Meier et al. 2006).

In the human pancreas, 65-80% of islet cells are beta cells, where insulin is exclusively synthesized and secreted in response to increased blood glucose and other nutrients (Elayat et al. 1995). Besides insulin, C-peptide and amylin are released by beta cells as well. C-peptide is a byproduct when proinsulin is cleaved into mature insulin. C-peptide plays a role in preventing certain complications associated with type I diabetes, especially diabetic nephropathy. Animal studies suggest that the C-peptide reduces diabetes-induced glomerular hyperfiltration resulting in the regression of fibrosis (Rebsomen et al. 2008). Amylin exhibits glucose regulatory effects through suppressing glucagon secretion, delaying the absorption of glucose into the circulation, and limiting nutrient consumption (Ratner et al. 2004). Alpha cells constitute 15-20% of islet cells and secrete glucagon to increase blood glucose concentration through the release of glucose

from glycogen in liver and skeletal muscle. Somatostatin-producing delta cells make up 3-10% of islet cells, and the rest are pancreatic polypeptide-secreting cells and ghrelin-producing epsilon cells (Elayat et al. 1995) (Figure 1-1).

B. Insulin synthesis, secretion, and function

Insulin is a peptide hormone composed of two polypeptide chains that are linked together by two disulfide bonds. About 10-15% total mRNA in pancreatic beta cells is insulin mRNA, which is translationally quiescent in low glucose concentration (Goodge and Hutton 2000). During the synthesis of insulin, the messenger RNA transcript is translated into an inactive precursor preproinsulin. In the short term, glucose can increase translation initiation and elongation, transfer of free mRNA from a cytoplasmic pool to translationally active membrane-bound polysomes on the rough ER, and release of signal recognition particle (SRP) from the SRP receptors (Itoh and Okamoto 1980; Welsh et al. 1986; Wicksteed et al. 2001). Preproinsulin bears an N-terminal hydrophobic signal that is required for preproinsulin to pass through the rough ER membrane for post-translational processing. Upon translocating into the lumen of the ER, the preproinsulin signal sequence is proteolytically cleaved to produce proinsulin. Once the proper disulfide bonds are formed, proinsulin is transported into the Golgi apparatus (Shoelson et al. 2006). Proinsulin is converted into mature and active insulin and C-peptide by two endoproteases PC1 (prohormone convertase) and PC2, and exoprotease CPH (carboxypeptidase H). The conversion occurs in the calcium-rich acidic environment of secretory granules in beta cells. PC1 and PC2 are calcium-dependent proteases, whereas catalytic activity of CPH depends on zinc (Jackson et al. 1997; Kahn and Halban 1997).

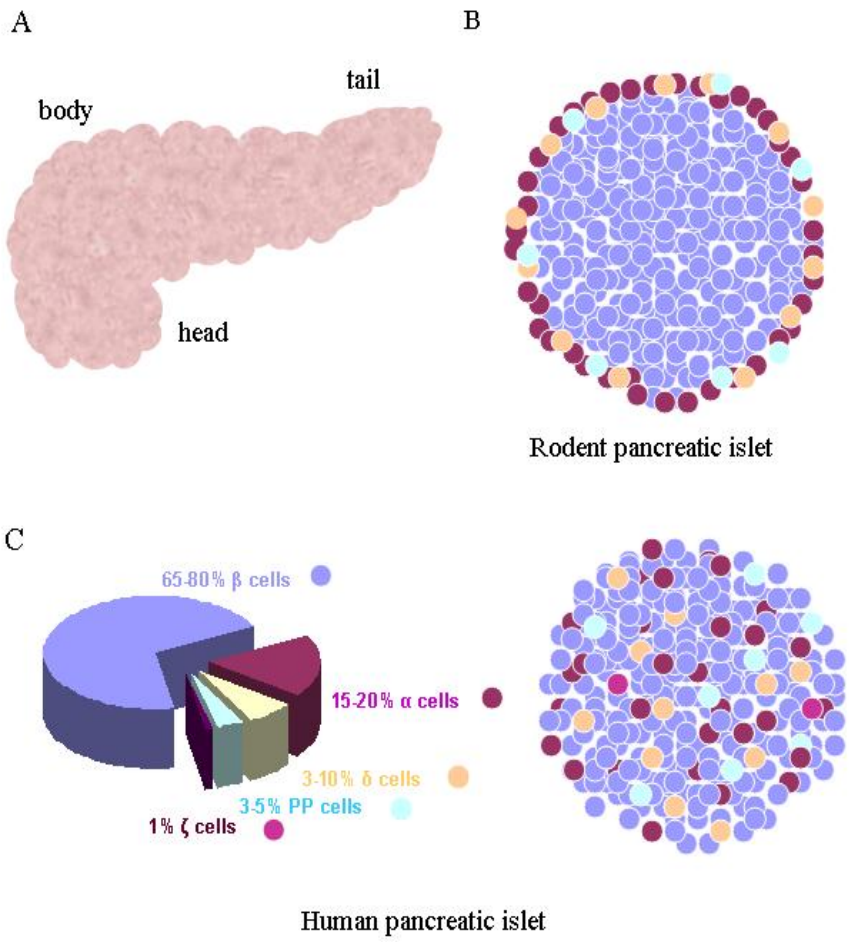


Figure 1-1 Architecture of the pancreas and cell distribution within the islets. Schematic diagrams of the human pancreas (A), rodent pancreatic islet (B), human pancreatic islet (C), and cell distribution in human islet (C).

Glucose regulates the conversion of proinsulin to insulin at least in part through the modulation of biosynthesis of PC1 and PC2 (Martin et al. 1994). The synthesized insulin is then packed in secretory granules accumulating in the cytoplasm, some of which are in a readily releasable pool. Every beta cell contains 10,000-13,000 secretory granules, each comprising about 10^6 insulin molecules, polypeptides, low molecular weight compounds like ATP, and a high concentration of metal ions, such as Zn^{2+}. A majority of granule zinc associates with hexameric insulin (Olsen et al. 2003).

Beta cells are known as "glucose sensors" and secrete insulin in response to an increase in circulatory glucose. Circulating glucose equilibrates rapidly across beta-cell membranes because of the expression of high capacity, low affinity type 2 glucose transporters (GLUT2) (Thorens 1992). Upon uptake into beta cells, glucose is rapidly phosphorylated by a rate-limiting enzyme, glucokinase, then undergoes glycolysis and enters the respiratory cycle to create ATP. The closure of ATP-sensitive potassium channels in beta-cell membranes, caused by an increased ratio of ATP to ADP, prevents intracellular potassium ions from flowing across the membrane. The ensuing rise of intracellular positive charge leads to beta-cell depolarization, followed by the activation of voltage-sensitive calcium channels, through which calcium ions enter the cell. The brisk increase of intracellular calcium triggers insulin granule exocytosis (Newgard and McGarry 1995) (Figure 1-2). Under fasting conditions, the rate of insulin secretion is around 2 pmol/kg/min and this rate increases by 5-10 fold after meal (Porksen et al. 1996).

Glucose-stimulated insulin secretion (GSIS) from beta cells is a process coordinated by glucose uptake, metabolism and Ca^{2+}-triggered insulin exocytosis.

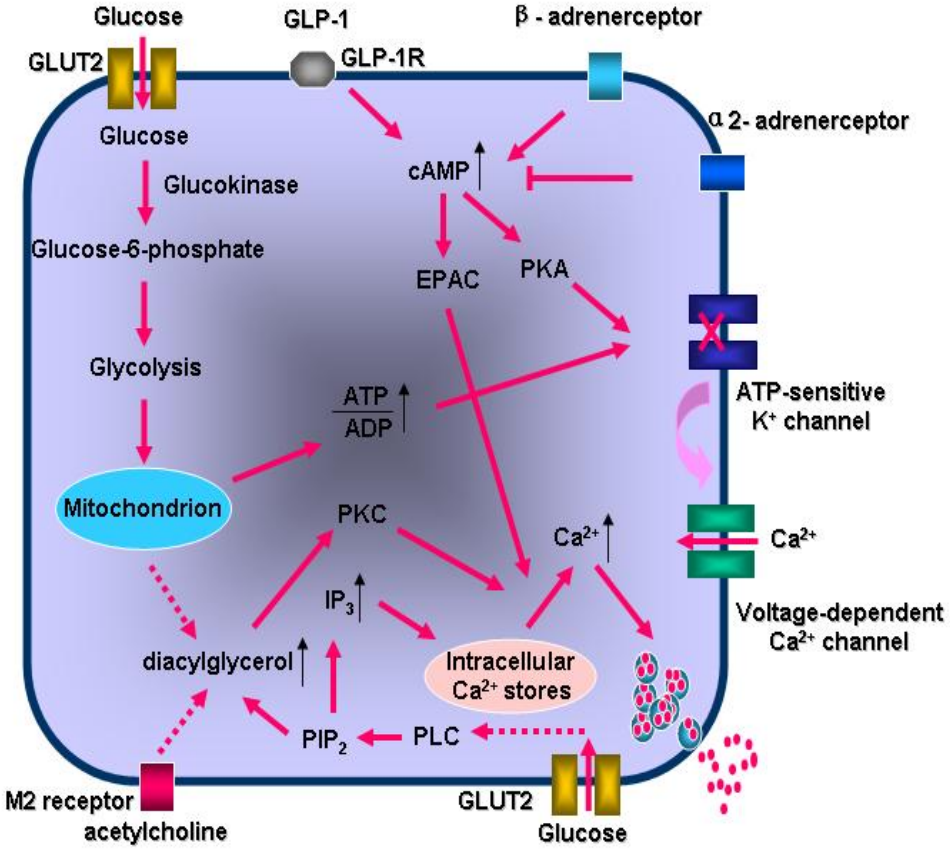


Figure 1-2 A diagrammatic representation of glucose signaling pathways in beta cells.

Impaired GSIS is a hallmark of type II diabetes, in which the perturbation of expression of various genes, such as *GLUT2* and *Rab3*, may be involved (Iezzi et al. 1999). GSIS is proportional to both glucose concentration and the rate of change of the glucose concentration. Insulin secretion in mammals as well as from the perfused rodent pancreas follows a typical biphasic process. The initial phase develops rapidly and lasts 3-10 minutes, the amount of released insulin depends upon the amount in storage. Once exhausted, a second phase of insulin release is triggered, which develops slowly and is sustained for the duration of a rise in blood glucose (Daniel et al. 1999).

Although the amino-acid sequence of insulin varies among species, certain segments of the molecule are highly conserved, thereby leading to the similar three dimensional structure of insulin. It has also been discovered that specific cells in the fruitfly's brain secret a hormone similar to insulin, which mimics the functions of its human counterpart (Rulifson et al. 2002). Insulin has a vast array of metabolic functions such as decreasing blood glucose concentration and regulating the metabolism of fat and proteins. It is the primary hormone that modulates the storage and utilization of glucose in target tissues including muscle, liver and adipose tissue. Insulin binds and activates the insulin receptor causing the latter to phosphorylate itself as well as docking proteins such as insulin receptor substrate 1 (IRS1). IRS1 then binds adaptor proteins like Grb2 and phosphatidylinositol-3 kinase (PI3K) to transduce the signal to downstream effectors either to inhibit the breakdown of glycogen and triglyceride or to stimulate glucose uptake and storage as well as other anabolic events. Among the cellular responses controlled by insulin, the key step in regulating glucose homeostasis is the translocation of type 4 glucose transporters (GLUT4) from cytoplasmic vesicles to cell membranes. In

adipose tissue and striated muscle, GLUT4 effectively removes glucose from the blood stream by facilitating the transport of glucose into cells (Saltiel and Pessin 2002). Plasma insulin has a half-life of about six minutes. Insulin may be inactivated through internalization, but most of insulin is degraded in the liver and kidney by insulin protease or by reduction of the disulfide bond (Duckworth et al. 1998). Because hormone degradation is as important as hormone secretion, this short half-life makes it possible to precisely control glucose homeostasis.

C. Beta-cell apoptosis and diabetes

Cell death occurs mainly as a result of a passive necrotic process or an active apoptotic process. Apoptosis is the most common mechanism by which the body removes infected, transformed or damaged cells through activating an intrinsic suicide program. This is also a fundamental mechanism leading to cell loss in disease (Chandra et al. 2001). Pancreatic beta cells are sensitive to a number of proapoptotic stimuli shown to be involved in the pathogenesis of diabetes, a disease affecting about one in twenty of the world's adult population.

Histological studies using pancreas specimens from patients with new-onset type I diabetes demonstrated that beta-cell mass is reduced by 70-80% at the time of diagnosis. Accumulating data suggest that in type I diabetes pancreatic beta cells are the target of autoimmune assault and that apoptosis is the major form of beta-cell death. In at least three animal models of type I diabetes, macrophages are found to be the predominant infiltrating immune cells during the early stage of insulitis. Other immune cells participating in the pathogenesis are CD4+ and CD8+ lymphocytes, NK (nature killer)

cells and B cells. The major secreted proinflammatory cytokines are IL-1β, interferon (IFNγ) and tumor necrosis factor (TNF-α) (Cnop et al. 2005). Ironically, beta cells themselves produce some of these cytokines and chemokines, such as IL-1β, monocyte chemotactic protein-1 (MCP-1), IFNγ inducible protein-10 (IP-10) and TNF-α (Maedler et al. 2002; Piemonti et al. 2002). Beta-cell apoptosis is triggered by either direct contact with activated macrophages and T-cells or exposure to soluble cytokines, nitric oxide (NO), and/or reactive oxygen species (ROS). However, the underlying mechanisms by which beta-cell apoptosis is initiated remain controversial. Perforin, FasL, IL-1β, IFNγ and NO have been shown as the effector molecules. Unfortunately, inhibiting an individual signaling pathway has not been shown to be adequate to prevent beta-cell loss (Figure 1-3).

Type II diabetes is characterized by combination of insulin resistance and beta-cell dysfunction. Obesity and insulin resistance are considered the major factors causing type II diabetes. Recently, a body of studies suggests that the hyperglycemia in type II diabetes is in large part a consequence of a deficit in beta-cell mass. Although a 25-50% reduction in beta-cell mass is found at the time of diagnosis of type II diabetes, about 65% of beta cells are found to be lost by the time of autopsy of patients with long-standing type II diabetes. These suggest that beta-cell loss could be a major contributor to pathogenesis in the late stage of type II diabetes (Maedler 2008). The remarkably high level of beta-cell loss could result from either an impaired proliferative capability or an elevated rate of cell death.

As shown in human pancreas autopsy specimens and in isolated islets, beta-cell loss in type II diabetes is accompanied by a marked increase in beta-cell apoptosis.

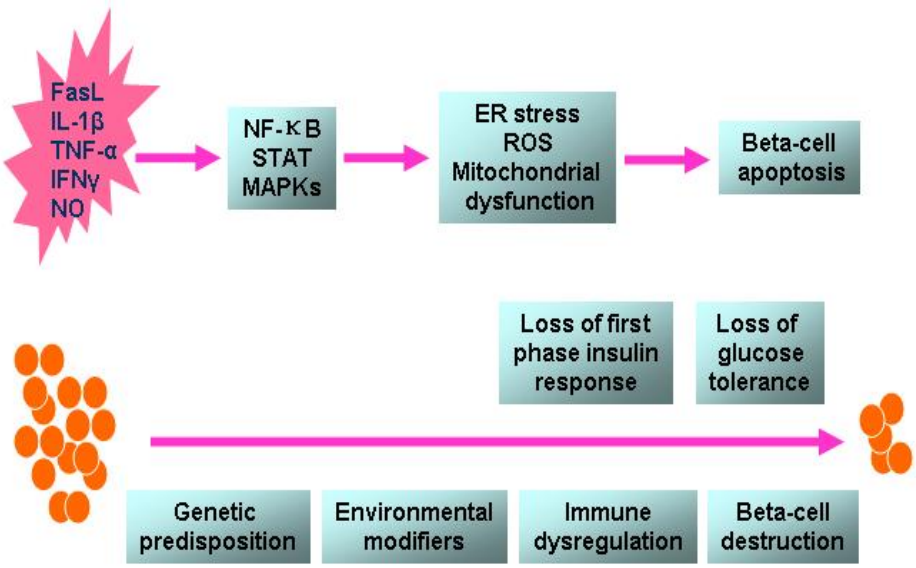
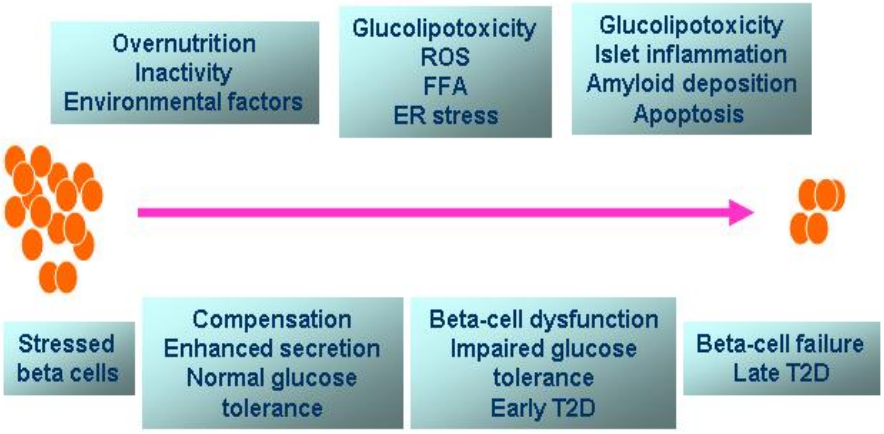


Figure 1-3 Islet beta-cell death and type I diabetes.

However, the molecular mechanisms by which beta-cell apoptosis is triggered during the development of type II diabetes are largely unknown. In humans, many factors have been proposed to be the triggers of increased beta-cell apoptosis, such as prolonged exposure to high concentration of glucose and lipid, toxic oligomers of human islet amyloid polypeptide, ROS, ER stress and proinflammatory cytokines (Prentki and Nolan 2006) (Figure 1-4).

In cultured human islets, short-term elevated glucose induces beta-cell proliferation, whereas chronically high glucose enhances beta-cell apoptosis. Interestingly, apoptosis occurs frequently in proliferating beta cells, suggesting that cells undergoing proliferation are highly susceptible to apoptosis. Chronically high glucose causes increased glucose metabolism through oxidative phosphorylation, leading to mitochondrial dysfunction and the production of ROS. Beta cells have a limited capability to defend against extra ROS due to low levels of ROS-detoxifying enzymes. The generation of ROS will activate stress-induced pathways, including NF-κB and JNK pathways (Robertson and Harmon 2006). In a similar manner, a low level of IL-1β promotes beta-cell proliferation, but a high concentration of IL-1β is proapoptotic by activating NF-κB and JNK pathways and perhaps others. Additionally, the ER is highly developed in pancreatic beta cells due to the high secretory demand, which also makes beta cells susceptible to ER stress. ER stress can induce beta-cell apoptosis through the actions of JNK, activating transcription factor 3 (ATF-3) and proapoptotic members of the Bcl-2 (B-cell CLL/lymphoma 2) family (Kaneto et al. 2004). An increase in circulating free fatty acids is another abnormality often observed in type II diabetes. Exposure of rat pancreatic islets to 2 mmol/L fatty acids (oleate/palmitate 2:1) for 48 h induces beta-cell apoptosis probably



Type II diabetes

Figure 1-4 Islet beta-cell failure and type II diabetes

through oxidative stress because it can be counteracted by nicotinamide (Lupi et al. 2002; Piro et al. 2002). Although the mechanisms of beta-cell apoptosis induced by free fatty acids are not delineated, multiple studies have shown that elevated free fatty acids cause ER stress and expression of uncoupling protein-2 (UCP-2), the generation of ROS, and activation of the JNK pathway. Thus, in the presence of metabolic overload and deleterious substances, signals are sensed, integrated and transmitted through activating a network composed of mitogen-activated protein kinases (MAP kinases), NF-κB and other pathways, leading to the high frequency of beta-cell apoptosis during the pathogenesis of type II diabetes.

Destruction of beta cells is now considered to play an essential role in both type I and type II diabetes. This provides a rationale for therapeutic strategies aimed at either preventing beta-cell loss, if the mechanisms of beta-cell apoptosis are elucidated, or regenerating functional beta cells.

II. Regulation of insulin gene expression

A. Insulin gene structure

In most species the precursor preproinsulin exists as a single gene except for rat and mouse, which possess two nonallelic insulin genes. The two nonallelic insulin genes in each species are about 90% identical in the coding regions. The human insulin gene is located on the short arm of chromosome 11, whereas the rat insulin I and II genes are colocalized on chromosome 1 and the mouse genes are found on chromosomes 19 and 7, respectively (Owerbach et al. 1980; Soares et al. 1985; Davies et al. 1994). The rodent

insulin II and the human genes contain three exons separated by two introns, whereas insulin I lacks the second intron (Steiner et al. 1985). In adult rodent islets the nonallelic genes are coordinately expressed and regulated. Although sequences up to 4 kb upstream of the start site have been implicated in regulating insulin transcription, the proximal 400 bp of the insulin gene promoter confers both tissue-specific expression and metabolic regulation of the insulin gene. Thus, most studies on the regulation and function of the insulin promoter have focused on this highly conserved 400 bp region, which is organized in a complex arrangement of discrete cis-acting elements. Although the rat insulin I, II and human insulin promoters share a number of conserved regulatory motifs, the human promoter also contains certain unique elements (Melloul et al. 2002). Among the common elements in these promoters, E, A and RIPE3b elements are the major determinants of beta-cell specific insulin gene expression (Figure 1-5).

Systematic mutagenesis and transfection studies indicated that two E-box elements (E1 and E2) containing the consensus sequence, CANNTG, of E boxes are located between -104 and -112 bp (E1) and between -233 and -241 bp (E2) in the rat insulin I promoter. Deletion of either the E1 or E2 element causes more than a 90% reduction of transcriptional activity in insulin-secreting HIT-T15 cells, while a double mutation completely abolishes insulin gene promoter activity (Karlsson et al. 1987). The proximal E1 element is highly conserved throughout mammals. However, human and rat insulin II 5' flanking regions do not contain the E2 box. A related sequence in the human insulin promoter binds the basic helix-loop-helix (bHLH) protein upstream stimulatory factor (USF) (Read et al. 1993).

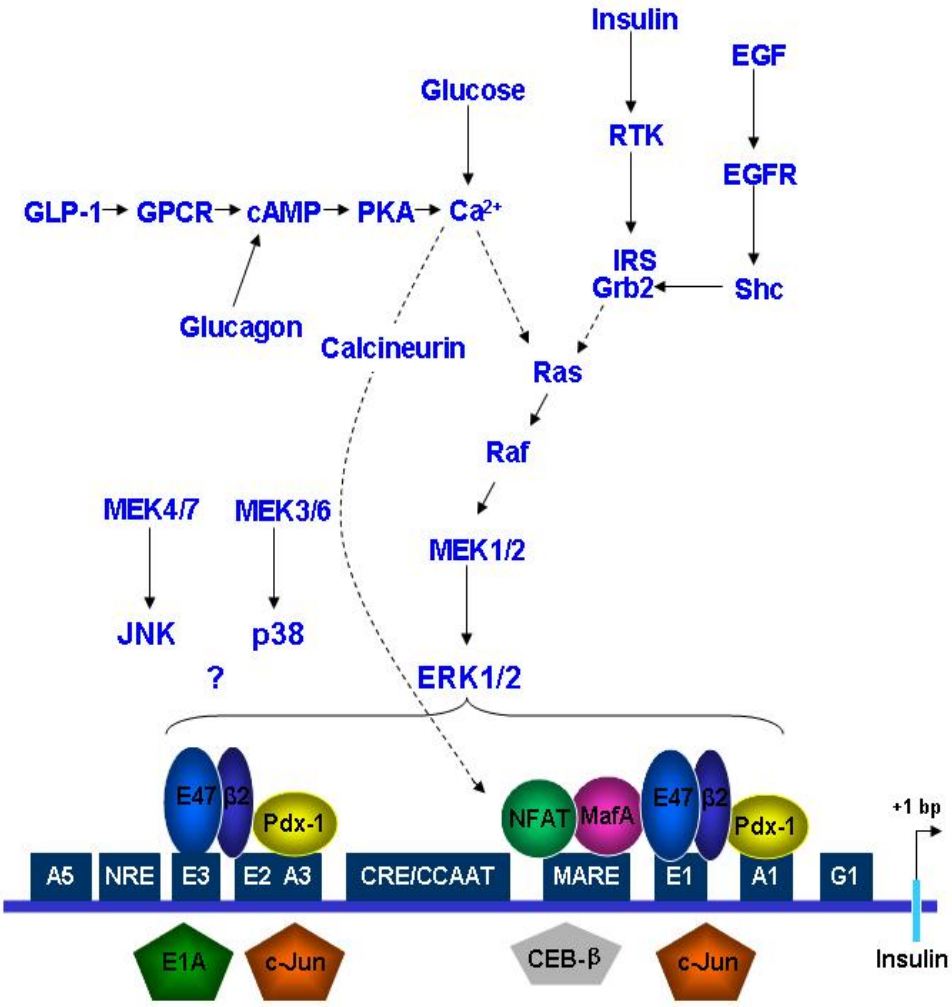


Figure 1-5 A diagrammatic representation of the human insulin gene promoter and signaling pathways regulating insulin transcription.

A boxes (A1-A5) bearing the core sequence TAAT are additional prominent regulatory elements in the insulin gene promoter. Homeodomain-containing proteins bind A boxes except for A2 (GGAAAT). The rat insulin I gene contains four A elements (A1-A4), among which A1 and A3 boxes are the most conserved. The human promoter contains an A5 element but lacks the A4 element that is only found in the rat insulin I promoter (German et al. 1995). Deletion of the rat insulin I A3 regions or the corresponding A3 region in the human insulin promoter results in a dramatic reduction of transcriptional activity. The A1 element also contributes modestly to insulin gene transcription (German and Wang 1994).

The rat insulin II gene enhancer, RIPE3b/C1 (-126 to -101 bp) has been shown to mediate beta-cell specific activity in transfection experiments. RIPE3b was initially suggested to contain two overlapping sequences, the A2 (-126 to -113 bp) and the C1 (-116 to -107 bp) elements. Two groups determined that both C1 and A2 elements together constitute a large binding site named the Maf response element (MARE) and that the transactivator is the beta-cell specific Maf family protein MafA (Kataoka et al. 2002; Olbrot et al. 2002). Due to the overlapping of MARE and A2 elements, transient transfection experiments demonstrated that both MafA and A2.2, which is a beta-cell specific activator lacking the biological identity, cooperatively activate insulin gene expression (Nishimura et al. 2005).

Both rodent insulin I, II and the human insulin promoter contain a cAMP-response element (CRE). The second messenger cAMP regulates the transcription of genes carrying CREs through binding the transcription factor CRE binding protein (CREB) to this motif. In beta cells, certain hormones including glucagon and glucagon like protein 1

(GLP-1) increase intracellular cAMP. However, reporter assays revealed that cAMP-induced transcription through insulin promoter CRE is weak when compared to the responses of CREs in other gene promoters, such as glucagon and somatostatin. The insulin CRE is not responsive to membrane depolarization and calcium influx (Oetjen et al. 1994). In the human insulin gene, four CRE sites were identified by deletion analyses: two lie in the promoter region, whereas the other two are localized in exon1 and intron1. Mutational analyses of the four CREs revealed that all elements contribute additively to insulin gene expression (Inagaki et al. 1992).

The C2 element, containing a CAGG repeat, was first identified in the human insulin gene promoter from -253 to -244 bp. Although no equivalent motif was found in the rat promoters, a related CACC sequence was detected between -329 and -307 bp of the insulin I gene (Read et al. 1997). This C2 element binds transcription factors containing a paired domain and a homeodomain, such as PAX6 and PAX4 (paired homeobox gene) (Sander et al. 1997; Fujitani et al. 1999). Deletion analyses also identified a G1 element (G/GAGA) in all three promoters partially overlapping with the A2 element. Mutation of the G1 element in the rat insulin II promoter causes a dramatic loss of activity (Karlsson et al. 1987). The ubiquitous zinc finger protein human MAZ (myc-associated zinc finger protein), and its mouse homologue, Pur-1 (purine binding factor-1), binds to the G1 element and stimulates promoter activity (Kennedy and Rutter 1992). Other elements such as Sp1, insulin-linked polymorphic region (ILPR), and negative regulatory element (NRE) are only found in the human insulin promoter (Figure 1-5).

B. Insulin gene transcription factors

Numerous transcription factors and cofactors have been described to act on the insulin gene promoter. The highly dynamic and mutlicomponent complexes, including transcription factors, DNA structural proteins, cofactors and RNA polymerase, are formed to ensure the precise regulation under various conditions (Ohneda et al. 2000; Lawrence et al. 2008). Their expression levels and posttranslational modifications are important in regulating insulin gene expression.

i. Basic helix-loop-helix proteins

E boxes bind bHLH family proteins, which are transcription factors of tissue-specific genes through the formation of heterodimers between ubiquitous (class A) and cell-specific (class B) family members (Massari and Murre 2000). E1 binds insulin enhancer factor 1 (IEF1), a heterodimer including one of two alternatively spliced *E2A* gene transcripts E12/E47. E12/E47 interacts with the bHLH protein NeuroD1, also known as Beta2, which is expressed in beta cells and other pancreatic endocrine cells, the intestine, the pituitary, and a subset of neurons. NeuroD1 is an important regulator of both insulin gene expression and pancreatic development (Naya et al. 1995; Naya et al. 1997). Homozygous *NeuroD1-/-* mice are hyperglycemic, contain fewer beta cells, fail to develop a mature pancreas, and die within 5 days after birth (Naya et al. 1997). Similarly, mice bearing NeuroD1 mutations with impaired DNA binding or transactivating ability develop diabetes (Malecki et al. 1999). Specific mutations in NeuroD1 have been linked to maturity onset diabetes of the young type 6 (MODY6) (Kristinsson et al. 2001). A polymorphism, Ala45 to Thr, has also been associated with Type I diabetes in certain

populations. Additionally, recent studies suggested that NeuroD1 may regulate insulin containing granule release from beta cells by directly inducing the expression of genes involved in exocytosis, such as SNAP25, Munc18, syntaxin1 and Sec24D (Cinek et al. 2003; Ishizuka et al. 2007)

p300, a transcriptional coactivator, binds directly to NeuroD1 and enhances the ability of NeuroD1 to activate the insulin gene transcription. Besides interacting with coactivators, E-box transcription factors also bind repressors of insulin transcription. For example, NeuroD1 has been shown to interact with small heterodimer partner (SHP), an orphan nuclear receptor functioning as a transcriptional repressor. The basic leucine zipper transcription factor, c-Jun, inhibits insulin transcription by reducing the transactivation potential of E12/E47 proteins (Robinson et al. 1995).

ii. Homeodomain-containing proteins

Pancreatic duodenal homeobox-1 (Pdx-1), one of the homeodomain-containing family members, binds to A boxes in the insulin gene promoter. The transactivation domain is located at the N-terminus, which is also required for unique protein-protein interactions with other transcription factors such as NeuroD1 and p300. Pdx-1 is first detected in the primitive gut tube around E8.5 during embryonic development of the rodent pancreas and is mainly restricted to beta cells and delta cells in the mature pancreas (Guz et al. 1995). Pdx-1 is essential for pancreatic development, beta-cell differentiation and maintenance of mature beta-cell function. It can regulate several beta-cell genes, such as insulin, GLUT2, islet amyloid polypeptide (IAPP), glucokinase, MafA and somatostatin (Babu et al. 2007). *Pdx-1* knockout mice fail to develop a pancreas and

die shortly after birth (Offield et al. 1996). Heterozygous *Pdx-1* mice have a normal pancreas but are hyperglycemic due to inadequate insulin secretion. A targeted disruption of *Pdx-1* in beta cells causes age-related diabetes, indicating that Pdx-1 is crucial for maintenance of beta-cell function (Ahlgren et al. 1998). These results are concordant with the finding that certain individuals with MODY are heterozygous for an inactivating mutation of *Pdx-1*, thus its designation as the MODY4 gene (Stoffers et al. 1997).

iii. Basic leucine zipper proteins

MafA, a basic leucine zipper family member, controls beta-cell specific insulin gene expression through binding the RIPE3b/C1 element and functions as a potent transactivator of the insulin gene (Figure 1-5). While both NeuroD1 and Pdx-1 are expressed in various types of islet cells, MafA is only expressed in beta cells in islets. During mouse pancreatic development, MafA expression is detected at the late stage of beta-cell development around E13.5 (Matsuoka et al. 2004). *MafA* knockout mice display glucose intolerance and develop age-dependent pancreatic islet abnormalities and diabetes mellitus. It was also observed that in *MafA*-deficient mice, expression of insulin I, II, Pdx-1, NeuroD1 and GLUT2 is decreased, and glucose-, arginine- and KCl-stimulated insulin secretion is severely impaired (Zhang et al. 2005). In addition to regulation of insulin gene expression, MafA also plays a role in modulating the transcription of other genes, such as GLUT2, Nkx6.1 (NK6 homeobox 1), Pdx-1, pyruvate carboxylase, and CHOP.

CCAAT/enhancer-binding protein (C/EBP) also belongs to the basic leucine zipper family and preferentially binds to a consensus sequence of T(T/G)NNGNNAA(T/G) as a

dimer. A putative binding motif for C/EBP (CEB box) is found in rat insulin I, II and human insulin promoters and C/EBP-β binds to the CEB element in these promoters. When beta cells are chronically exposed to high glucose, the expression of C/EBP-β increases and insulin content decreases over time. C/EBP-β inhibits rat insulin I promoter activity by binding to the heptad leucine repeat in the activation domain of E47, which greatly reduces the ability of E47 to bind DNA (Lu et al. 1997).

Another key insulin gene transcription factor is NFAT (nuclear factor of activated T cells), which has five isoforms NFATC1-C5 encoded by different genes in human. NFATC1-C4 proteins are expressed in pancreatic beta cells. NFAT activity is regulated by calcineurin, a Ca^{2+} and calmodulin-dependent phosphatase, through dephosphorylation of numerous sites within the regulatory region. Calcineurin activates NFAT proteins and stimulates their translocation to the nucleus and subsequent DNA binding. Lawrence et al. showed that the synergistic effect of glucose and GLP-1 on insulin gene transcription is mediated through the calcineurin-NFAT signaling pathway in beta cells (Lawrence et al. 2002). Furthermore, transgenic mice with a beta-cell specific deletion of the calcineurin regulatory subunit b1 have reduced beta-cell mass and pancreatic insulin content and develop age-dependent diabetes. These defects can be rescued by conditional expression of active NFATc1 (Heit et al. 2006). In addition, aged *Nfatc2*$^{-/-}$ *Nfatc4*$^{-/-}$ mice display a defect in fat accumulation and remain lean. *Nfatc2*$^{-/-}$ *Nfatc4*$^{-/-}$ mice are also resistant to high fat diet-induced obesity. Deficiency of NFATc2 and NFATc4 increases insulin sensitivity at least through sustained activation of the insulin signaling pathway (Yang et al. 2006).

iv. Synergistic effect of the transcription factors

MafA is a weak transactivator of the insulin promoter when expressed alone, as are Pdx-1 and NeuroD1. However, when these three factors are coexpressed, they synergistically and strongly activate the insulin promoter. Potentiation by Pdx-1 and NeuroD1 is dependent upon MafA, and MafA binding to the insulin enhancer is increased by Pdx-1 and NeuroD1. In addition, neither insulin I nor II mRNA is induced in the liver by infection with an adenovirus harboring a MafA expression construct alone, but both insulin I and II are induced by Ad-MafA combined with Ad-Pdx-1 or Ad-NeuroD1. Larger amounts of Insulin I and II mRNA are induced in the liver if animals are infected with all three. Interestingly, in triply infected streptozotocin-induced diabetic mice, blood glucose level is dramatically reduced (Kaneto et al. 2005). Furthermore, it has been reported that ectopic expression of MafA is sufficient to induce a small amount of endogenous insulin expression in a variety of other cell types such as AR42J pancreatic acinar cells (Matsuoka et al. 2007). Recently, Melton and colleagues identified a specific combination of Neurogenin-3, Pdx-1 and MafA that reprograms differentiated pancreatic exocrine cells in adult mice into cells that remarkably resemble beta cells not only in morphology but also in function (Zhou et al. 2008).

C. Regulation of insulin gene expression by glucose and other nutrients

A variety of factors, such as nutrients, hormones and neurotransmitters, can regulate insulin secretion from beta cells. Among these glucose is the predominant regulator of beta-cell function and coordinately regulates insulin gene transcription, biosynthesis and secretion. Enhancing insulin gene transcription by glucose is conducted

through a number of complementary mechanisms, including modifications and recruitment of transcription factors to regulatory sites, modifications of histones on the promoter, and initiation of transcription. As mentioned above, A3, E1 and MARE are the major glucose-responsive transcription regulatory elements. Upon glucose stimulation, MafA, Pdx-1, NeuroD1/E47 and other factors bind their specific elements, interact with each other and induce a synergistic effect on insulin transcriptional activity.

Changes in glucose levels have been shown to regulate Pdx-1 nuclear localization, DNA binding, interaction with co-regulators and transactivating activity at least through post-translational modifications. Under low glucose conditions, Pdx-1 is localized mainly to the nuclear periphery and interacts with histone deacetylases (HDAC-1 and 2). Increases in glucose disrupt the interaction of Pdx-1 with HDACs and promote its association with the histone acetylase (HAT) p300, accompanied by its relocalization throughout the nucleus. Blocking localization of Pdx-1 to the nuclear periphery, by treatment with the phosphatase inhibitor okadaic acid, disrupts the ability of Pdx-1 to interact with HDACs and promotes its association with the HAT. These findings suggest that Pdx-1 phosphorylation is required for its function as the transactivator of the insulin gene (Andrali et al. 2008). Several signaling pathways including p38, ERK, and PI3K pathways have been implicated in Pdx-1 phosphorylation. However, the exact phosphorylation residues and their physiological significance still remain to be determined.

Glucose has also been shown to control the nuclear localization and transactivating activity of NeuroD1 through post-translational modifications, including phosphorylation by ERK1/2 and glycogen synthase kinase 3 (GSK3β), acetylation by p300 and

glycosylation by an O-linked GlcNAc transferase. In the presence of high glucose, NeuroD1 becomes phosphorylated by ERK2 at multiple sites on its transactivation domain, which is correlated with an increase in NeuroD1 transactivating capacity. Glucose-mediated phosphorylation has also been implicated in regulating NeuroD1 subcellular localization. NeuroD1 localizes mainly in the cytoplasm in low glucose, whereas high glucose causes its translocation into the nucleus, which can be blocked by the MEK (MAP/ERK kinase) inhibitor PD98059 (Petersen et al. 2002; Khoo et al. 2003). Acetylation of NeuroD1 by p300 seems to regulate its DNA binding and transactivating capacity in insulinoma cell lines as well.

Both MafA mRNA and protein levels increase in response to high glucose. Biochemical studies and bioinformatic analyses indicated that Pdx-1, FoxA2, HNF3β, Nkx2.2, NeuroD1 and FoxO1 are critical factors in regulating glucose-dependent MafA expression (Raum et al. 2006). The phosphorylation of MafA at multiple sites by GSK3β leads to its degradation by the proteasome (Han et al. 2007). In addition, phosphorylation of MafA can modulate its binding to the insulin gene promoter.

Besides its major effect on the rate of transcription, glucose markedly stabilizes preproinsulin mRNA. Two elements located in the 3' untranslated region of the mRNA molecule, the conserved UUGAA sequence and a pyrimidine-rich sequence, are proposed to mediate this effect. Stabilization appears to involve glucose-regulated binding of a polypyrimidine tract binding protein to the pyrimidine-rich sequence (Tillmar et al. 2002).

However, chronically elevated glucose becomes toxic to insulin gene expression and secretion and beta-cell survival, which is known as glucotoxicity. Impaired insulin gene expression is reportedly associated with both reduced binding activity and

expression of Pdx-1 and MafA. *In vivo*, Pdx-1 binding activity is decreased in islets from Zucker Diabetic Fatty (ZDF) rats and its expression is also reduced in hyperglycemic rats. The reduction in MafA binding activity in HIT-T15 cells chronically exposed to high glucose is due to a loss of protein expression. Consistently, MafA expression is reduced or lost in certain diabetic models, such as FoxO307 mice (overexpression of constitutively nuclear FoxO1 (forkhead box O1) in liver), insulin receptor mutant mice, and New Zealand obese mice (Nakae et al. 2002; Okamoto et al. 2004; Kitamura et al. 2005).

In addition, C/EBP-β may directly bind E47 and prevent formation of the NeuroD1/E47 activator complex under glucotoxic conditions. Lawrence and colleagues showed that C/EBP-β prevents MafA from binding to its cognate sequence and forms a complex with NFAT in an ERK1/2-dependent manner in beta cells exposed to chronically elevated glucose (Lawrence et al. 2005). Long-term high glucose can cause oxidative stress, thought to be a significant cause of beta-cell dysfunction. Decreased insulin gene transcription and MafA protein expression is prevented by antioxidants in glucotoxic insulin-secreting cells. Importantly, treatment of ZDF rats with antioxidants normalizes plasma glucose levels and restores insulin secretion, insulin content and insulin mRNA levels (Harmon et al. 2005).

As suggested by the above, free fatty acids are another key regulator. Exposure of isolated islets and insulin-secreting cells to elevated levels of fatty acids *in vitro* impairs insulin gene expression when glucose concentrations are concomitantly elevated. The deleterious effects of free fatty acids on insulin gene expression in isolated islets have been ascribed to several factors, such as decreased insulin promoter activity, reduced

DNA binding of Pdx-1, increased fatty acid oxidation leading to the production of ROS, and an accumulation of long-chain fatty acyl-CoAs.

Individual amino acids have variable potency in stimulating insulin secretion. Certain combinations of amino acids can actively promote insulin secretion and increase GSIS. Leucine is believed to stimulate insulin secretion through either the allosteric activation of glutamate dehydrogenase causing oxidation of glutamate to the Krebs cycle intermediate α-ketoglutarate, or the transamination of leucine to α-ketoisocaproate (Panten et al. 1972; Sener and Malaisse 1980). Induction of insulin release by L-Arginine occurs by depolarization of beta cells via the uptake of this charged amino acid through the amino-acid transporter mCAT2A (murine cationic amino acid transporter) (Brosnan 2003). Although the mechanisms by which amino acids influence insulin secretion are not well understood, amino acid uptake and metabolism is interconnected with glucose and lipid metabolism.

Hormones including GLP-1 and gastric inhibitory polypeptide (GIP) potentiate GSIS through elevating intracellular cAMP levels. High levels of cAMP activate protein kinase A (PKA) and cAMP-dependent guanine nucleotide exchange factors EPACs, and induce an increase in intracellular calcium through activation of L-type calcium channels, which trigger insulin secretion (Kang et al. 2001) (figure 1-2). Another important downstream target of GLP-1 is Pdx-1. Increase of Pdx-1 expression caused by GLP-1 has been demonstrated both *in vivo* and *in vitro*. GLP-1 also promotes the cytoplasmic to nuclear translocation of Pdx-1 in a PKA-dependent manner in beta-cell lines (Buteau et al. 1999; Wang et al. 2001). In addition, GLP-1 can increase beta-cell proliferation and decrease beta-cell apoptosis.

III. Signaling pathways in pancreatic beta-cells

A. ERK1/2 pathway in pancreatic beta cells

The MAP kinases are components of highly conserved protein kinase cascades important for transmitting extracellular signals to coordinate cellular responses. MAP kinases have been implicated in many physiological events ranging from cell proliferation and differentiation to cell survival. There are four major groups of MAP kinases in mammalian cells: ERK1/2, JNK, p38, and extracellular signal-regulated kinase 5 (ERK5). MAP kinase pathways feature a core, triple-kinase cascade: the MAP kinases are activated by phosphorylation on Thr and Tyr by dual-specificity MAP kinase kinases (MAP2Ks), which in turn are activated by Ser/Thr phosphorylation by MAP kinase kinase kinases (MAP3Ks). ERK1/2 are often described as activated by mitogenic stimuli such as growth factors and hormones, whereas JNK and p38 are generally activated by stress stimuli. Once activated, MAP kinases can directly phosphorylate overlapping subsets of proteins containing exposed Ser/Thr-Pro motifs in both the cytoplasm and the nucleus (Raman et al. 2007).

ERK1/2 are involved in regulating diverse beta-cell functions. Among these perhaps the most significant function is to control insulin gene transcription. ERK1/2 alter the activity of transcription factors, modulate histone modifications on the insulin gene promoter, and organize the formation of complexes to initiate transcription. In cultured pancreatic beta cells and human islets, ERK1/2 are activated by glucose and other nutrients that induce or potentiate insulin secretion. As mentioned above, glucose

uptake and metabolism cause the production of ATP, which triggers the closure of ATP-sensitive K^+ channels leading to the activation of voltage-dependent Ca^{2+} channels and calcium influx. Elevated intracellular calcium induces insulin gene expression (Frodin et al. 1995; Khoo et al. 2003). Glucose induces ERK1/2 activation in a calcium-dependent manner in beta cells through both calcium influx and the subsequent release of calcium from intracellular calcium stores. Phosphorylation of ERK1/2 is inhibited by agents that interfere with these events, such as the L-type Ca^{2+} channel blocker verapamil. By using calcineurin inhibitors FK506 or cyclosporin A, it was found that the calcium requirement is at least in part due to an essential action of calcineurin on the ERK1/2 pathway. The requirement for calcineurin has been further confirmed by showing that the expression of calcineurin (also known as protein phosphatase 2B) inhibitory protein, a specific calcineurin binding protein, also inhibits ERK1/2 activation (Benes et al. 1998; Rothermel et al. 2000; Arnette et al. 2003). This may be a reason why there is an increase in the incidence of diabetes among post transplantation patients who are taking phosphatase 2B modulating drugs such as cyclosporine A. However, the factor which responds to calcium to initiate the activation of ERK1/2 remains to be identified.

Glucose-induced intracellular calcium oscillations are thought to trigger insulin granule exocytosis (Westerlund et al. 1997). The opening of voltage-sensitive calcium channels admits extracellular calcium into the intracellular pool of free calcium. In addition, glucose stimulation increases phosphoinostide hydrolysis and production of IP_3 (inositol-1,4,5-triphosphate) and DAG (diacylglycerol). IP_3 binds to the ER IP_3 receptors and promotes calcium release from intracellular stores in the ER (Blondel et al. 1994; Vadakekalam et al. 1996). DAG, in synergy with IP_3-induced elevation of intracellular

calcium, activates classical PKC (protein kinase C). The novel PKC is activated by DAG alone. Elevated intracellular calcium augments insulin exocytosis through the activation of serine/threonine kinases mediated by calmodulins (Breen and Ashcroft 1997). Inhibition of Ca^{2+}/calmodulin-dependent protein kinase II impairs nutrient-induced insulin secretion (Wenham et al. 1992).

Glucagon-like peptide 1 (GLP-1) also activates ERK1/2 through events that are sensitive to calmodulin antagonists and FK506. GLP-1 binds to the GLP-1 receptor, a G-protein coupled receptor expressed in many tissues including beta cells. Activation of the GLP-1 receptor stimulates cAMP formation, activates downstream targets, and increases intracellular calcium, which triggers insulin secretion (Arnette et al. 2003). Similarly, it is not understood how GLP-1 leads to the activation of ERK1/2 in beta cells.

It has been demonstrated that ERK1/2 are required for glucose-induced insulin gene transcription in cultured beta cells and in isolated islets. In reporter assays, glucose affects the activity of a luciferase reporter linked with the insulin gene promoter in a biphasic manner. In the first 2-6 h, glucose stimulates the transcription in an ERK1/2-dependent manner, which has been confirmed by inhibition of the effect of glucose by the MEK inhibitor U0126 or by coexpression of a kinase-dead ERK2. After 12 h, glucose has no effect on the insulin gene promoter, and 24 h later, glucose exhibits an inhibitory effect on the promoter activity. Interestingly, the inhibitory effect of glucose is also ERK1/2-dependent (Lawrence et al. 2005).

In analyzing the stimulatory effects of glucose, electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (CHIP) assays showed that ERK1/2 can regulate the transcriptional activity of NeuroD1, Pdx-1, MafA, E2A and NFAT in

beta cells. *In vitro* phosphorylation assays and mutagenesis indicated that NeuroD1, Pdx-1, MafA and E2A are direct ERK2 substrates. ERK1/2 are essential in DNA binding of all five proteins in CHIP assays, suggesting that post-translational modifications of these factors are required in modulating insulin gene expression. In order to understand the inhibitory effects of chronic glucose exposure, Lawrence and colleagues found that long-term glucose stimulation of human islets reduces the binding of MafA and NFAT to the insulin promoter in EMSA experiments. ERK1/2 are required for the binding of the insulin gene repressor, C/EBP-β, to the CEB element in CHIP assays (Lawrence et al. 2005).

Although the mechanisms by which ERK1/2 influence insulin gene promoter activity are not completely understood, it has been suggested that ERK1/2 can regulate insulin gene expression through controlling the localization of transcription factors, modulating their interactions with other cofactors, and altering the DNA binding and transactivation capability. Phosphorylation of transcription factors by ERK1/2 can regulate nuclear localization, which has been suggested for Pdx-1 and NeuroD1. As mentioned before, an important ERK1/2 phosphorylation site in NeuroD1 has been suggested and probable sites in Pdx-1 were also found (Elrick and Docherty 2001; Khoo et al. 2003). In addition, NeuroD1 phosphorylation is required for its dimerization with E2A proteins. Transactivation activity was substantially reduced in NeuroD1 mutants lacking ERK1/2 phosphorylation sites (Lawrence et al. 2008). However, their phosphorylation and exact physiological functions in intact cells remain to be defined.

Histone modifications, such as acetylation, phosphorylation, sumoylation and methylation, are important in controlling gene expression. CHIP assays indicate that

ERK1/2 are components of transcriptional complexes on the insulin gene promoter, where ERK1/2 could either directly phosphorylate the transcription factors and/or histone proteins or function as scaffolding proteins to recruit other components required for insulin transcription (Lawrence et al. 2008).

In order to further identify the functions of ERK1/2 in beta cells, real-time PCR was used to screen genes, whose mRNA changes are ERK1/2-dependent. In the presence or absence of the MEK inhibitor U0126, relative quantitative differences in mRNA of certain genes important in beta-cell function or implicated in the pathogenesis of diabetes were examined, such as CHOP, peroxisome proliferator activated receptor γ (PPARγ), UCP-2, phosphoprotein enriched in astrocytes (PEA-15), NFATc2, hepatic nuclear factor-1beta (HNF1-β), glucokinase, and Pdx-1. Expression of the CHOP gene in pancreatic beta cells was significantly increased in the presence of the MEK inhibitor U0126 to block ERK1/2 activation (Lawrence et al. 2007).

CHOP, also known as growth arrest- and DNA damage-inducible gene 153 (GADD153), is one of the most commonly used indicators of ER stress, which is an important event in triggering beta-cell apoptosis in diabetes. CHOP is present in the cytoplasm under nonstressed conditions, and stress leads to induction of CHOP and its accumulation in the nucleus. Beta cells isolated from CHOP knockout mice are resistant to apoptosis caused by nitric oxide or accumulation of a folding-defective mutant of proinsulin (Oyadomari and Mori 2004). CHOP deletion in *Lepr*$^{db/db}$ mice increases expression of antioxidative stress response genes and decreases expression of proapoptotic genes.

Sequence alignment shows two regions in the CHOP gene, one in the 5' flanking region and one in the first intron, that are similar to the region of the insulin gene that binds MafA. CHIP assays demonstrated that MafA binds to both regions in beta cells isolated from human islets, but not other factors that interact with the insulin gene promoter. Coexpression of the reporter construct coupled to the promoter element with MafA dramatically repressed activity in HEK293 cells. In contrast, activity of the intron element was enhanced by MafA. These findings suggest that MafA expression and regulatory activity is a key step in CHOP regulation in beta cells under various conditions. In addition, C/EBP-β bound to the CHOP gene when beta cells had been chronically exposed to high glucose (Lawrence et al. 2007). This is consistent with the increase in C/EBP-β expression that occurs under high glucose conditions. Interestingly, binding of MafA and C/EBP-β to the CHOP gene is dependent upon ERK1/2 activity.

Nerve growth factor (NGF) was shown to stimulate the activities of these protein kinases in beta cells (Frodin et al. 1995). Insulin, serum, phorbol esters and epidermal growth factor (EGF) also activate ERK1/2. In rat insulinoma cells maintained in the physiological glucose concentration range, IGF-1 (insulin-like growth factor 1) acts synergistically with glucose to enhance proliferation, which appears to be mediated through the sustained activation of downstream signaling pathways including ERK1/2 and PI3K and subsequent activation of mTOR/p70S6K (mammalian Target of Rapamycin/ ribosomal protein S6 protein kinase) (Hugl et al. 1998). Growth factors regulate ERK1/2 in beta cells in a largely calcium-independent manner because FK506 and other calcineurin inhibitors have little effect on ERK1/2 stimulation by growth factors (Arnette et al. 2003).

B. JNK signaling pathway in pancreatic beta cells

JNK isoforms 1-3 are highly related but are encoded by three distinct genes. They can regulate multiple activities through their ability to control gene expression by phosphorylating transcription factors such as c-Jun, a component of activator protein-1 (AP-1). JNK1 and 2 are ubiquitously expressed whereas JNK3 is predominantly expressed in brain, pancreatic islets, heart and testis (Kyriakis and Avruch 2001; Johnson and Lapadat 2002). Recent studies have demonstrated that JNK is a central component in modulating insulin action and in the pathogenesis of both type I and type II diabetes. JNK can directly phosphorylate IRS-1 at several sites including the inhibitory site Ser307 (Aguirre et al. 2000). Therefore, JNK activation can suppress the signaling transduction by the insulin receptor. In fact, JNK1 activity is markedly elevated in muscle, liver and adipose tissue in obese mice. *JNK1*- but not *JNK2*- deficient mice exhibit dramatic protection against insulin resistance and defective insulin receptor signaling (Hirosumi et al. 2002). However, another study showed that disruption of the *JNK2* gene in NOD (non-obese diabetic) mice decreases destructive insulitis and reduces the progression to diabetes (Jaeschke et al. 2005). It seems that both JNK1 and JNK2 are involved in insulin resistance (Tuncman et al. 2006).

JNK interacting proteins (JIPs) are scaffolding molecules that can regulate and localize JNK. Increased JNK activity caused by a point mutation in the human homolog of *JIP1*, also known as islet brain 1 (IB1), has been identified in a rare Mendelian form of diabetes in humans, providing crucial genetic support of the role of JNK in the pathogenesis of type II diabetes (Waeber et al. 2000). *JIP1* knockout mice fail to activate

JNK in adipose tissue and display resistance to the effects of a high-fat diet on obesity and insulin resistance (Jaeschke et al. 2004). IB1 also functions as a transactivator of the insulin and GLUT2 genes, indicating that regulation of JNK activity by IB1 could be an important mechanism in controlling pancreatic gene expression (Bonny et al. 1998).

It was reported that the JNK pathway is activated and preceds the decrease of insulin gene expression in isolated rat islets exposed to oxidative stress. Suppression of the JNK pathway can protect beta cells from oxidative stress. Administration of a JNK inhibitor results in lower blood glucose and higher insulin levels in the leptin-receptor-deficient model of diabetes (Bennett et al. 2003). Ozan and colleagues have shown that obesity causes ER stress, which leads to suppression of insulin receptor signaling through hyperactivation of JNK (Ozcan et al. 2004). Adenoviral overexpression of dominant-negative JNK1 protects insulin gene expression and secretion from oxidative stress. Moreover, overexpression of wild type JNK suppresses both insulin gene expression and secretion (Kaneto et al. 2002). These results correlate with changes in the binding of the important transcription factor Pdx-1 to the insulin promoter. Adenovial overexpression of dominant negative JNK preserved Pdx-1 DNA binding activity in the presence of oxidative stress, while wild type JNK overexpression decreased Pdx-1 DNA binding activity and insulin gene expression. As a potential mechanism for JNK-mediated Pdx-1 inactivation, it was recently reported that Pdx-1 was translocated from the nucleus to cytoplasm in response to oxidative stress in HIT-T15 cells.

Oxidative stress and activation of JNK can alter the expression of genes associated with diabetes, which may be mediated through transactivation of c-Jun/AP-1 but also via the regulation of other factors. For example, there is evidence that JNK can

phosphorylate and thereby downregulate the activity of PPARγ (Camp et al. 1999). In addition, ROS-activated JNK is believed to mediate the inhibition of Bcl_2 and Bcl_{XL} (BCL2-like 1) families of apoptosis suppressor proteins, either directly or through the phosphorylation of an allosteric modulator such as BAD (Bcl-xL/Bcl-2-associated death promoter protein) (Donovan et al. 2002) (Figure 1-6). This leads to the permeabilization of the mitochondral membrane and release of cytochrome c. It has been demonstrated that inhibit of JNK by small peptides can protect beta cells from apoptosis (Bonny et al. 2001).

C. NF-κB signaling pathway in pancreatic beta cells

The transcription factor NF-κB is crucial in diverse cellular processes, such as inflammation, immunity, cell proliferation and apoptosis. Five members of the NF-κB family, p50, p52, p65 (RelA), c-Rel and RelB, form various homo- and heterodimers. Dimers of these NF-κB subunits produce different DNA-binding specificities and transcriptional potential (Hayden and Ghosh 2008). In the resting state, inactive NF-κB complexes are sequestered in the cytoplasm via their non-covalent interaction with inhibitory proteins called IκBs. Upon stimulation, IκB proteins can be phosphorylated by the IKK (IκB kinase) complex and degraded by the proteasome. NF-κB dimers then translocate into the nucleus to regulate the expression of target genes through binding to the specific NF-κB binding sites on the promoter.

In pancreatic beta cells, NF-κB is a central component in various networks that control different processes, such as attraction and activation of immune cells and ER stress-induced apoptosis. However, the role of NF-κB in the pathogenesis of type I

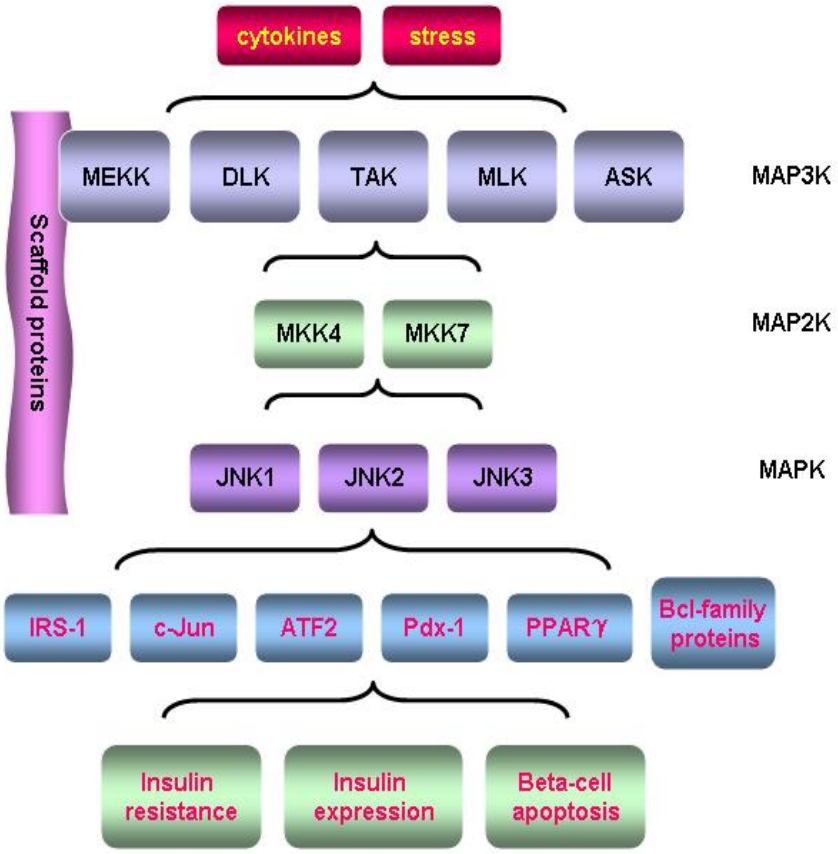


Figure 1-6 Schematic representation of JNK signaling pathway.

diabetes, whose activity is mainly stimulated by TNF-α and IL-1β, has been controversial. Most reports have described a proapoptotic effect of NF-κB in pancreatic beta-cell death in the presence of IL-1β, TNF-α and/or IFNγ, whereas the development of autoimmune diabetes is accelerated in the NOD transgenic mouse expressing a degradation-resistant form of IκBα in beta cells (Eldor et al. 2006; Kim et al. 2007). Indeed, in most other cell types NF-κB exhibits antiapoptotic function. However, the NF-κB inhibitor sodium salicylate has been shown to protect cultured human islets against apoptosis (Zeender et al. 2004). Thus, the ultimate effects of NF-κB activation may rely on the level of activation, the expression of other genes, and tissue or culture conditions.

Recent studies have also revealed the role of NF-κB in insulin resistance during the development of type II diabetes. It has been demonstrated that IKKβ is the main subunit of the IKK complex in mediating proinflammatory signal-induced phosphorylation of IκBα, leading to the degradation of IκBα and the consequent activation of NF-κB (Shoelson et al. 2006). Yuan and colleagues found that increased IKK activity promotes insulin resistance in obese rodents, and that a reduction in either IKK activity or expression (via disrupting a single IKKβ allele) is able to reverse the insulin resistance (Yuan et al. 2001). In addition, activation of NF-κB by selectively overexpressing constitutively active IKKβ in liver causes moderate systemic insulin resistance. On the contrary, attenuation of NF-κB by overexpression of IκBα in the liver not only diminishes the expression of NF-κB-dependent genes, but also reverses the phenotypes of type II diabetes (Cai et al. 2005). Systemic neutralization of IL-6 exhibits a significant improvement in insulin resistance in the transgenic mice overexpressing IKKβ in the liver. Larsen et al. have reported that anakinra, a receptor antagonist of the inflammatory

cytokine IL-1, improved blood glucose control and insulin secretion in patients with type II diabetes (Maybee et al. 2007). Thus, these results suggest that NF-κB and its downstream target genes, such IL-1, IL-6 and TNF-α, are critical in the development of insulin resistance. At the same time, NF-κB may be essential for compensatory expansion of beta-cell mass during the loss of insulin sensitivity. NF-κB has also been implicated as a required transcription factor for GLUT2, which contributes to glucose-stimulated insulin secretion by beta cells. In conclusion, the complex nature of NF-κB action suggests that suppression of NF-κB may be beneficial on insulin resistance and type II diabetes, in spite of the fact that it also exacerbates type II diabetes.

D. SUMO modification in pancreatic beta cells

SUMO proteins belong to the family of ubiquitin-like peptides. SUMO-1 shares only about 20% amino-acid sequence identity with ubiquitin; nevertheless, the structure of SUMO-1 resembles the three dimensional structure of ubiquitin. However, SUMO-1 has different surface charge distribution, which undoubtedly accounts in part for the marked functional distinctions from ubiquitin. SUMO is widely expressed and evolutionarily conserved among diverse species. Some organisms, such as yeast, have a single SUMO protein; whereas the human genome encodes four isoforms, SUMO-1-4. Among them, SUMO-2 and 3 are highly homologous and functionally equivalent. SUMO-1 - 3 are ubiquitously distributed; but SUMO-4 is only found in the immune system and kidney (Hay 2001; Geiss-Friedlander and Melchior 2007). Hundreds of proteins have been reported as SUMO modification targets; the majority are nuclear

proteins. A consensus sumoylation motif, ψKXE (ψ is a large hydrophobic amino acid and X is any amino acid), has also been identified.

Like ubiquitination, sumoylation results in the formation of an isopeptide bond between the C-terminal Gly residue of SUMO and the ε-amino group of a Lys residue in the target protein. Sumoylation requires a cascade involving three classes of enzymes. The first step is the activation of a mature SUMO protein by the SUMO-specific E1 activating enzyme composed of AOS1 and UBA2 (ubiquitin-like modifier activating enzyme 2). SUMO is then transferred from UBA2 to the E2 conjugating enzyme Ubc9 (ubiquitin-conjugating enzyme 9). Finally, Ubc9 transfers SUMO to the substrate and an isopeptide bond is formed, facilitated by SUMO E3 ligases (Figure 1-7). In contrast to ubiquitination, SUMO E3 ligases are not essential for sumoylation, but enhance the efficiency of this reaction (Guo et al. 2007; Liu and Shuai 2008). In most cases, sumoylation results in the addition of a single SUMO molecule to an individual Lys residue. However, the formation of poly-SUMO chains of SUMO-2/3 has been reported both *in vivo* and *in vitro*. Recently, SUMO-1 was also shown to form chains *in vitro*. It remains unknown what the functional significance of SUMO chains are. Modification by SUMO is a reversible process, which is facilitated by specific proteases, sentrin-specific proteases SENP1-3 and SENP5-7 (Mukhopadhyay and Dasso 2007).

SUMO modification of a given target could regulate many functions of the protein, such as localization, stability, activity, or protein-protein interactions. The majority of known SUMO substrates are transcription factors. Sumoylation has been reported to have both positive and possible negative effects on their transcriptional activities. Based on the analyses of amino-acid sequences, several insulin gene transcription factors, such as

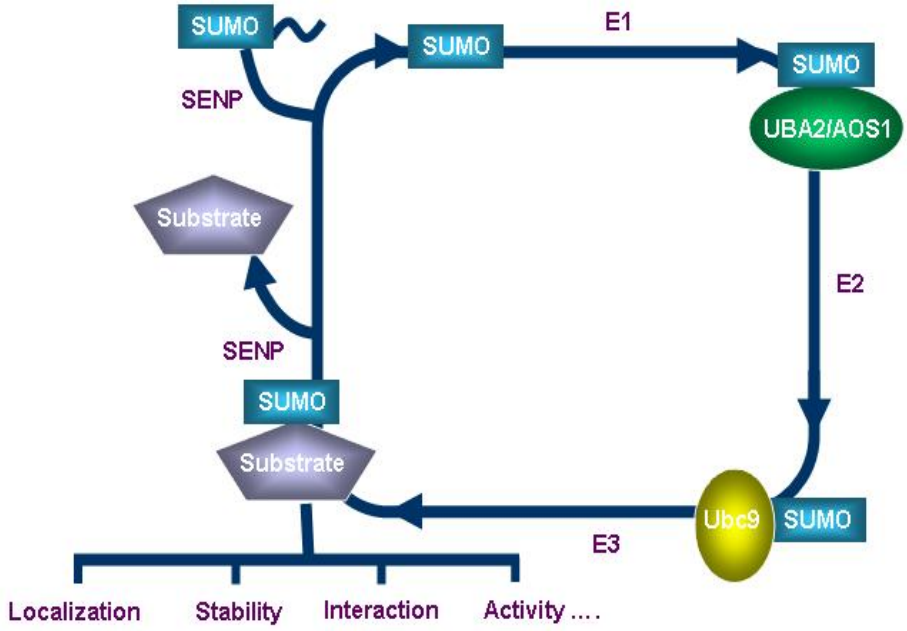


Figure 1-7 Schematic representation of the mechanism of sumoylation/desumoylation.

NFAT, Pdx-1 and MafA, contain putative SUMO modification consensus motifs. NFAT is activated in response to T cell activation signals. Treatment with phorbol 12-myristate 13-acetate (PMA) and ionomycin induces SUMO modification of NFAT1 on residues K684 and K897. Among them, sumoylation of K684 is required for NFAT1 transcriptional activity and subsequent sumoylation of K897, whereas sumoylation of K897 is only required for nuclear retention of NFAT1 (Terui et al. 2004). However, sumoylation of NFAT and other insulin gene transcription factors and the biological significance in pancreatic beta cells remain to be identified.

The autoimmune destruction of beta cells in the development of type I diabetes requires the expression of certain immune-related genes, such as proinflammatory cytokines, chemokines and adhesion molecules, the majority of which are regulated by NF-κB pathway. Furthermore, cytokines are important in mediating beta-cell death mainly through Janus Kinases (JAK)/Signal Transducers and Activators of Transcription (STAT) and NF-κB signaling pathways. As one of the key posttranslational modifications of these modulators, sumoylation could be implicated in the onset of type I diabetes.

Chapter Two: Sumoylation Regulates the Transcriptional Activity of MafA in Pancreatic Beta Cells.

I. Abstract

MafA is a transcriptional regulator expressed primarily in pancreatic beta cells. It binds to the RIPE3b/C1 binding site within the insulin gene promoter, which plays a critical role in regulating insulin gene expression in response to glucose. Here, I show that MafA is post-translationally modified by SUMO-1 and 2 in both beta cells and HEK293 cells. Mutation of a single site in MafA, lysine 32, blocks its SUMO modification. Incubation of beta cells in low glucose (2 mM) or exposure to hydrogen peroxide increases sumoylation of MafA. Forced sumoylation of MafA results in reduced transcriptional activity towards the insulin gene promoter and increased suppression of the CHOP gene promoter. Sumoylation of MafA has no apparent effect on its nuclear localization in beta cells, its DNA binding to the insulin promoter, or its ubiquitin-dependent degradation. These studies suggest that modification of MafA by SUMO modulates gene transcription and thereby beta-cell function.

II. Introduction

Insulin is essential for maintaining glucose homeostasis and is synthesized in and secreted from beta cells in the islets of Langerhans. Glucose sensitive *cis*-regulatory elements on the insulin gene promoter are critical in regulating insulin gene expression (German et al. 1995). Several transcription factors have been identified that stimulate insulin gene transcription through A and E boxes of the promoter, including Pdx-1 and Beta2/NeuroD1, both MODY genes (Malecki 2005). MafA also stimulates insulin gene transcription by binding to the RIPE3b/C1 glucose-sensitive element, a Maf-recognition element (MARE) (Olbrot et al. 2002; Matsuoka et al. 2003). MafA belongs to the family of large Maf proteins, basic leucine zipper transcription factors. MafA contains a transactivation domain at its N terminus, a DNA binding domain at its C terminus, and homodimerizes through its basic leucine zipper domain. Within pancreatic islets MafA expression is limited to beta cells and is involved in transcription not only of the insulin gene but also of other genes involved in beta cell-specific functions (Aramata et al. 2007). As might be expected for a regulator of insulin gene transcription, MafA-deficient mice display glucose intolerance and develop diabetes, although impaired insulin secretion appears to be the primary defect (Zhang et al. 2005). Additionally, islet structure is abnormal in these mice. Together these findings indicate that MafA is required for the development and maintenance of mature insulin producing pancreatic beta cells.

Both transcription and post-translational modifications have been implicated in the regulation of MafA under diverse conditions. Transient exposure of beta cells to high

glucose has been reported both to increase and decrease MafA mRNA and protein (Zhao et al. 2005; Ye et al. 2006), while chronic exposure to high glucose or lipids has been reported to decrease MafA protein with or without a loss of MafA mRNA (Hagman et al. 2005; Harmon et al. 2005). Glucose and oxidative stress are reported to regulate MafA expression at the transcription level through FoxO1 (Kitamura et al. 2005). FoxA2, HNF3β, Nkx2.2 (NK2 homeobox 2), and Pdx-1 modulate MafA expression through conserved sequences in the distal region of the MafA promoter (Raum et al. 2006). Phosphorylation is thought to be critical for MafA transcriptional activity. *In vitro* kinase assays suggest that MafA may be phosphorylated by ERK2 and p38 MAP kinases (Benkhelifa et al. 2001; Sii-Felice et al. 2005). Two groups observed that following phosphorylation of Ser65 of MafA, it is sequentially phosphorylated on Ser61, Thr57, Thr53, and Ser49 by glycogen synthase kinase 3β (GSK3β) (Han et al. 2007; Rocques et al. 2007). Phosphorylation on Ser14 has also been reported (Benkhelifa et al. 2001). MafA phosphorylation may also lead to its ubiquitination and degradation by the proteasome (Rocques et al. 2007). These findings suggest that multiple covalent modifications of MafA control its function.

Covalent post-translational modification with SUMO regulates diverse cellular processes, including DNA repair, cell cycle, gene transcription, and nucleocytoplasmic transport (Gill 2005; Kerscher et al. 2006). Mammals express four SUMO isoforms. Sumoylation with all isoforms occurs in a stepwise process that involves a cascade of SUMO specific enzymes, an E1 activating enzyme, an E2 conjugating enzyme, and an E3 ligase. SUMO proteases remove SUMO from their targeting proteins. Sumoylation is dynamic, with substrates undergoing rapid conjugation and deconjugation. Only small

fractions of the substrates are thought to be subjected to sumoylation at steady state, although the underlying mechanism is still in question (Kerscher et al. 2006). Among the best characterized SUMO substrates are transcription factors, many of which display repressed transcriptional activity upon sumoylation (Kerscher 2007). It has been proposed that sumoylation of transcription factors contributes to the assembly of promoter complexes and the recruitment of chromatin-modifying enzymes (Yang and Gregoire 2006).

Several insulin gene transcription factors have consensus sumoylation motifs (ψKXE); these include Pdx-1, C/EBP-β, NFAT, and MafA. Modification of C/EBP-β and NFAT1 by SUMO-1 is important in regulating their transcriptional activity and/or localization (Eaton and Sealy 2003; Terui et al. 2004). However, little is known about the sumoylation of insulin gene transcription factors or the possible biological significance of this modification in beta cells.

Based on the amino acid sequence, two sumoylation consensus motifs, VK32KE and LK296LE, are present on MafA. Because I am examining mechanisms of glucose-induced changes in transcription in beta cells, I tested the idea that SUMO modification of MafA may affect insulin gene expression and beta cell function. Herein, I provide evidence that MafA is subjected to SUMO-1 or 2 modifications in beta cells. Hypoglycemia and oxidative stress are potential modulators of MafA sumoylation. Sumoylation regulates MafA transcriptional activity towards both insulin and CHOP gene promoters. These findings demonstrate that MafA is sumoylated in beta cells and suggest that sumoylation impacts beta-cell function.

III. Materials and methods

Cell culture and harvest. Early passages of the pancreatic beta cell lines, rat INS-1 and mouse Min6, were kindly provided by Chris Newgard (Duke University) and Gene Webb (University of Chicago), respectively. Cells were maintained as described (Lawrence et al. 2007). Briefly, INS-1 cells were cultured in RPMI1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 11 mM glucose, 2 mM glutamine, 1 mM sodium pyruvate, 50 μM beta-mercaptoethanol and 100 μg/ml each of penicillin and streptomycin at 37 ˚C. Min6 cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 25 mM glucose and the same supplements as for INS-1 cells. Cells at about 80% confluence were incubated for 2 h in Krebs-Ringer bicarbonate HEPES (KRBH) buffer containing 2 mM glucose and 0.1% bovine serum albumin (BSA) before stimulation with 30 mM glucose for Min6 cells and 25 mM glucose for INS-1 cells. After treatment with the reagents indicated in figure legends, medium was removed, and cells were washed with cold phosphate-buffered saline (PBS) and harvested in cold lysis buffer (50 mM HEPES, pH 7.5, 0.15 M NaCl, 1% Triton X-100, 0.2 mg/ml phenylmethylsulfonyl fluoride (PMSF), 0.1 M NaF, 2 mM Na_3VO_4, 10 μg/ml aprotinin, 5 μg/ml pepstatin A, 5 μg/ml leupeptin, and 20 μM N-ethylmaleimide). After 15 min on ice, lysates were sedimented for 15 min at 16,000 x *g* in a microfuge at 4°C. Supernatants were stored at –80°C until further analysis. Protein concentrations were measured using the BioRad Bradford reagent. HEK293 cells were grown in DMEM containing 10% FBS, 1 mM L-glutamine, and 100 units/ml penicillin and streptomycin at 37°C in 10% CO_2. The procedure of harvesting HEK293 cells is similar to beta cells.

Materials. The Dual-Luciferase Reporter Assay System and passive lysis buffer were purchased from Promega (Madison, WI). The ERK1/2 antibody Y691 was as described (Boulton and Cobb 1991). The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): c-Maf (M-153), SUMO-1, GFP, rabbit IgG, and Myc. The following reagents were purchased from Sigma-Aldrich (St Louis, MO): hydrogen peroxide, ionomycin, anisomycin, N-acetyl-L-cysteine, catalase, N-ethylmaleimide. A vector harboring full-length human MafA was kindly provided by Michael German (University of California, San Francisco). The plasmid expressing K32R mutant MafA was provided by Michael Lawrence. The MafA coding sequence was subcloned into pCMV5 with an N-terminal myc tag. Vector pCS2 containing N-terminal GFP-SUMO-1, SUMO-1-ΔGG, SUMO-2, HA-SUMO-1-specific peptidase 2 (SENP2), FLAG-Ubc9 and C93S dominant negative Ubc9 were gifts from Hongtao Yu (University of Texas Southwestern Medical Center). HA-SUMO-1 in pSFFV was a gift from Kim Orth (University of Texas Southwestern Medical Center). Site-directed mutagenesis was done with the QuikChange kit (Stratagene) according to the manufacturer's instructions. Complementary oligonucleotides from regions –320 to –300 bp of the CHOP gene, and A2E1 and MARE regions of the insulin gene were synthesized (Integrated DNA Technologies, Coralville, IA) and blunt-end ligated as 3X repeats into the SmaI site of the pGL3-Luc reporter (Promega). All constructs and mutants were confirmed by sequencing.

Immunoprecipitation and immunoblotting. Aliquots of cell extracts containing 20 μg of protein were subjected to SDS/polyacrylamide gel electrophoresis. Proteins were electrotransferred to nitrocellulose membranes (Millipore, Billerica, MA) and blocked

with 5% nonfat powdered milk in 20 mM Tris, pH 7.5, 0.15 M NaCl (TBS) containing 0.1% Tween-20 for 2 h at room temperature and incubated with primary antibody overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody diluted 1:3000 in TBS for 30 min at room temperature. Proteins were detected by enhanced chemiluminescence. For densitometric analyses, films were scanned by ScanJet 5300C and the density of each band was measured by using multi Gauge V2.3 software. For immunoprecipitation, lysates (0.5-1 mg) were incubated with the indicated antibody and protein A Sepharose beads overnight at 4°C. The beads were washed 3X with 25 mM Tris, pH 7.4, 0.1 M NaCl, and 0.1% Triton X-100.

Transfections and reporter assays. INS-1, Min6, or HEK293 cells were grown in 6-well plates to 80% confluence and cotransfected with either pGL3-MARE, pGL3-CHOP (–320 to –300 bp) or pGL3-A2E1, together with pRL-SV40, and the indicated plasmids using the FuGENE HD reagent (Roche Molecular Biochemicals). After 48 h, cells were harvested with passive lysis buffer that was supplemented with 100 mM beta-glycerophosphate, 2 mM Na_3VO_4, 100 mM NaF, 10 µg/ml aprotinin, 5 µg/ml pepstatin A, 5 µg/ml leupeptin, 0.2 mg/ml PMSF. The lysates were frozen in liquid nitrogen, thawed, and then vortexed for 30 s prior to centrifugation at 16,000 x *g* for 15 min at 4°C. Promoter activity in the supernatants was assayed with the Dual Luciferase Assay System using a TD-20/20 bioluminometer (Turner Designs) and protein expression was assessed by immunoblotting.

Immunofluorescence. Cells were plated onto cover slips in 12-well dishes and transfected with the indicated plasmids. After 48 h, cells were fixed with ice-cold methanol for 1 h at -20 °C, rinsed three times with PBS, and permeabilized with 0.2%

Triton X-100 in PBS for 15 min at 4 °C. Prior to addition of antibodies, cells were incubated in 0.1% Triton X-100, 4% BSA in PBS for 2 h. Primary antibody in the same solution was incubated with cells overnight at 4 °C. After washing, cells were incubated with secondary antibody (1:5000) and DAPI (4',6-diamidino-2-phenylindole) (1:10000) for 1 h. Fluorophores were visualized using a Deltavision RT deconvolution microscope (Applied Precision, Issaquah, WA). Z-stacks were deconvolved and processed with Image J software (Rasband, W.S., Image J, National Institutes of Health, Bethesda, Maryland, USA, 1997-2006).

Subcellular fractionation. Min6 cells transfected with the indicated plasmids were washed with PBS, and harvested in hypotonic lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, 1 mM NaF, 20 μM N-ethylmaleimide) with other protease inhibitors as above. After swelling for 15 min on ice, NP-40 was added giving a final concentration of 0.6% followed by vortexing for 10 sec. A cytosolic fraction was obtained by centrifugation at 5000 x *g* for 5 min. The pellet was resuspended in hypertonic extraction buffer (20 mM Hepes, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM NaF, 20 μM N-ethylmaleimide) plus 1 mM PMSF and other protease inhibitors as above and incubated on ice for 30 min with vortexing every 5 min. The nuclear extract supernatant was obtained by sedimenting the insoluble material at 16,000 x *g* for 15 min.

Electrophoretic mobility shift assays (EMSA). Complementary oligonucleotides containing wild type MARE (5'- TACAGCTTCAGCCCCTCTCGCCATC) and mutated MARE (5'- TACAGCTGACTACCCTCTCGCCATC) consensus sites of the rat insulin I gene promoter were synthesized (IDT Integrated DNA Technologies). The

oligonucleotides were hybridized and end-labeled with T4 polynucleotide kinase (New England Biolabs) in the presence of [γ-^{32}P]ATP. Equal amounts of nuclear extract proteins (20 μg) were incubated for 30 min with double-stranded ^{32}P-labeled MARE probe (20,000 cpm) in reaction buffer (10 mM Tris, pH 8.0, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 6% glycerol, 20 μM N-ethylmaleimide). As indicated, antibodies were added 2 h before incubating with the labeled probe. The reactions were subjected to electrophoresis on 5% non-denaturing polyacrylamide gels and bands were detected on film by autoradiography.

Statistical analysis. Results are expressed as means ± SEM determined from at least three independent experiments. Statistical significance was calculated by one-way ANOVA followed by two-by-two comparisons with post-hoc adjustments as indicated in the legends.

IV. Results

A. MafA is modified by SUMO-1 in pancreatic beta cells.

Previous studies showed that Maf family members MafG and MafB can be modified by SUMO (Motohashi et al. 2006; Tillmanns et al. 2007). Because there are two sumoylation consensus motifs, VK32KE and LK296LE, in the activation and DNA binding domains of MafA (Figure 2-1A), I investigated whether MafA undergoes sumoylation in pancreatic beta cells. In beta cells cultured in standard medium, no slowly migrating products, typical of ubiquitin- or SUMO- protein conjugates, were detected by

immunoblotting. Because only a relatively small percentage of SUMO substrates are usually found sumoylated *in vivo*, I transiently expressed epitope-tagged MafA in both Min6 and INS-1 cells. Upon coexpression of myc-MafA with HA- or GFP-SUMO-1, I observed a myc-MafA species of apparent molecular weight increased by an amount expected upon modification by SUMO-1 (Figure 2-1B, Figure 2-2C). Under these conditions, a relatively small amount of MafA was modified. The more slowly migrating band was absent when myc-MafA was coexpressed with SUMO-1-ΔGG, which lacks the C-terminal diglycine motif and cannot be conjugated to substrates (Figure 2-1B). To provide additional evidence that the slowly migrating band is indeed the SUMO-1 modified form of MafA, INS-1 cells were cotransfected with myc-MafA and GFP-SUMO-1 and MafA was immunoprecipitated with the anti-Maf antibody. Immunoblotting with an antibody to SUMO-1 detected GFP-SUMO-1 linked to MafA in cells transfected with SUMO-1 but not in cells transfected with SUMO-1-ΔGG (Figure 2-1C). A comparable profile of MafA sumoylation was observed in HEK293 cells (Figure 2-14). The extent of MafA sumoylation was similar in the presence or absence of overexpression of Ubiquitin-like conjugating enzyme 9 (Ubc9), a SUMO conjugating enzyme (E2) that activates the sumoylation pathway (Figure 2-1D). This indicates that Ubc9 is not rate-limiting, and suggests that a SUMO E3 ligase would be required to enhance the efficiency of MafA sumoylation. In contrast, coexpression of dominant negative Ubc9 or the SUMO specific protease SENP2 reduced detection of sumoylated MafA (Figure 2-1D). Thus, SUMO-1 can be covalently coupled to MafA in beta cells.

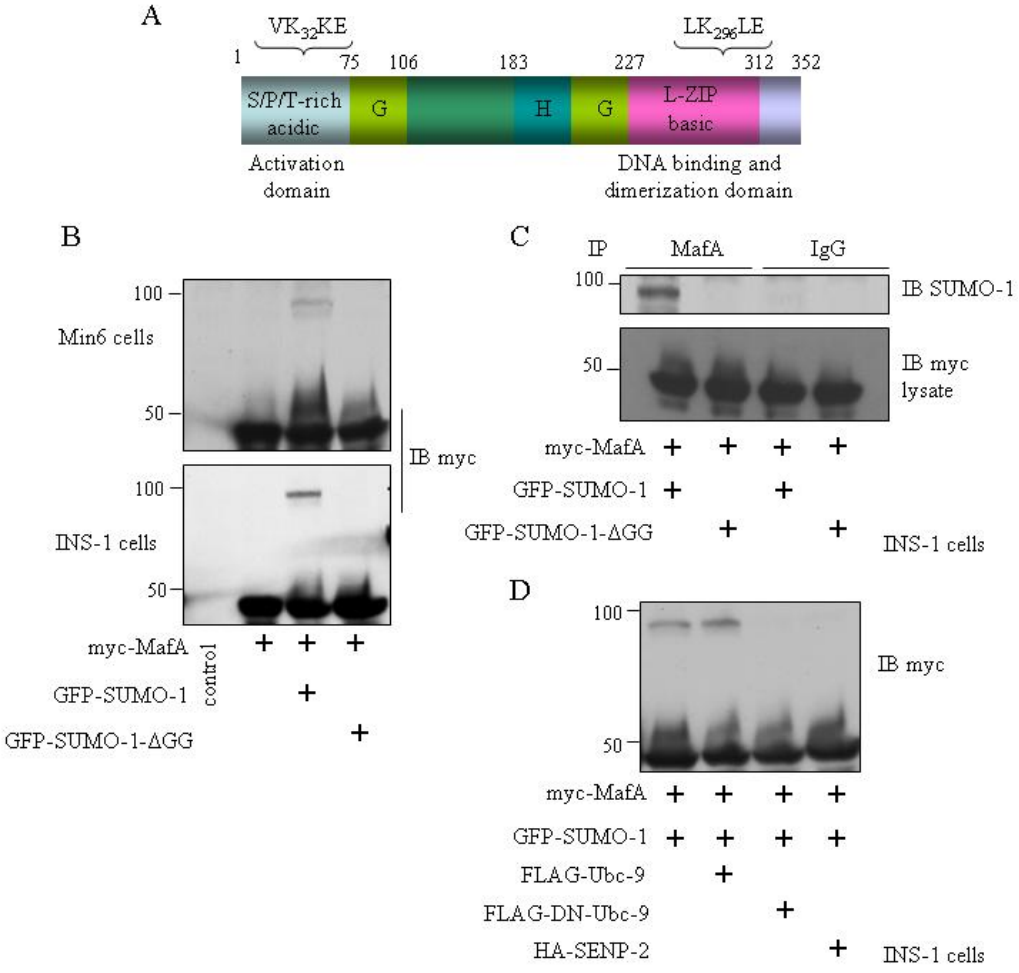


Figure 2-1. Sumoylation of MafA in pancreatic beta cells.
A. Human MafA with its known domains and functions. VK32KE and LK296LE are two consensus sumoylation motifs. B. Myc-MafA was coexpressed with GFP-SUMO-1 or GFP-SUMO-1-ΔGG in beta cells. Cell extracts were immunoblotted with an antibody to myc. C. Lysates from INS-1 cells transfected with the indicated plasmids were immunoprecipitated with anti-c-Maf or control IgG and then detected by immunoblotting with an antibody to SUMO-1. The lower panel shows an anti–myc immunoblot of the lysates. D. Lysates from INS-1 cells transfected with the indicated plasmids were immunoblotted with an antibody to myc. Experiments in each panel were repeated at least three times.

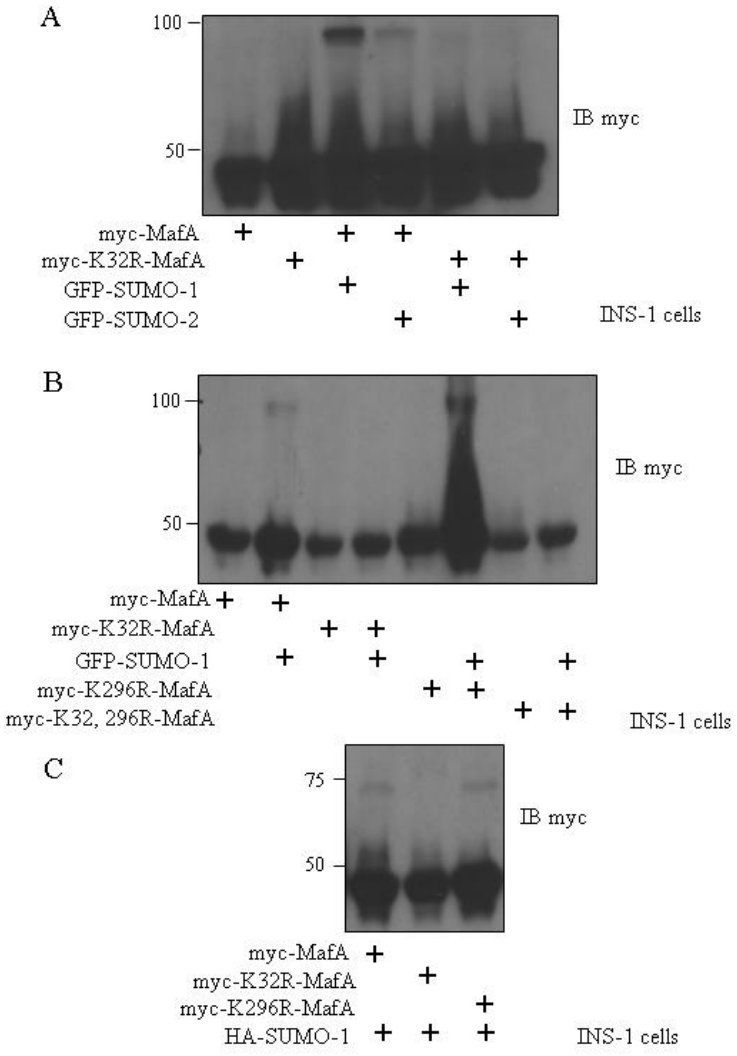


Figure 2-2. MafA mutants in pancreatic beta cells.
A-C. Immunoblot with an antibody to myc of the lysates from INS-1 cells expressing wild type myc-MafA, K32R, K296R, or K32, 296R mutant of MafA and GFP-SUMO-1, GFP-SUMO-2, or HA-SUMO-1 as indicated. All experiments were repeated three or more times.

B. MafA is modified by SUMO-1 and SUMO-2 on Lys32.

SUMO-1 consists of 101 amino acids and shares about 50% sequence identity with SUMO-2/3 (Ulrich 2005). To test whether MafA could be modified by SUMO-2, I coexpressed myc-MafA and GFP-SUMO-2 in INS-1 cells. Immunoblotting with an antibody to myc showed that MafA was modified by SUMO-2, but to a lesser extent than by SUMO-1 (Figure 2-2A). To identify the sumoylation sites, K32R MafA, K296R MafA, and the double mutant were made by site-directed mutagenesis. Sumoylation of K32R and K32R/K296R MafA was no longer detected in the presence of overexpressed GFP-SUMO-1 or SUMO-2. However, K296R MafA was still modified (Figure 2-2B). Coexpression with HA-SUMO-1 showed similar results (Figure 2-2C). These data are consistent with the conclusion that K32 is the major site of sumoylation on MafA in pancreatic beta cells.

C. Exposure of pancreatic beta cells to low glucose increases sumoylation of endogenous MafA.

To establish the relevance of MafA sumoylation in beta cell function, I examined the effect of varying glucose concentration on endogenous MafA sumoylation. Immunoblotting of immunoprecipitated MafA with SUMO antibodies showed a small amount of endogenous sumoylated MafA in both Min6 and INS-1 cells maintained in standard medium (i.e., in 25, and 11 mM glucose, respectively) (Figure 2-3A). I confirmed that the 100 kD MafA species was sumoylated by immunoprecipitating with a

SUMO-1 antibody and immunoblotting with a Maf antibody (Figure 2-3B). As a negative control, normal rabbit IgG did not immunoprecipitate these proteins (Figure 2-3C). Placing beta cells in 2 mM glucose for 2 h enhanced MafA sumoylation. Sumoylation increased further with longer times in low glucose (Figure 2-3A, B). These conditions also dramatically reduced the total amount of MafA, consistent with previous reports. Subsequent stimulation of beta cells with 30 mM glucose for 15 min following incubation in 2 mM glucose did not decrease the amount of sumoylated MafA (Figure 2-3D). These results indicate that varying the glucose concentration can affect the extent of MafA sumoylation in pancreatic beta cells.

D. Oxidative stress enhances MafA sumoylation in pancreatic beta cells.

Protein sumoylation can be induced by heat shock, oxidative stress, electric stimulation, and ethanol in some systems (Bossis and Melchior 2006; Geiss-Friedlander and Melchior 2007). To identify factors which could affect MafA sumoylation in pancreatic beta cells, I exposed beta cells to different stimuli, including KCl, anisomycin, ionomycin, hydrogen peroxide, TNF-α, heat shock, and ethanol. Only hydrogen peroxide stimulated the sumoylation of overexpressed MafA to a measurable extent in both Min6 and INS-1 cells (Figure 2-4A and data not shown). To explore this finding further, I tested effects of different concentrations of hydrogen peroxide (0.1, 0.5, and 2 mM) and different times of exposure (3, 10, and 30 min). As little as 0.1 mM hydrogen peroxide increased MafA sumoylation, the greatest effect observed after 10 min (Figure 2-5A). That the band was the sumoylated form of MafA was confirmed by immunoblotting of

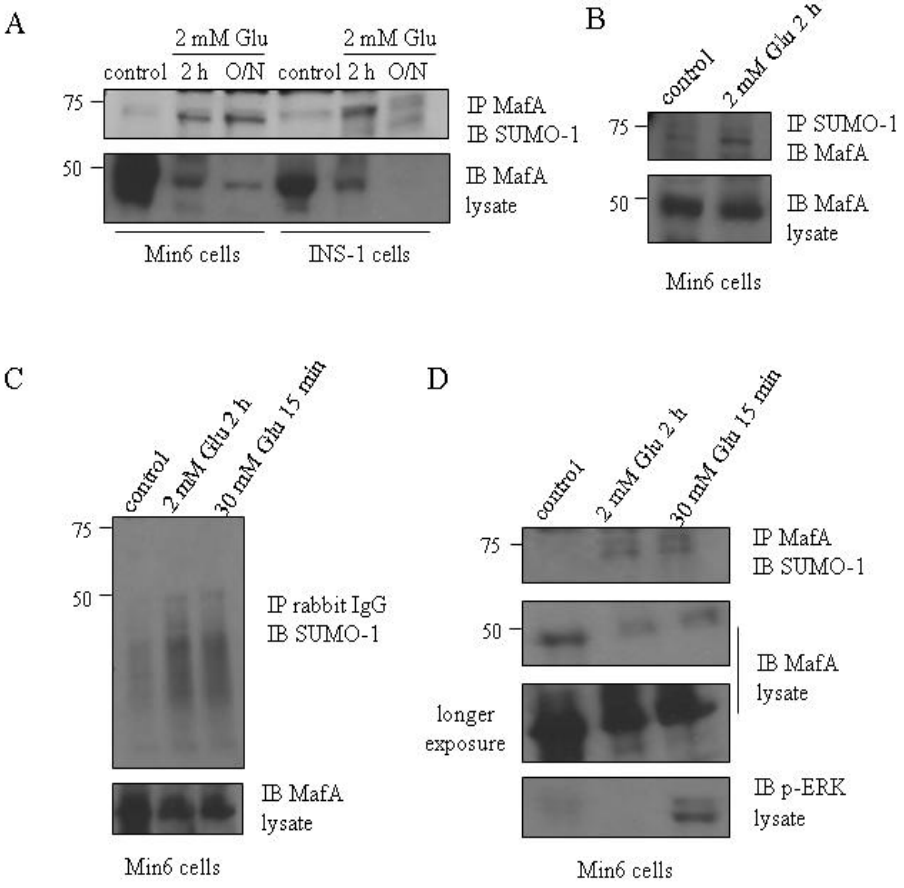


Figure 2-3. Effects of glucose on MafA sumoylation in pancreatic beta cells.
A. Lysates from INS-1 or Min6 cells placed in 2 mM glucose for 2 h or overnight were immunoprecipitated with an antibody to c-Maf and immunoblotted with an antibody to SUMO-1. The lower panel shows an anti–Maf immunoblot of the lysates. B. Lysates from Min6 cells starved for 2 h were immunoprecipitated with an antibody to SUMO-1 and immunoblotted with an antibody to c-Maf. The lower panel is an anti–Maf immunoblot of the lysates. C, D. Lysates from Min6 cells starved for 2 h or stimulated with 30 mM glucose for 15 min after starvation were immunoprecipitated with an antibody to c-Maf or control rabbit IgG and immunoblotted with anti-SUMO-1 antibody. The lower panel is an anti-Maf immunoblot of the lysates. All experiments were repeated three times.

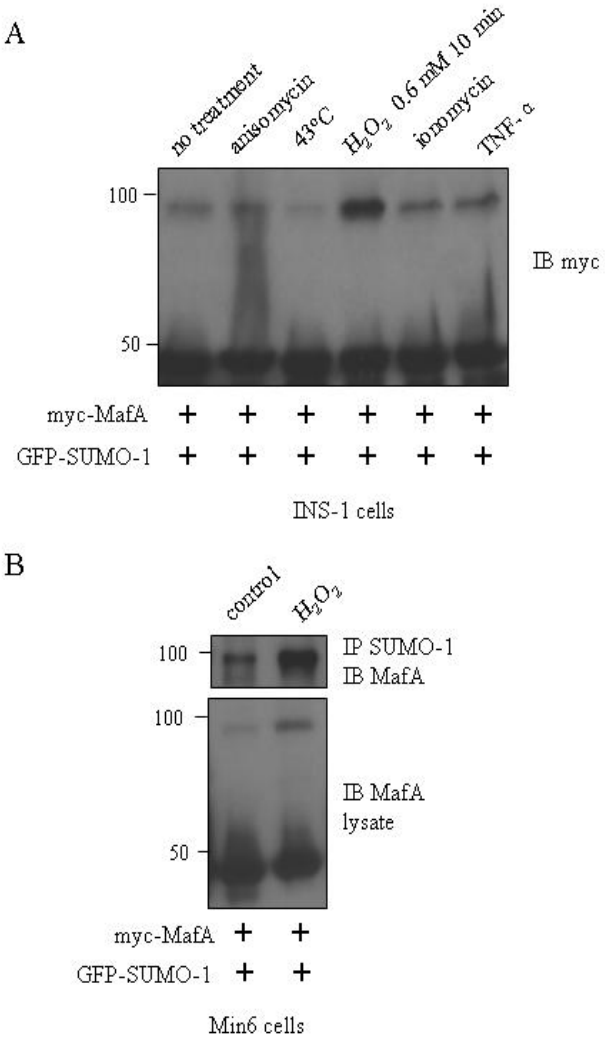


Figure 2-4. Hydrogen peroxide affects MafA sumoylation in pancreatic beta cells.
A. INS-1 cells were cotransfected with myc-MafA and GFP-SUMO-1 followed by treatment with 50 μM anisomycin, 1 μM ionomycin, 100 ng/ml TNF-α, 0.6 mM H_2O_2 for 10 min or 43°C for 30 min. Lysates were analyzed by immunoblotting with an antibody to myc. B. Lysates from Min6 cells transfected with myc-MafA and GFP-SUMO-1 and treated with 0.5 mM H_2O_2 for 10 min were immunoprecipitated with an anti-SUMO-1 antibody followed by immunoblotting with an antibody to c-Maf. The bottom panel shows an immunoblot of the lysates with anti-c-Maf antibody. All experiments were repeated three times.

anti-SUMO-1 immunoprecipitates with an anti-Maf antibody (Figure 2-4B). I exposed beta cells to catalase and the antioxidant N-acetyl-L-cysteine (NAC) after hydrogen peroxide treatment. Both agents modestly reduced the amount of sumoylated MafA (Figure 2-5B). Placing beta cells in fresh culture medium for 30 min after hydrogen peroxide treatment more effectively reduced MafA sumoylation (Figure 2-5B).

E. Preventing sumoylation of MafA alters its transcriptional activity.

To assess functional consequences of MafA sumoylation, I compared wild type MafA and K32R MafA, the mutant lacking the SUMO-1 acceptor site, in transcriptional reporter assays. INS-1 cells were cotransfected with a reporter plasmid containing the rat insulin I MARE and the MafA constructs. The K32R mutant induced greater reporter activity than an equal amount of wild type MafA under several conditions, consistent with the idea that sumoylation inhibits MafA activity towards the insulin gene promoter (Figure 2-6A, B). Further support for this hypothesis came from coexpression of MafA with the protease SENP2, which increased reporter activity induced by wild type MafA, presumably due to reversal of its fractional sumoylation. In contrast, SENP2 expression had no effect on the activity of K32R MafA (Figure 2-6B). Min6 cells were transfected with another reporter construct containing the rat insulin I A2E1 region, which includes MafA as well as other transcription factor binding sites, along with MafA. Increasing amounts of SUMO-1 reduced reporter gene expression (Figure 2-7A), also consistent with an inhibitory effect of MafA sumoylation on insulin gene transcription.

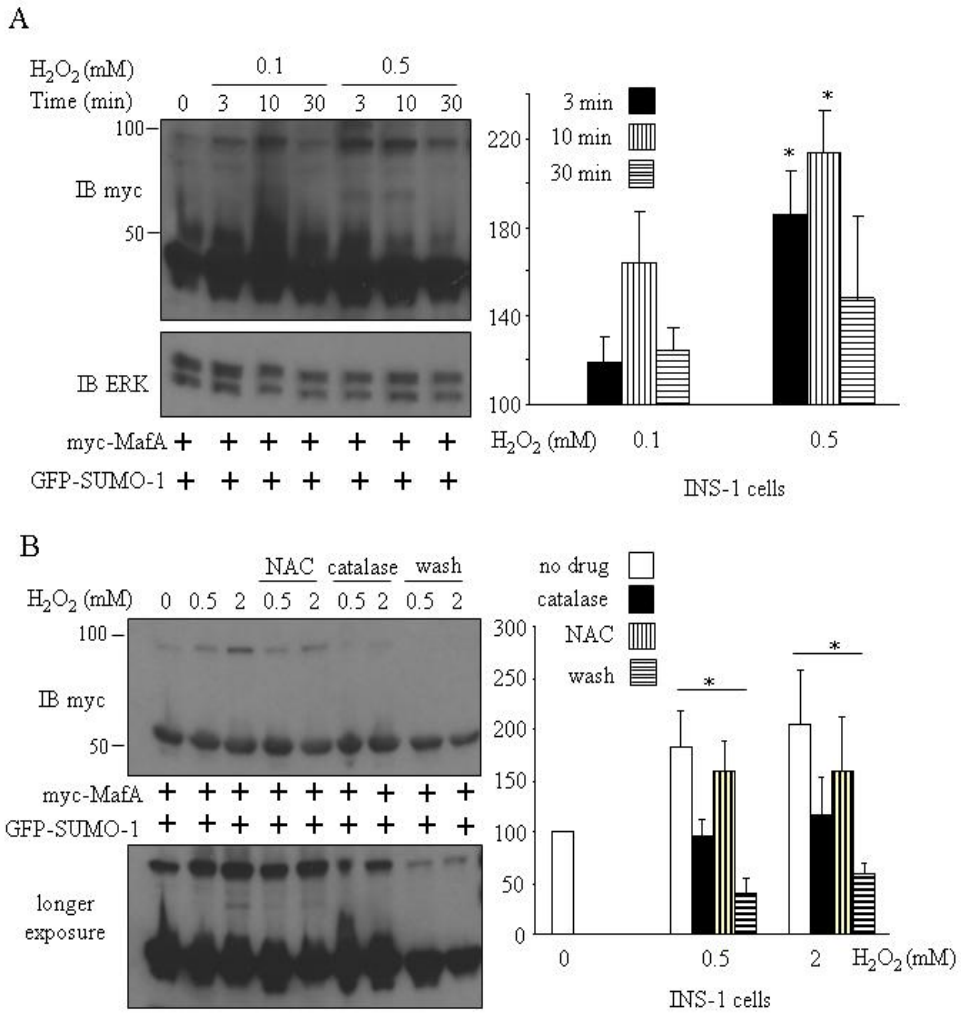


Figure 2-5. Effects of oxidative stress on MafA sumoylation in pancreatic beta cells. A. INS-1 cells transfected with myc-MafA and GFP-SUMO-1 were treated with 0.1 or 0.5 mM H_2O_2 for 3, 10, or 30 min. Lysates were subjected to immunoblotting with an anti-myc antibody. The lower panel shows an anti-ERK immunoblot of the lysates. B. INS-1 cells transfected with myc-MafA and GFP-SUMO-1 were treated with 100 μM NAC for 1 h followed by 0.5 or 2 mM H_2O_2 for 10 min, or with 1000 U/ml Catalase and H_2O_2 for 10 min, or with H_2O_2 for 10 min followed by washing with fresh medium for 30 min. Lysates were immunoblotted with an antibody to myc. Right panels show results expressed as means relative to the control set at 100% ± SEM of three independent experiments. Differences between groups were analyzed using one way ANOVA followed by Holm-Sidak method (* $P<0.001$).

Disruption of the CHOP gene delays beta-cell death caused by ER stress (Oyadomari et al. 2002). Lawrence et al. previously showed that CHOP gene expression is induced in pancreatic beta cells maintained in 5.5 mM glucose and repressed by higher concentrations of glucose (Lawrence et al. 2007). This is due in part to MafA, which represses CHOP transcription by binding to a MARE-CEB site, -320 to -300 bp, in the promoter. I asked whether overexpression of MafA or K32R MafA had an effect on transcriptional activity towards the CHOP promoter in pancreatic beta cells. Using luciferase linked to the MARE-CEB site from the CHOP promoter, I found that K32R MafA had little or no ability to repress reporter gene expression. In contrast, wild type MafA caused a small but statistically significant repression (Figure 2-7B), suggesting that transcriptional repression of this promoter requires modification, presumably sumoylation, of MafA on K32. To explore this observation further, MafA wild type and K32R were expressed along with the MARE-CEB site from the CHOP promoter in HEK293 cells, which express no detectable endogenous MafA. Wild type MafA was able to repress expression of the reporter, while K32R MafA did not (Figure 2-8). Furthermore, expression of SENP2 reversed repression induced by wild type MafA. These results support the conclusion that repression of CHOP requires sumoylated MafA.

F. MafA sumoylation has no apparent effect on its DNA binding to the insulin promoter.

Although Lys32 is in the activation domain of MafA, I asked whether sumoylation has any effect on MafA binding to the insulin gene promoter. Wild type or K32R mutant

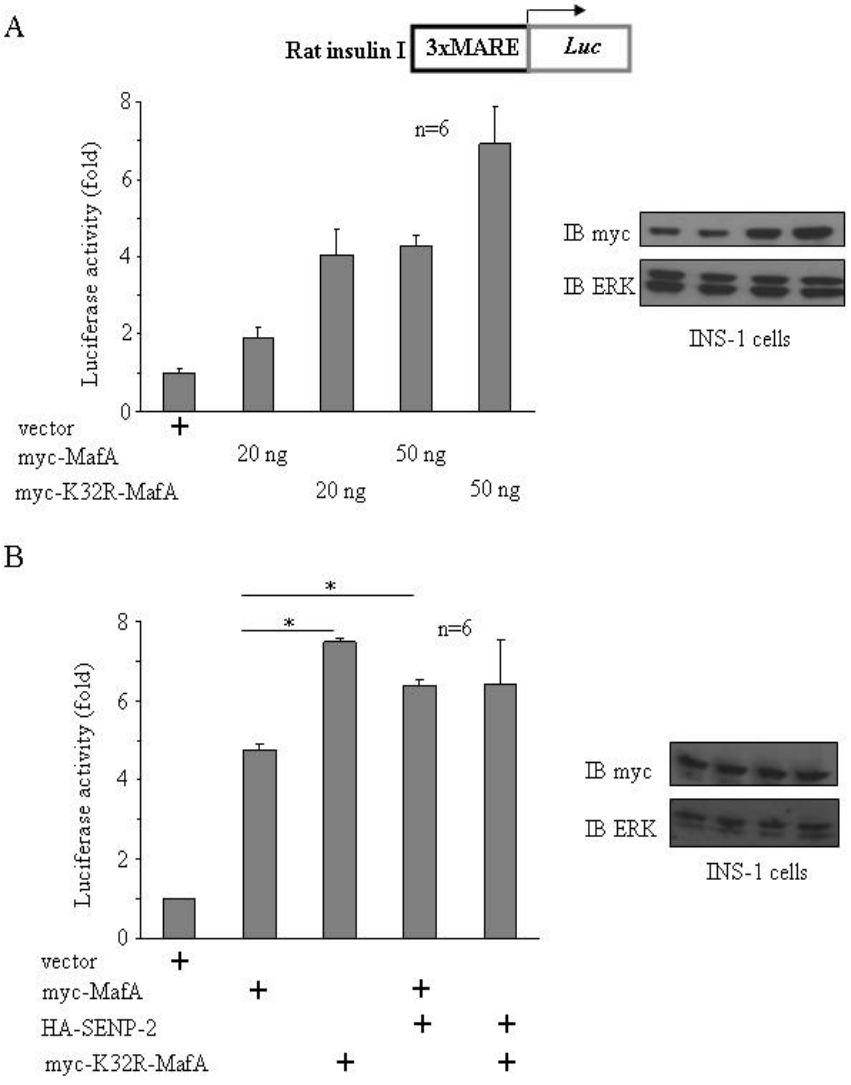


Figure 2-6. Effects of MafA sumoylaiton on its transcriptional activity in beta cells. (1) Luciferase assays of rat insulin I promoter region 3x MARE in INS-1 cells transfected with the indicated plasmids. Right panels show immunoblots of the lysates with antibodies to myc and ERK. Results are expressed as means ± SEM of six replicates. Differences between groups were analyzed using one way ANOVA followed by Tukey test in B (* $P<0.05$).

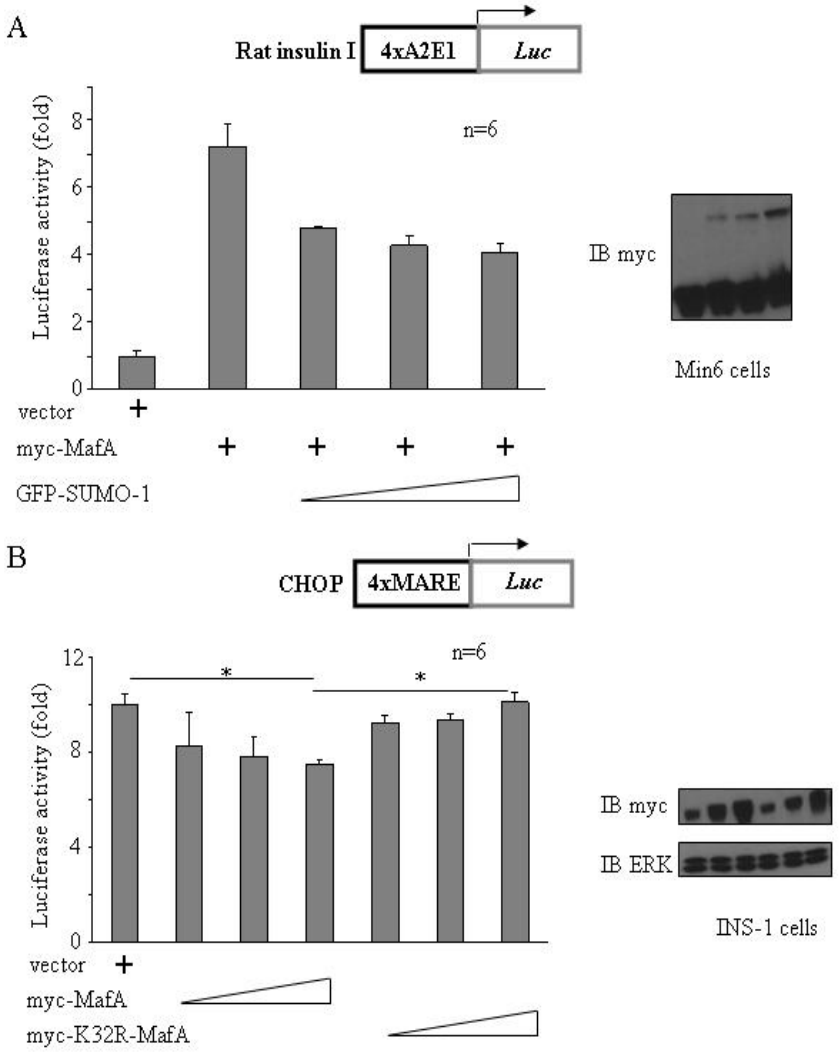


Figure 2-7. Effects of MafA sumoylaiton on its transcriptional activity in beta cells. (2) Promoter-reporter assays of rat insulin I promoter region 4X A2E1 (A) and CHOP gene promoter region 3X -320 to -300 bp (B) in beta cells overexpressing the indicated plasmids. Immunoblots in right panels show myc expression. Results are expressed as means ± SEM of six replicates. Differences between groups were analyzed using one way ANOVA followed by Tukey test in D (* $P<0.05$).

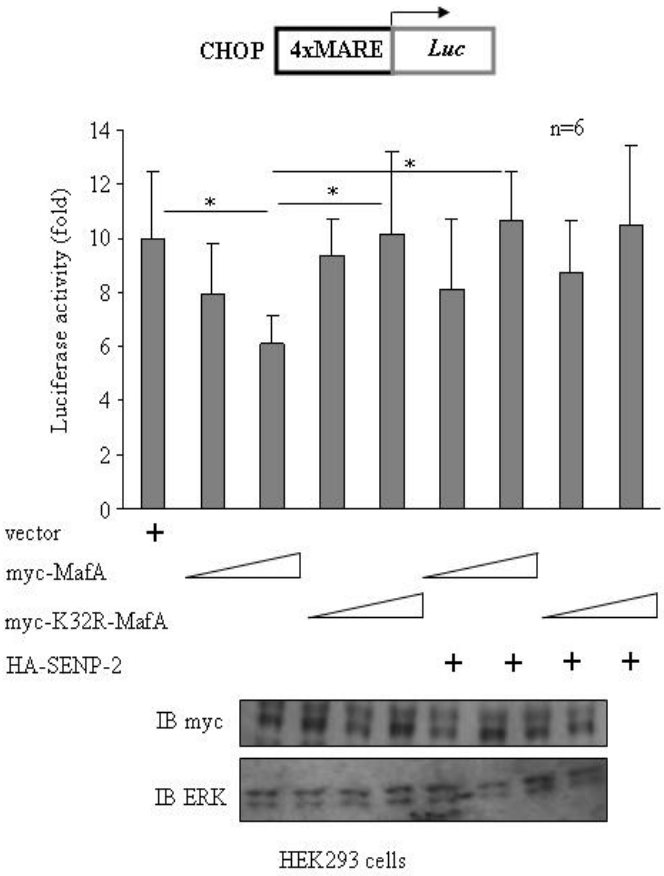


Figure 2-8. Effects of MafA sumoylaiton on its transcriptional activity in HEK293 cells.

Luciferase assays of the CHOP promoter region 3x MARE in HEK293 cells overexpressing the indicated plasmids. Lower panels show myc expression. Results are expressed as means ± SEM of six replicates. Differences between groups were analyzed using one way ANOVA followed by Duncan's method in E (* $P<0.05$).

of MafA were overexpressed in INS-1 cells with or without the coexpression of GFP-SUMO-1. Nuclear extracts were used in electrophoretic mobility shift assays. Both wild type and the K32R mutant of MafA bound to a [γ-^{32}P]ATP-labeled probe containing insulin promoter MARE in the presence or absence of overexpressed SUMO-1 (Figure 2-9). In competition assays, both wild type and mutant MafA bound to the wild type probe but not the mutant one (Figure 2-9A). This binding was competed with a 50-fold excess of an unlabeled wild type probe (Figure 2-9B). Due to the relatively low amount of sumoylated MafA, I did not observe the binding of sumoylated MafA to the probe. In supershift assays, the MafA antibody bound to both wild type and the K32R mutant of MafA, thereby causing the supershift (Figure 2-9C). These data do not prove but are consistent with the idea that sumoylation of MafA does not impair MafA DNA binding to the insulin gene promoter in beta cells.

G. MafA sumoylation has no detectable impact on its nuclear localization in beta cells.

Sumoylation has been shown to alter the subcellular localization of certain proteins. To investigate whether MafA sumoylation grossly alters its subcellular localization in beta cells, I expressed wild type MafA, K32R MafA, and SUMO-1 in different combinations in Min6 cells followed by indirect immunofluorescence staining. In agreement with previous studies, endogenous MafA was detected in the nucleus of beta cells (Figure 2-10A). Overexpressed SUMO-1 was also localized in the nucleus. Expression of SUMO-1 did not detectably alter the localization of endogenous MafA

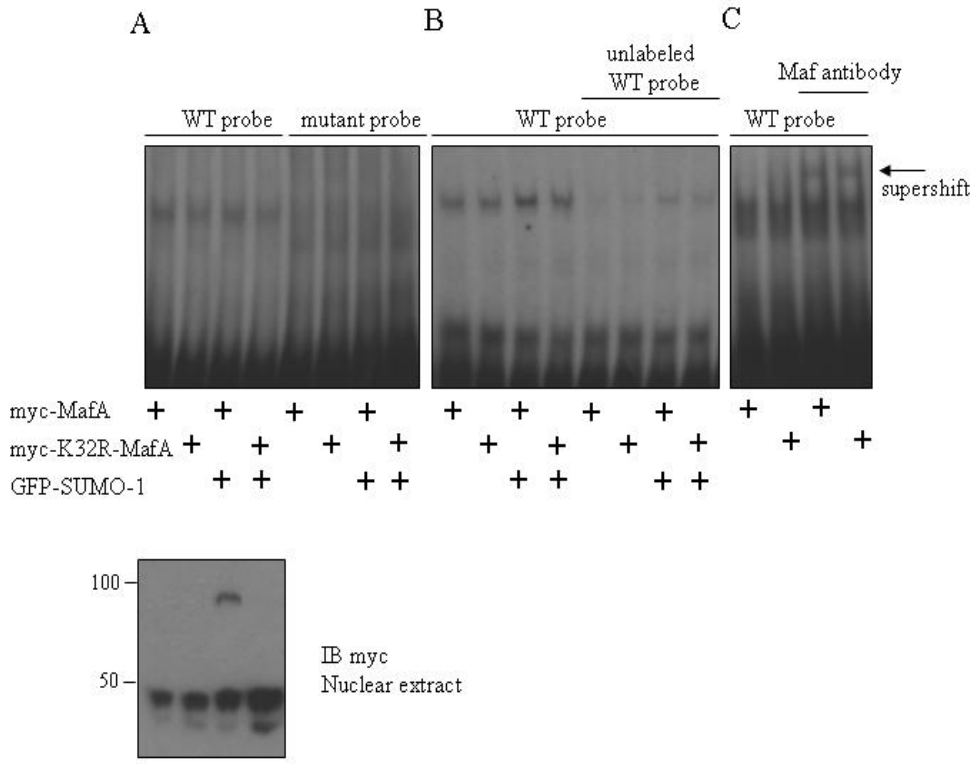


Figure 2-9. Effects of MafA sumoylation on its DNA binding to the insulin gene promoter.

A. Nuclear extracts of INS-1 cells transfected with the indicated plasmids were incubated with [γ-^{32}P]ATP-labeled wild type MARE or mutant probe followed by electrophoresis. The bottom panel shows the myc expression. B. EMSA using INS-1 nuclear extracts with or without preincubation with a 50-fold excess of unlabeled MARE probe. C. EMSA using INS-1 nuclear extracts incubated without or with the c-Maf antibody. Experiments in A and B are representative of three and C of two experiments.

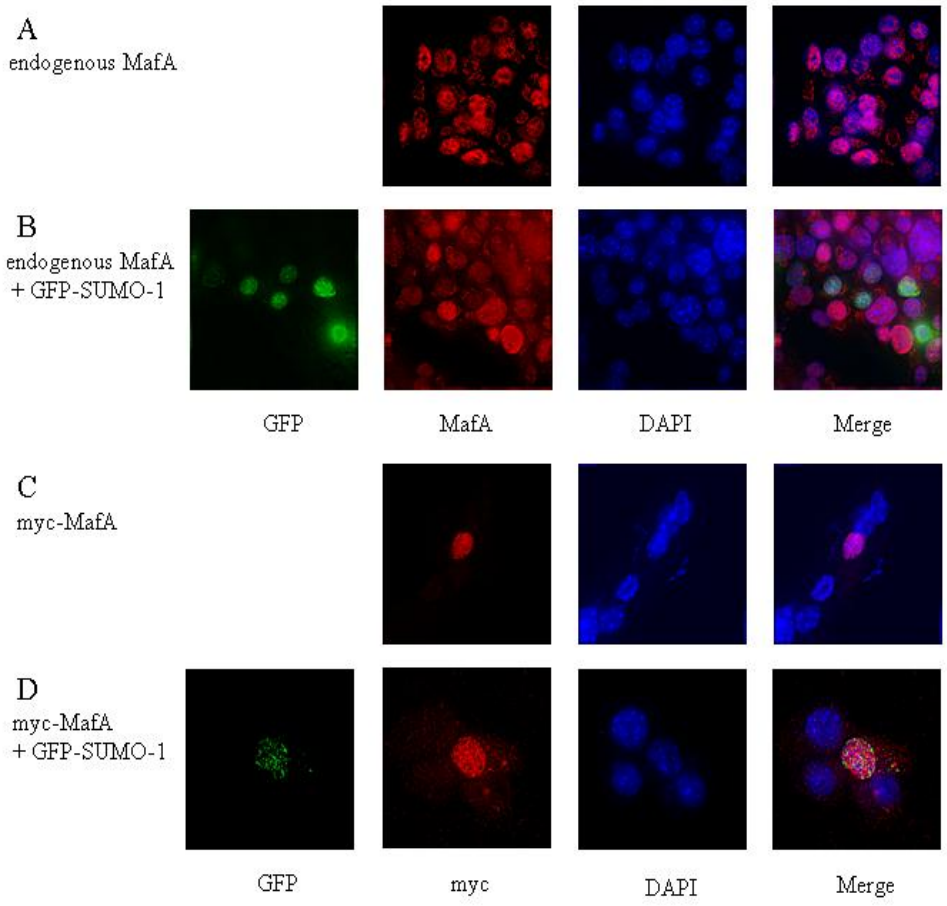


Figure 2-10. Effects of MafA sumoylation on its nuclear localization in beta cells. Indirect immunofluorescence staining showed the subcellular localization of endogenous MafA (red) (A, B), overexpressed myc-MafA (red) (C), and GFP-SUMO-1 (green) in Min6 cells. Signals are merged in the right panels. All experiments were repeated three times.

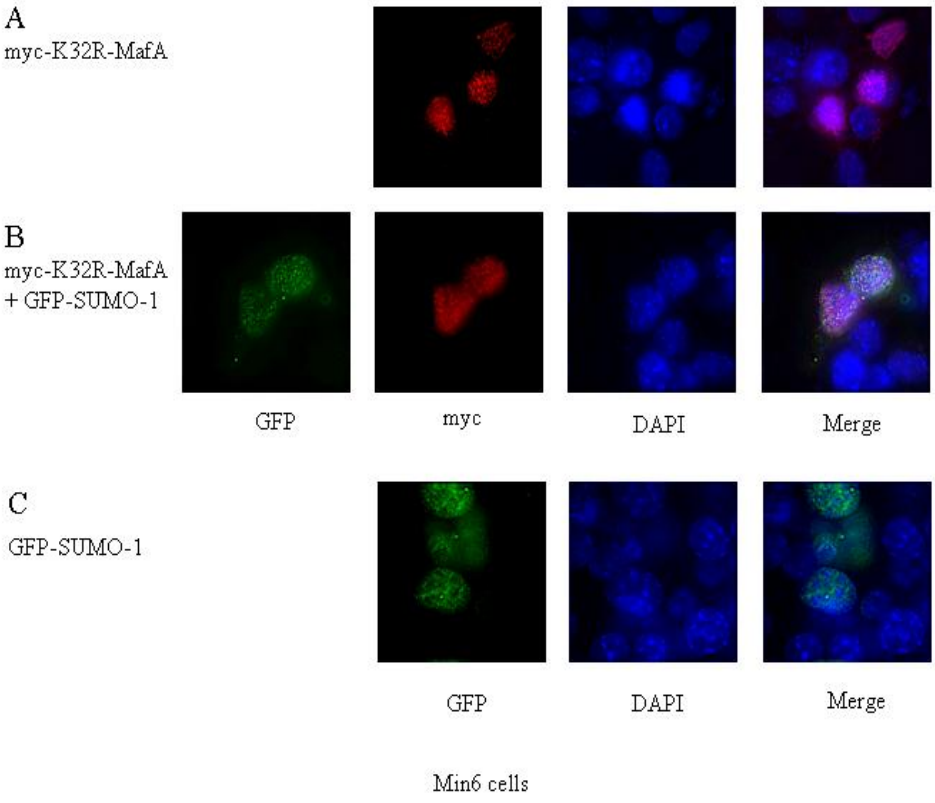


Figure 2-11. K32R-MafA localization in beta cells.
A-C. Min6 cells overexpressing myc-K32R-MafA (red) and/or GFP-SUMO-1 (green) were detected by immunofluorescence staining. Signals are merged in the right panels. All experiments were repeated three times.

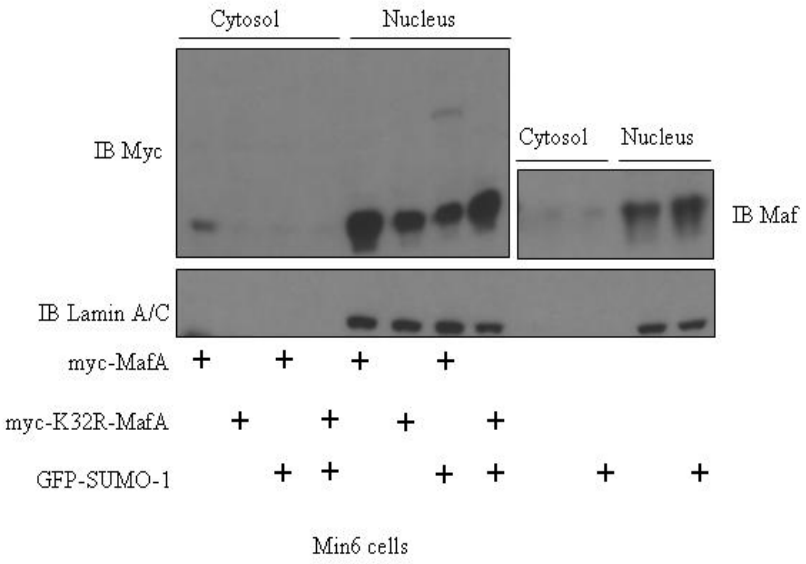


Figure 2-12. MafA subcellular localization in pancreatic beta cells.
Min6 cells transfected with the indicated plasmids were fractionated into nuclear and cytosolic parts. Extracts were immunoblotted with anti-myc, Maf, and LaminA/C antibodies. All experiments were repeated three times.

(Figure 2-10B, 2-11C). Similar localization patterns were also observed with both wild type myc-MafA and the K32R mutant in the presence or absence of coexpressed SUMO-1 (Figure 2-10C, D, 2-11A, B). Using wild type MafA plus SUMO-1, the fraction of tagged MafA that was sumoylated should have been greater; using the mutant, the sumoylated species should have been eliminated, and in neither case were differences observed. Immunoblotting of SUMO-MafA in subcellular fractions also revealed no differences in its subcellular distribution profile relative to the form that could not be modified (Figure 2-12).

H. MafA sumoylation has little effect on its stability in beta cells.

To confirm that MafA is degraded by the proteasome, I treated INS-1 cells with MG132, a proteasome inhibitor. Immunoblotting showed that the amount of endogenous MafA protein increased in MG132-treated cells (Figure 2-13A). I investigated whether sumoylation might affect ubiquitin-dependent degradation of MafA. INS-1 cells were either untransfected or transfected with GFP-SUMO-1 and/or myc-K32, 296R-MafA followed by treatment with 20 μg/ml cycloheximide (CHX) for different times. MafA decay curves were plotted after densitometric analyses. Coexpression of SUMO-1 did not greatly affect the half life of endogenous MafA. Wild type MafA and the double mutant of MafA had similar steady-state expression in the presence of the protein synthesis inhibitor (Figure 2-13B-D). These results suggest that SUMO and ubiquitin modifications of MafA do not compete for the same lysine residue.

I. MafA is modified by SUMO in HEK293 cells.

To compare the profile of MafA sumoylation in HEK293 cells, which do not contain detectable endogenous MafA, I performed parallel experiments. Immunoblotting showed that overexpressed MafA was sumoylated to a comparable level in HEK293 cells as in beta cells. MafA sumoylation was diminished by coexpressing SENP2 or dominant negative Ubc9 (Figure 2-14A, B). These results are confirmed by reciprocal immuno-precipitation with anti-myc or SUMO-1 antibodies (Figure 2-14C, D). Similarly, exogenous MafA was modified by both SUMO-1 and SUMO-2 on lysine 32 in HEK293 cells (Figure 2-15A). EMSA experiments demonstrated that wild type and K32R mutant MafA have similar DNA binding activity to the insulin gene promoter (Figure 2-15C). However, not only hydrogen peroxide but also ionomycin and TNF-α dramatically increased MafA sumoylation in HEK293 cells coexpressing MafA and SUMO-1 (Figure 2-15B). These data suggest that the biological significance of MafA sumoylation in pancreatic beta cells may be distinguished from other systems.

V. Discussion

I show here that endogenous MafA is sumoylated in pancreatic beta cells. To identify functions associated with this modification, I examined effects of MafA sumoylation on the insulin gene and CHOP gene promoters. Reporter activity driven by the MafA-responsive sequence from the insulin gene promoter is enhanced by preventing sumoylation of MafA, leading to the idea that sumoylation of MafA inhibits its ability to

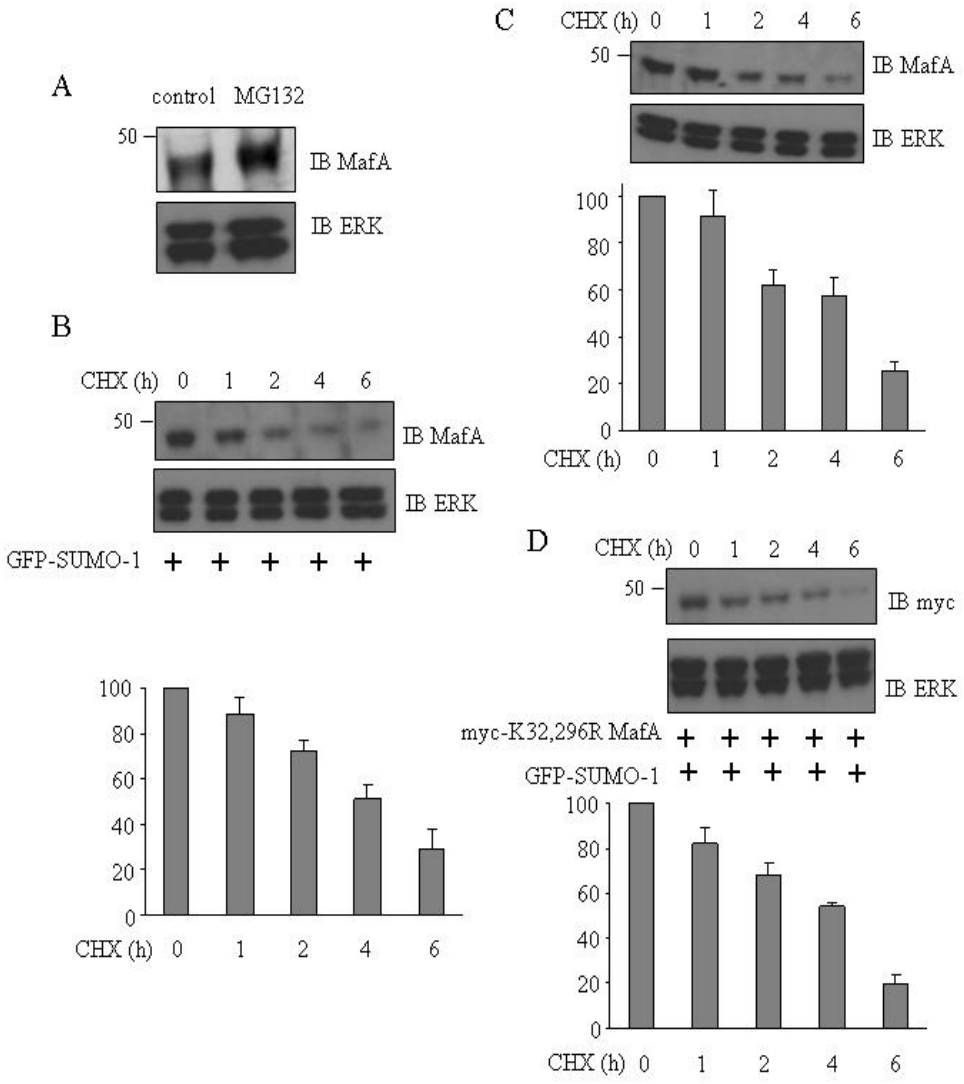


Figure 2-13. Effects of MafA sumoylation on its stability in beta cells.
A. Lysates of INS-1 cells treated with 25 μM MG132 for 6 h were analyzed by immunoblotting with antibodies to c-Maf and ERK. B-D. INS-1 cells were transfected or untransfected with the indicated plasmids. After 48 h, cells were treated with 20 μg/ml CHX and harvested at 0, 1, 2, 4, or 6 hours. Lysates were subjected to immunoblotting with antibodies to myc, c-Maf, or ERK. Experiments were repeated three times. MafA decay curves were plotted from densitometric scans from each experiment.

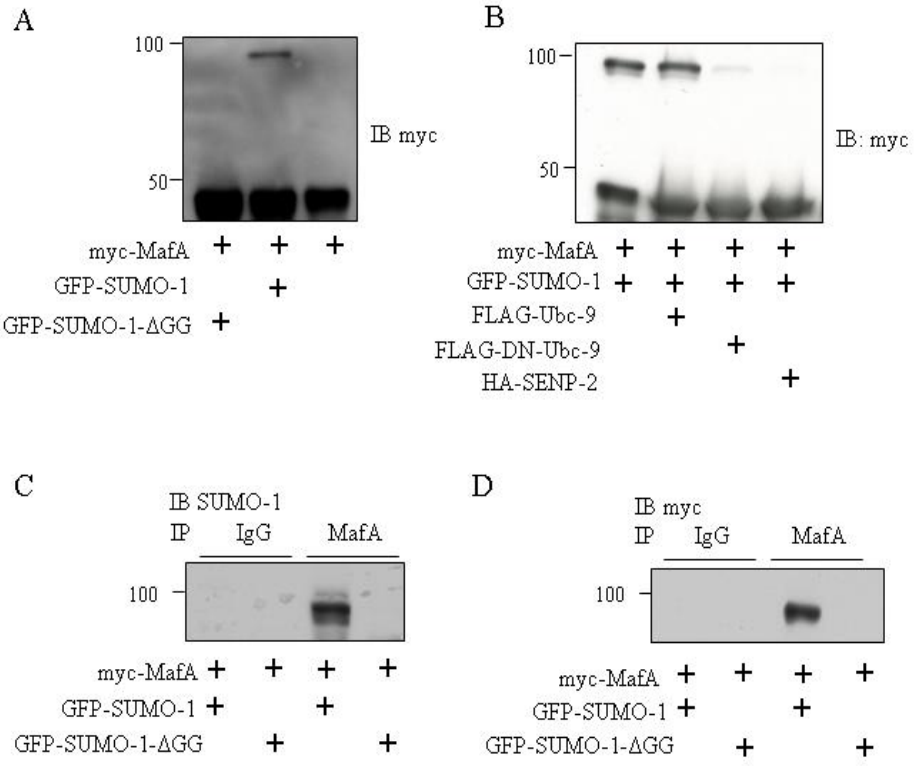


Figure 2-14. Sumoylation of MafA in HEK293 cells.
A. Myc-MafA was coexpressed with GFP-SUMO-1 or GFP-SUMO-1-ΔGG in HEK293 cells. Cell extracts were immunoblotted with an antibody to myc. B. HEK293 cells were transfected with the indicated plasmids.Cell lysates were immunoblotted with an antibody to myc. Lysates from HEK293 cells expressing myc-MafA and GFP-SUMO-1 or GFP-SUMO-1-ΔGG were immunoprecipitated with anti-c-Maf or control IgG and then detected by immunoblotting with anti-SUMO-1 (C) or myc (D) antibody.

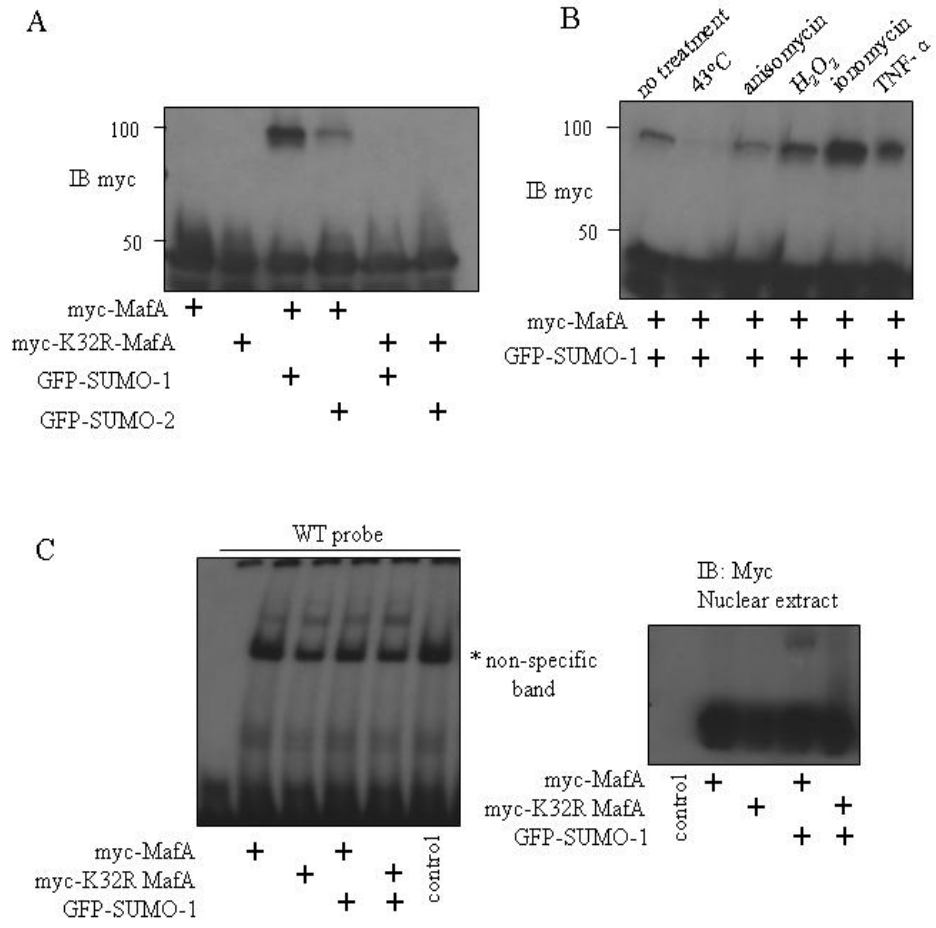


Figure 2-15. MafA mutant in HEK293 cells.
A. Immunoblot with an antibody to myc of the lysates from HEK293 cells transfected with the indicated plasmids. B. HEK293 cells were cotransfected with myc-MafA and GFP-SUMO-1 followed by treatment of 50 μM anisomycin, 1 μM ionomycin, 100 ng/ml TNF-α, 0.6 mM H_2O_2 for 10 min or 43°C for 30 min. Lysates were analyzed by immunoblotting with an antibody to myc. C. Nuclear extracts of HEK293 cells transfected with the indicated plasmids were incubated with [γ-^{32}P]ATP-labeled wild type MARE probe followed by electrophoresis. Right panel shows myc expression in nuclear extracts. All experiments were repeated three times.

stimulate transcription. Lawrence et al. found previously that MafA binding to the CHOP promoter is associated with an inhibition of CHOP expression (Lawrence et al. 2007). Here I find that blocking sumoylation of MafA prevents transcriptional repression of the CHOP promoter in beta cells or in a reconstituted system lacking endogenous MafA, consistent with the conclusion that inhibition of CHOP gene expression by MafA requires its sumoylation. Taken together, these results suggest that sumoylation of MafA regulates important biological activities of this protein that may enhance beta cell survival as well as modulate insulin gene expression.

Previous chromatin immunoprecipitation experiments demonstrated that MafA is bound to the CHOP promoter under conditions in which CHOP promoter activity is repressed (Lawrence et al. 2007). MafA lacking the sumoylation site is not capable of inhibiting CHOP promoter activity in cells lacking endogenous MafA. From these experiments, I conclude that reduced CHOP promoter activity requires binding of sumoylated MafA to the CHOP promoter. Because MafA binds to the promoter under conditions in which it represses transcription and sumoylated MafA is required for promoter inhibition, it seems most likely that neither altered localization nor degradation plays a substantial role in the functional effects detected on transcription. This hypothesis is supported by the lack of observable changes in these behaviors upon a forced increase in sumoylation.

Transcription repression is a function commonly associated with sumoylation (Yang and Sharrocks 2004; Gill 2005; Geiss-Friedlander and Melchior 2007). The molecular mechanisms by which SUMO modification represses gene transcription may be several. Sumoylation may recruit corepressors or other chromatin modulators, such as

histone deacetylases, to promoters. These modulators may assist in initiating the formation of repressive complex or in retaining the repressive chromatin state, which could account for the substantial effect of low-level sumoylated proteins.

Increased sumoylation of MafA is associated with a less robust stimulation of insulin promoter activity. For the insulin promoter, it is unclear if the decrease in stimulatory activity is due to effects of sumoylated MafA on the promoter complex, such as recruitment of deacetylases, or to lack of sumoylated MafA binding to the promoter. EMSA experiments suggest that sumoylation does not prevent DNA binding. Further examination of MafA interactions on the insulin promoter will be required to define the mechanism underlying its altered function in insulin gene transcription.

Mutagenesis indicates that a single site, K32, is the predominant, if not only, sumoylation site in MafA, consistent with what was recently reported for MafB (Tillmanns et al. 2007). K32 is in a region containing several reported phosphorylation sites, but is not immediately juxtaposed to these sites. Previous studies have not unambiguously delineated the effects of these putative phosphorylations on MafA activity; thus, predicting how these modifications may interact is not yet possible. Several studies of the ternary complex factor Elk1 have revealed interplay between phosphorylation and sumoylation that may well serve as a model for the regulation of MafA function. Phosphorylation enhances Elk-1 transcriptional activity while sumoylation inhibits this function (Yang et al. 2003).

Important questions remaining to be answered concern the mechanisms that determine the extent of MafA sumoylation in beta cells and the role of glucose concentration. Beta cells experience high levels of oxidative stress that are thought to

contribute to cell death in response to hyperglycemia (Oyadomari et al. 2002; Poitout and Robertson 2002). The increase in MafA sumoylation by hydrogen peroxide suggested that exposure to elevated glucose might also increase MafA sumoylation; however, greatest sumoylation of endogenous MafA was detected in cells in low not high glucose. Ongoing studies focus on defining these mechanisms.

Chapter Three: Regulation of IL-1β-induced CHOP Expression by JNK and NF-κB in Pancreatic Beta Cells

I. Abstract

Apoptosis is the main reason for immune-mediated pancreatic beta-cell destruction in type I diabetes. Exposure of beta cells to IL-1β induces ER stress and activates proapoptotic networks, which include the NF-κB and JNK signaling pathways. I was interested in elucidating the mechanisms by which JNK and NF-κB regulate the expression of CHOP, a mediator of ER stress-induced apoptosis, upon IL-1β treatment. It is well documented that IL-1β causes the activation and translocation of NF-κB into the nucleus, which produces proapoptotic effects in pancreatic beta cells. I found that both CHOP messenger RNA and protein greatly increase upon exposure of beta cells to IL-1β. EMSA experiments suggested that CHOP is a direct target of IL-1β-activated NF-κB. Furthermore, the AP-1 complex composed of c-Jun, a JNK target, and other factors promotes CHOP expression. Immunoblot data indicated that c-Jun expression markedly increases, and multiple residues on c-Jun are phosphorylated after IL-1β treatment. In addition, IL-1β increases c-Fos and C/EBP-β expression and reduces both MafA and Pdx-1 expression in beta cells. These results suggest that activated NF-κB and JNK resulting from IL-1β stimulation are involved in the regulation of CHOP gene expression in pancreatic beta cells, and that IL-1β influences beta-cell function through a variety of signaling pathways.

II. Introduction

Type I diabetes is an autoimmune disease characterized by a lack of insulin production due to the progressive depletion of pancreatic beta cells. During the course of insulitis, proinflammatory cytokines, such as IL-1β, TNF-α, and IFNγ, are released by invasive immune cells and contribute to beta-cell death (Lee et al. 2004; Cnop et al. 2005). Accumulating data demonstrate that apoptosis is probably the main cause of beta-cell destruction during the onset of type I diabetes (Mathis et al. 2001; Wellen and Hotamisligil 2005). Apoptosis is achieved through the release of intracellular proapoptotic proteins that activate caspases, a group of proteases that ultimately destroy critical structural proteins and stimulate the fragmentation of chromosomal DNA. Long-term exposure of beta cells to cytokines regulates genes related to beta-cell function and promotes the activation of proapoptotic signals. IL-1β alone can induce apoptosis of rat and mouse beta cells, while addition of TNF-α and IFNγ might be required to trigger human beta-cell apoptosis (Eizirik and Mandrup-Poulsen 2001). Among the variety of proapoptotic networks induced by IL-1β, NF-κB and JNK pathways play important roles in the pathogenesis of type I diabetes (Kaneto et al. 2006; Fornoni et al. 2008; Mokhtari et al. 2008).

A. NF-κB signaling pathway in IL-1β-induced beta-cell apoptosis.

NF-κB is formed by homo- or hetero-dimers of five NF-κB family members, which bind to a group of inhibitory kappaB (IκB) proteins and remain inactive in the cytoplasm (Gloire et al. 2006; Hayden and Ghosh 2008). IL-1β activates NF-κB through

degradation of IκB proteins and translocation of NF-κB dimers to the nucleus. NF-κB induces antiapoptotic effects in most cell types (Gloire et al. 2006; Ortis et al. 2008). However, many reports indicate that IL-1β-induced NF-κB activation promotes apoptosis in pancreatic beta cells (Melloul 2008). Elegant studies done by the groups of Melloul and of Robbins showed that the inhibition of IL-1β-induced NF-κB activation by adenovirus-mediated expression of phosphorylation-defective mutant of IκBα prevents apoptosis in human and rat primary beta cells and insulin-producing cell lines (Giannoukakis et al. 2000). They also demonstrated that conditional and specific NF-κB blockade *in vivo* prevents diabetes caused by streptozotocin-induced beta-cell loss (Eldor et al. 2006). Microarray analyses and other techniques predicted that IL-1β-induced NF-κB activation causes a variety of gene expression changes in beta cells, such as increases in nitric oxide synthase, c-Myc, and CHOP (Cardozo et al. 2001; Lamhamedi-Cherradi et al. 2003; Naamane et al. 2007; Eizirik et al. 2008). But the mechanisms by which IL-1β-activated NF-κB modulates gene transcription, such as CHOP, remain unknown.

B. CHOP expression in beta cells

CHOP, a 29kD protein, is a member of the CCAAT/enhancer binding proteins (C/EBPs). It is also known as growth arrest- and DNA damage-inducible gene 153 (GADD153), DNA-damage-inducible transcript 3 (DDIT3) and C/EBPζ. CHOP contains two known functional domains, an N-terminal transcriptional activation domain and a C-terminal basic-leucine zipper domain. The bZIP domain is essential for CHOP-induced apoptosis. Due to the high conservation in the bZIP domain, CHOP forms heterodimers

with other C/EBPs and inhibits gene transcription. In contrast, CHOP can enhance the transactivating effect of AP-1 by binding to the complex.

CHOP is a transcription factor mediating ER stress-induced apoptosis. It is ubiquitously expressed at a low level and robustly expressed in many cells under stressful conditions, such as glucose deprivation and ER calcium stores depletion. CHOP gene induction is mediated through the PERK/eIF2α/ATF4 (dsRNA-activated protein kinase–like ER kinase/eukaryotic initiation factor 2α/activating transcription factor 4), IRE1α/XBP1 (inositol-requiring protein 1α/X-box binding protein 1) and ATF6α pathways (Song et al. 2008). Many transcription factors have been reported to regulate CHOP expression. The 5' flanking region of the CHOP gene contains overlapping cis-acting CAAT enhancer binding (CEB), activating transcription factor (ATF), cyclic AMP response element and other DNA binding elements, which have been shown to bind to various complexes including C/EBP-β, ATF-2, ATF-3, XBP-1, and ATF-4 in different systems, depending on the mode of induction (Fawcett et al. 1999; Bruhat et al. 2000; Averous et al. 2004). We previously observed that in pancreatic beta cells MafA can repress CHOP promoter activity through a MARE, which overlaps with the CEB/ATF/CRE site (Lawrence et al. 2007). IL-1β induces AP-1 dimers composed of c-Fos, c-Jun, and/or JunB to bind to the AP-1 binding site, which increases CHOP promoter activity (Pirot et al. 2007). These findings implicate beta-cell-specific regulation of CHOP expression upon IL-1β treatment.

CHOP has been implicated in the pathogenesis of type I diabetes by promoting beta-cell death (Eizirik et al. 2008). Previous studies showed that CHOP expression is induced in cultured pancreatic beta cells maintained in 5.5 mM glucose and by

inflammatory cytokines via nitric oxide (NO) signaling (Cardozo et al. 2005; Lawrence et al. 2007). Moreover, CHOP-deficient islets are resistant to the toxic effects of the NO donor, S-nitroso-N-acetyl-D,L-penicillamine. Akita mice (bearing a point mutant in the insulin II gene) with a homozygous deletion of the CHOP gene show delayed diabetes onset, indicating that CHOP exacerbates the diabetic phenotype (Oyadomari et al. 2001; Oyadomari et al. 2002). It is recently reported that CHOP deficiency improves beta cell ultrastructure and promotes cell survival in high fat diet-fed *eIF2α*$^{S/A}$ mice. The high fat diet-fed *eIF2α*$^{S/A}$ mice are a model of beta cell failure caused by ER stress due to elevated proinsulin biosynthesis (Scheuner et al. 2005). And CHOP-null diebetic *Lepr*$^{db/db}$ (leptin receptor) mice exhibit increased expression of UPR (unfolded protein response) and antioxidative stress response genes (Song et al. 2008). However, little is known about the mechanism by which CHOP promotes beta-cell apoptosis although cytokines can activate caspase-3 in insulinoma cells of non-obese-diabetic mice and mice lacking caspase-8 in beta cells are protected from streptozotocin-induced type I diabetes (Augstein et al. 2004; Liadis et al. 2007).

C. JNK signaling pathway in IL-1β-induced beta-cell apoptosis

JNK is a MAP kinase that is activated mainly through cytokines and various environmental stresses. The mammalian JNKs are encoded by three distinct genes, each of which functions differently, according to knockout mice studies (David et al. 2002; Bogoyevitch 2006; Fukuda et al. 2008). JNK is activated by dual phosphorylation of T183/Y185 in JNK1 and JNK2 and T221/Y223 in JNK3 via the MAP kinase kinases, MEK7 and MEK4. Following activation, JNK phosphorylates a broad spectrum of target

proteins including c-Jun, Elk-1, ATF2, and members of the Bcl-2 family (Davis 2000; Bogoyevitch and Kobe 2006). It is well documented that IL-1β activates JNK signaling in beta cells (Ammendrup et al. 2000; Abdelli et al. 2007; Ferdaoussi et al. 2008). Increased JNK activity caused by a point mutation in islet-brain-1 (IB1), a JNK scaffold protein, has been identified in a rare Mendelian form of diabetes in humans (Waeber et al. 2000). Furthermore, cell-permeable peptide inhibitors of JNK prevent cytokine-induced apoptosis in insulin-producing cells (Bonny et al. 2001). Genetic studies showed that body weights of *Jnk1-/- ob/ob* mice were similar to lean wild-type and *jnk1-/-* control mice, but not to *ob/ob* controls (Hirosumi et al. 2002). In addition, IL-1β is able to activate two other MAP kinases, ERK1/2 and p38, in islets and rat insulinoma cells as well (Larsen et al. 2005; Chin-Chance et al. 2006; Larsen et al. 2006; Glauser and Schlegel 2007). ERK1/2 and p38 activation is necessary but not sufficient for IL-1β mediated beta-cell NO synthesis (Kwon et al. 1996).

As a major target of JNK, c-Jun is one of the components of AP-1, a transcription factor complex believed to be required in cell proliferation, survival, and death. The specific phosphorylation state of c-Jun is important in generating transactivation potential towards divergent genes (Morton et al. 2003). The phosphorylation of these residues is thought to be mediated by the isoforms of JNK and other kinases as well. For example, Ser63 and Ser73 are phosphorylated by JNK, Thr239, Ser243 and Ser249 are phosphorylated by GSK3β, Ser243 could be phosphorylated by ERK1/2, and Thr231 and Ser249 are phosphorylated by CkII (Casein kinase II) *in vitro* which prevents the binding

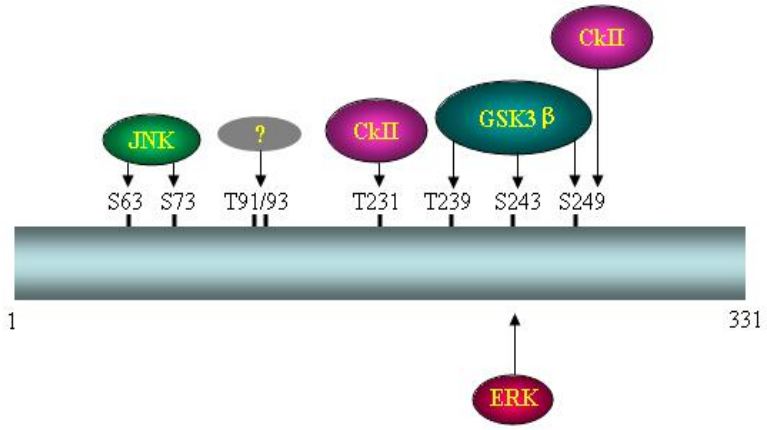


Figure 3-1. c-Jun: the known phosphorylation sites and proposed kinases.

of c-Jun to DNA (Boyle et al. 1991; Lin et al. 1992; Peverali et al. 1996) (Figure 3-1). Thus, it has been generally accepted that the activation of c-Jun requires the phosphorylation of Ser63/Ser73, as well as the dephosphorylation of one or more of the C-terminal residues (Morton et al. 2003). It now appears that site-specific c-Jun phosphorylation depends not only on activation but also on cell type and ultimate function (Manning and Davis 2003; Hotamisligil 2005; Liu and Lin 2005). However, the phosphorylation state of these residues and their kinases upon IL-1β stimulation are not identified to date. The analysis of IL-1β-induced c-Jun phosphorylation and the effect on CHOP gene expression should provide insights into the mechanism by which JNK induces beta-cell apoptosis.

Due to robust insulin secretory activity, pancreatic beta cells have a highly developed ER, which makes them particularly susceptible to ER stress caused by hyperglycemia and inflammatory cytokines. IL-1β stimulation inhibits the expression of the sarcoendoplasmic reticulum pump Ca^{2+} ATPase 2b, depletes ER calcium stores, induces CHOP expression, and eventually leads to beta-cell apoptosis (Cardozo et al. 2005; Kaneto et al. 2007; Eizirik et al. 2008). Uncovering the molecular mechanisms which initiate or exacerbate ER stress and contribute to beta-cell apoptosis will improve our ability to protect pancreatic beta cells from immune system destruction during the onset of type I diabetes.

III. Material and Methods

Cell culture, materials and immunoblotting. Maintenance and lysis of Min6 and INS-1 cells were as described in Chapter 2. For immunoblotting, I used 1:1000 dilution of the following antibodies: anti-phospho-IκBα (Ser32, 36) monoclonal antibody (Cell Signaling), anti-c-Jun polyclonal antibody (Santa Cruz), anti-phospho-c-Jun (Ser 63) monoclonal antibody (Santa Cruz), anti-c-Maf polyclonal antibody (Santa Cruz), anti-Pdx-1 polyclonal antibody (Upstate), anti-NeuroD1 polyclonal antibody (Santa Cruz), anti-CHOP monoclonal antibody (Santa Cruz), anti-phospho-ERK1/2 (Thr202/Tyr204) monoclonal antibody (Sigma), anti-phospho-p38 (Thr180/Tyr182) polyclonal antibody (Cell Signaling), anti-phospho-JNK (Thr183/Tyr185) polyclonal antibody (Cell Signaling), a 1:3000 dilution of anti-ERK1/2 polyclonal antibody (Y691), and a 1:500 dilution of anti-c-Fos polyclonal antibody (Santa Cruz).

Recombinant human IL-1β was purchased from Biosource (Carlsbad CA). Cells were treated with different concentrations of IL-1β for 5 min to 48 h as indicated in the legends. The inhibitors U0126, SP600125 and SB203580 were purchased from LC Laboratories (Woburn, MA).

Real time quantitative PCR. Min6 cells were cultured in normal medium as described in Chapter 2. Isolation of RNA from Min6 cells was done with TRI reagent solution according to the manufacturer's instructions (Ambion). Total RNA was reversely transcripted to cDNA with high capacity cDNA archive kit (Applied Biosystems). Reaction condition was 25°C for 10 min followed by 37°C for 120 min. SYBR green supermix with ROX was purchased from Bio-Rad (Hercules, CA). Syber green-based PCR was performed on the ABI 7500 DNA Sequence Detection System with

standard fluorescent chemistries and thermal cycling conditions specified by the manufacturer: 50°C for 2 min, 95°C for 10 min for one cycle, an additional 40 cycles at 95°C for 15 sec, 60°C for 1 min, and 62°C for 2 min for one cycle. 18S rRNA was used as an internal expression control. The primers are: insulin I forward 5'-TGTTGGTGCACTTCCTACCC, reverse 5'-CACTTGTGGGTCCTCCACTT, insulin II forward 5'-TTTGTCAAGCAGCACCTTTG, reverse 5'-GCTGGTGCAGCACTGA-TCTA, CHOP forward 5'-CCTAGCTTGGCTGACAGAGG, reverse 5'-GGGCACTGA-CCACTCTGTTT, MafA forward 5'-ATCATCACTCTGCCCACCAT, reverse 5'-ATGACCTCCTCCTTGCTGAA, Pdx-1 forward 5'-TGGATGAAATCCACCAAAGC, reverse 5'-TTCAACATCACTGCCAGCTC, NeuroD1 forward 5'-TTTGAAAGCCCC-CTAACTGA, reverse 5'-AAGATTGATCCGTGGC-TTTG, c-Jun forward 5'-CTGATC-ATCCAGTCCAGCAA, reverse 5'-GACACTGGGAAGCGTGTTCT, c-Fos forward 5'-CCAGTCAAGAGCATCAGCAA, reverse 5'-GTACAGGTGACCACGGGAGT, and 18s rRNA forward 5'-TTGACGGAAGG-GCACCACCAG, reverse 5'-GCACCACCA-CCCACGGAATCG.

Electrophoretic mobility shift assays (EMSA). Complementary oligonucleotides containing the wild type NF-κB consensus site of the rat CHOP gene promoter (5'-CTACCAAGGAAATGCCAGTACCTCA) were synthesized. The oligonucleotides were hybridized and end-labeled with T4 polynucleotide kinase as described in chapter 2. Equal amounts of INS-1 whole cell extract proteins (20 μg) were incubated for 30 min with double-stranded [γ-^{32}P]ATP-labeled probe (20,000 cpm) in reaction buffer. The

reactions were subjected to electrophoresis on 5% polyacrylamide gels and bands were detected on film by autoradiography.

IV. Results

A. IL-1β-induced the regulation of mRNA in pancreatic beta cells.

Recent reports of microarray studies have indicated that IL-1β induces the up- or down-regulation of a variety of genes which are predicted to be NF-κB downstream targets in primary rat beta cells, human pancreatic islets, and a rat beta-cell line (Cardozo et al. 2001). Therefore, real-time quantitative PCR was performed to test whether IL-1β induces changes in expression of genes, which are involved in beta-cell function and proapoptosis. Conditions were established by analyzing concentration-response curves and time courses of IL-1β treatment in Min6 cells. The mRNA was collected from Min6 cells cultured in normal medium and stimulated with 20 ng/ml of IL-1β for different times. CHOP gene mRNA increased after 10 min of IL-1β exposure and reached a peak around 60 min. The maximum enhancement is about 2.5 fold compared to the control (Figure 3-2A). Transcription of c-Jun started to increase at 10 min and dramatically increased to 16-fold over the control at 60 min after IL-1β addition (Figure 3-2B). With similar kinetics, IL-1β treatment augmented c-Fos transcription to about 3 fold at 30 min (Figure 3-3A).

To test whether IL-1β impairs beta-cell function, the transcription of beta-cell specific genes was monitored. Briefly, Pancreas Duodenum Homeobox (Pdx)-1 was

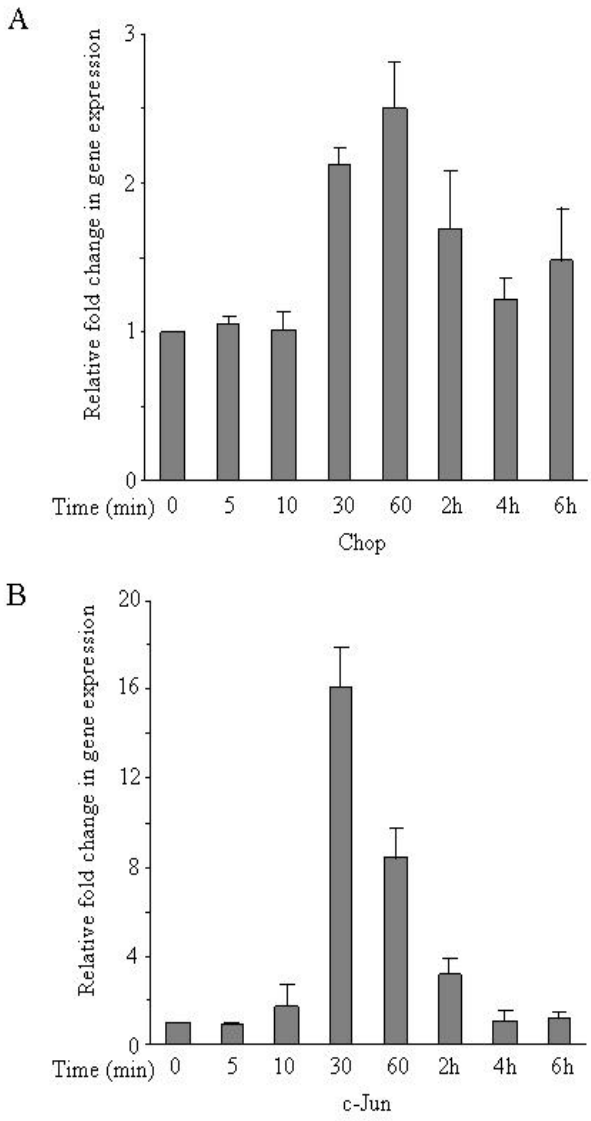


Figure 3-2. IL-1β-induced changes in mRNA of CHOP and c-Jun in beta cells.
Min6 cells were treated with 20 ng/ml IL-1β for different times. mRNA of Chop (A) and c-Jun (B) was measured by real-time PCR. Results were expressed as means relative to 18S rRNA expression levels ± SEM of three independent experiments.

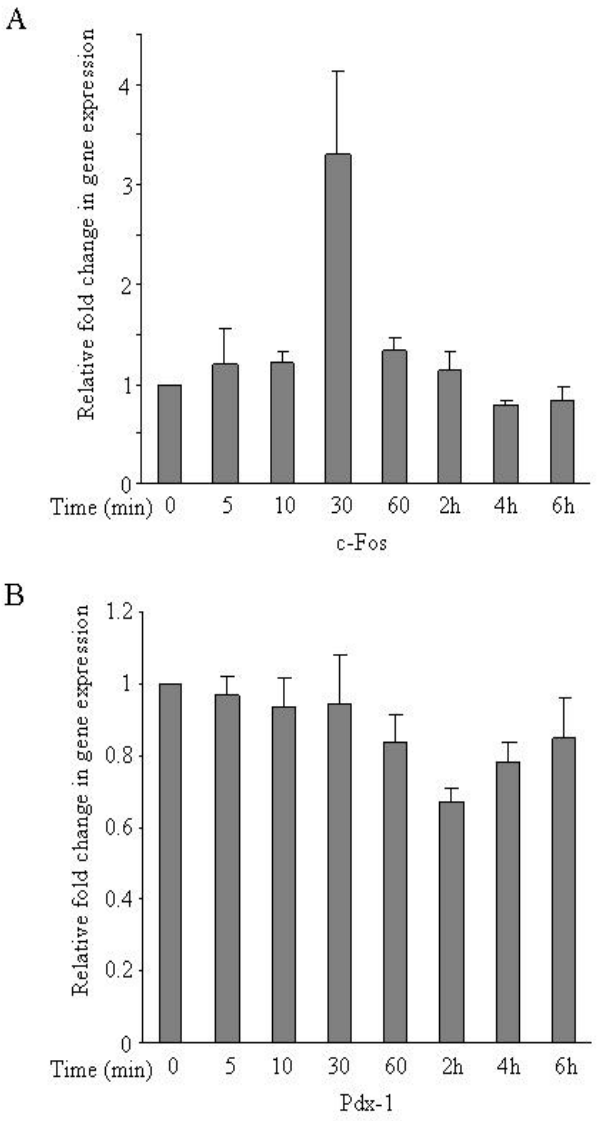


Figure 3-3. IL-1β-induced changes in mRNA of c-Fos and Pdx-1 in beta cells.
Min6 cells were treated with 20 ng/ml IL-1β for different times. mRNA of c-Fos (A) and Pdx-1 (B) was measured by real-time PCR. Results were expressed as means relative to 18S rRNA expression levels ± SEM of three independent experiments.

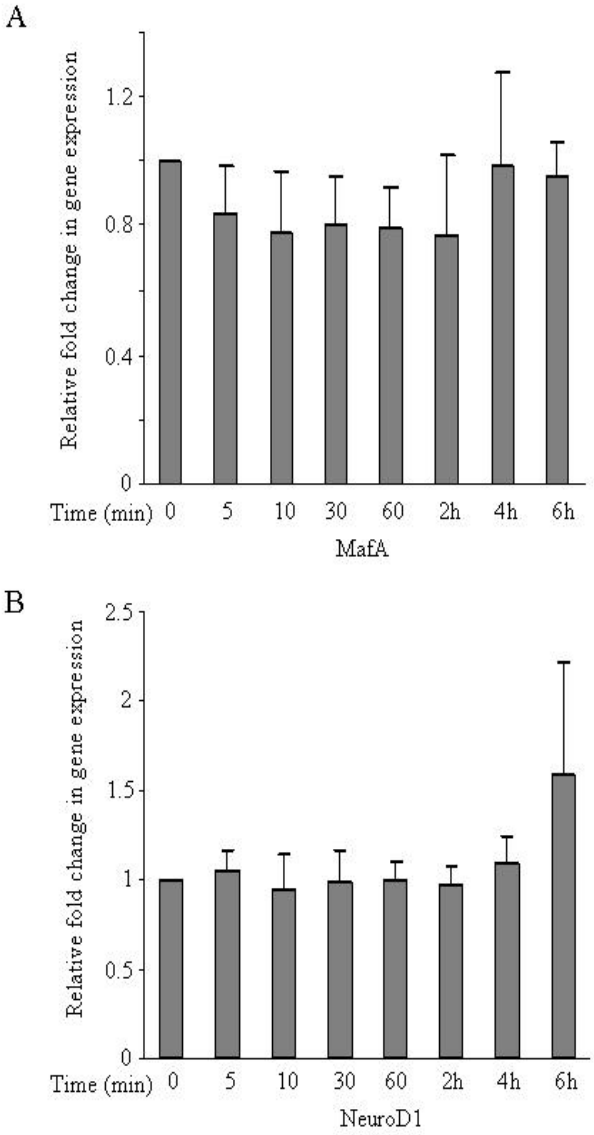


Figure 3-4. IL-1β-induced changes in mRNA of MafA and NeuroD1 in beta cells. Min6 cells were treated with 20 ng/ml IL-1β for different times. mRNA of MafA (A) and NeuroD1 (B) was measured by real-time PCR. Results were expressed as means relative to 18S rRNA expression levels ± SEM of three independent experiments.

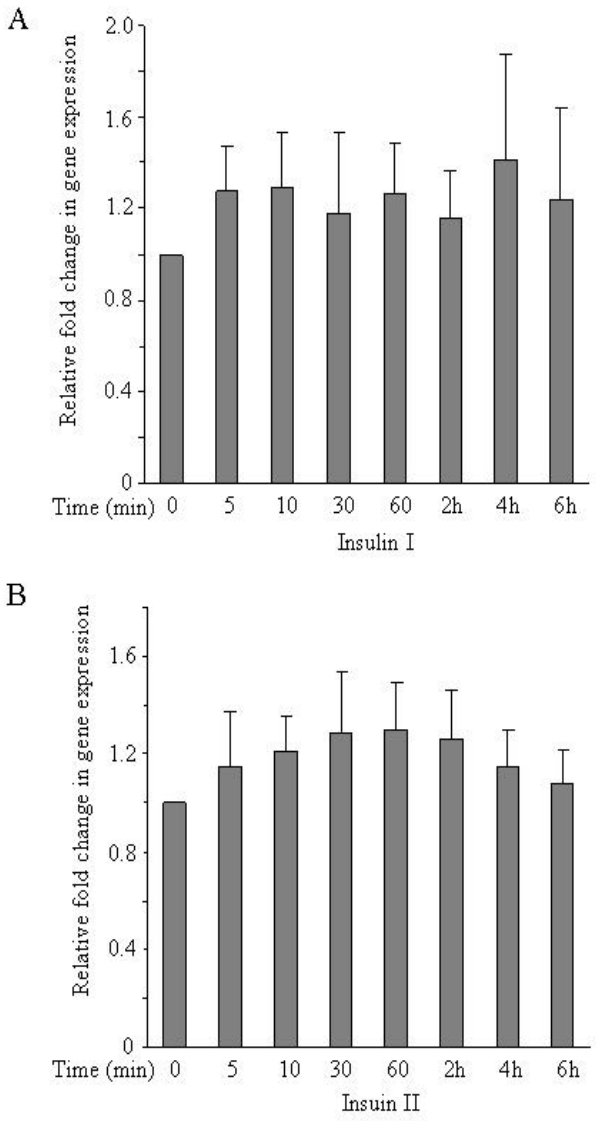


Figure 3-5. IL-1β-induced changes in mRNA of mouse insulin I and II in beta cells. Min6 cells were treated with 20 ng/ml IL-1β for different times. mRNA of insulin I (A) and insulin II (B) was measured by real-time PCR. Results were expressed as means relative to 18S rRNA expression levels ± SEM of three independent experiments.

downregulated to around 30% of the control at 2 h. The transcription of MafA and NeuroD1 was not significantly affected (Figure 3-3B, 3-4). In addition, there was no dramatic change in mRNA levels of either insulin I or II genes observed after IL-1β treatment (Figure 3-5). These data suggest that IL-1β may impair beta-cell function through regulation of certain beta-cell specific genes.

B. IL-1β-induced the regulation of protein expression in pancreatic beta cells.

Given these changes in gene expression, it is not surprising that IL-1β also regulates protein levels of these target genes. As expected, immunoblotting experiments showed that CHOP expression was hardly detected in untreated beta cells but significantly enhanced at 24 h of IL-1β treatment (Figure 3-6B). Addition of 1 ng/ml IL-1β was enough to promote CHOP expression. It has been established that low IL-1β concentrations (0.01-0.02 ng/ml) stimulate human islet cell proliferation and decrease apoptosis, whereas increasing amounts of IL-1β (2-5 ng/ml) have the opposite effects (Maedler et al. 2006). Expression of c-Jun was detected in untreated beta cells. An increase was observed around 10 min followed by a peak at 60 min, and then a return to untreated amounts around 24 h. The induction of c-Jun expression by IL-1β was dose-dependent (Figure 3-6A). Phosphorylation of c-Jun was greatly enhanced at 30 to 60 min after stimulation. Most importantly, immunoblotting indicated that multiple phospho-c-Jun species were induced (Figure 3-7B). In a manner similar to c-Jun, c-Fos expression dramatically increased at 30 to 60 min of IL-1β treatment (Figure 3-10). Additionally, the expression of Pdx-1 and MafA, but not NeuroD1, was downregulated after at least 6 h of

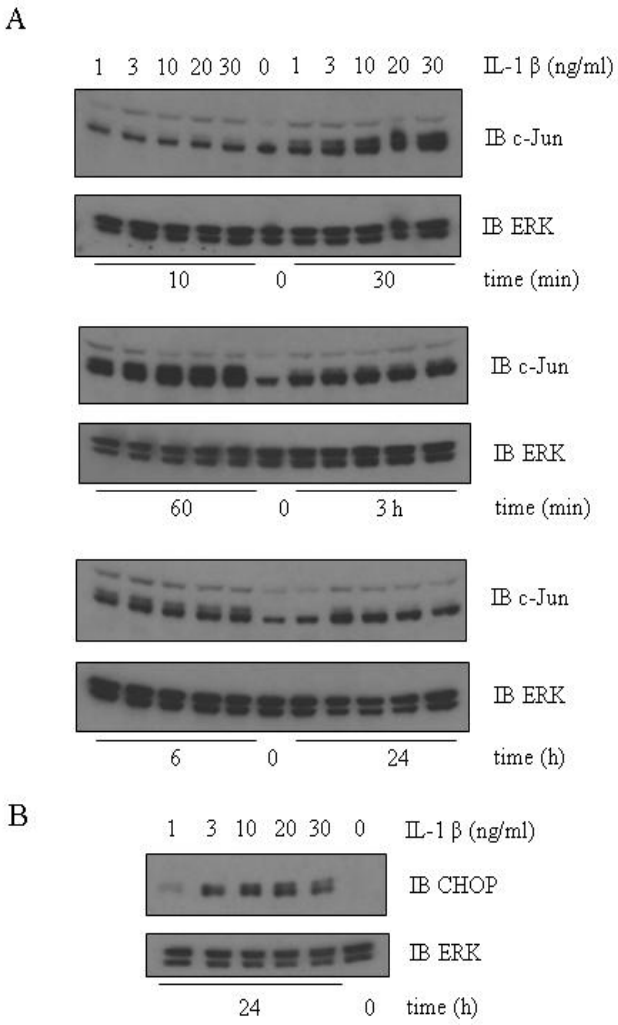


Figure 3-6. Effects of IL-1β on CHOP and c-Jun expression in beta cells.
A and B. INS-1 cells were stimulated with different concentrations of IL-1β for different times. Cell lysates were immunoblotted with antibodies to CHOP, c-Jun and ERK. Experiments in each panel were repeated three times.

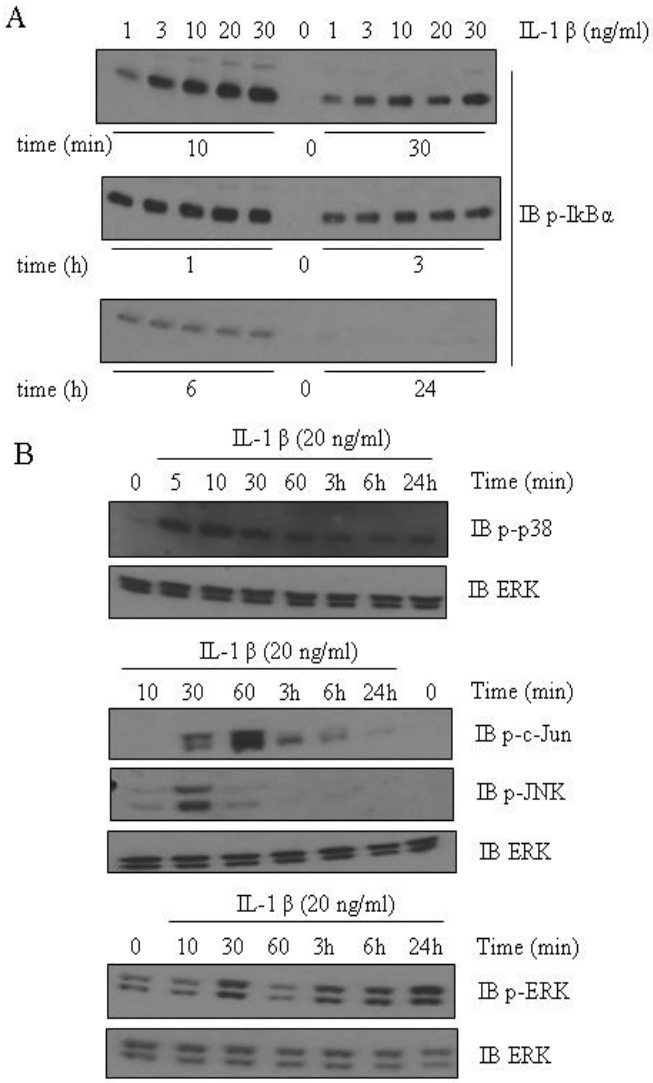


Figure 3-7. IL-1β-induced activation of MAP kinases and NF-κB in beta cells.
A and B. INS-1 cells were stimulated with different concentrations of IL-1β for different times. Cell lysates were immunoblotted with the indicated antibodies. Experiments in each panel were repeated three times.

treatment (Figure 3-8A). In contrast, IL-1β markedly enhanced C/EBP-β expression in a manner independent of ERK1/2 (Figure 3-8B).

A phospho-IκBα antibody was utilized to show the phosphorylation of IκBα as an indirect monitor of the activation of NF-κB. Phospho-IκBα was not detectable in control beta cells and was rapidly induced as early as 5 min after IL-1β treatment. The activation of NF-κB was dose-dependent, oscillated, and returned to control values after 24 h (Figure 3-7A). Furthermore, immunoblotting demonstrated that JNK, ERK1/2, and p38 were all activated by IL-1β in beta cells (Figure 3-7B). SP600125 (20 μM), a JNK inhibitor, inhibited the activity of JNK and also attenuated total c-Jun, phospho-c-Jun, and CHOP expression (Figure 3-9, 3-10A). U0126 (10 μM), a MEK inhibitor, blocked ERK1/2 activity, reduced c-Fos phosphorylation, and partially reduced c-Jun expression (Figure 3-9, 3-10B). Interestingly, SP600125 increased ERK1/2 phosphorylation. As expected, none of the three MAPK inhibitors appeared to have any effect on NF-κB activation (Figure 3-9, 3-8B).

C. IL-1beta-induced the binding of NF-κB to the CHOP gene promoter in beta cells.

As mentioned above, CHOP is predicted to be a downstream target of IL-1β-induced NF-κB signaling pathway. Because there is a putative NF-κB binding site on the 5' flanking region of the CHOP gene, I asked whether NF-κB could bind directly to the CHOP promoter. EMSA experiments were performed by using INS-1 cell lysates following treatment of the cells with IL-1β for different times. A relatively low level of

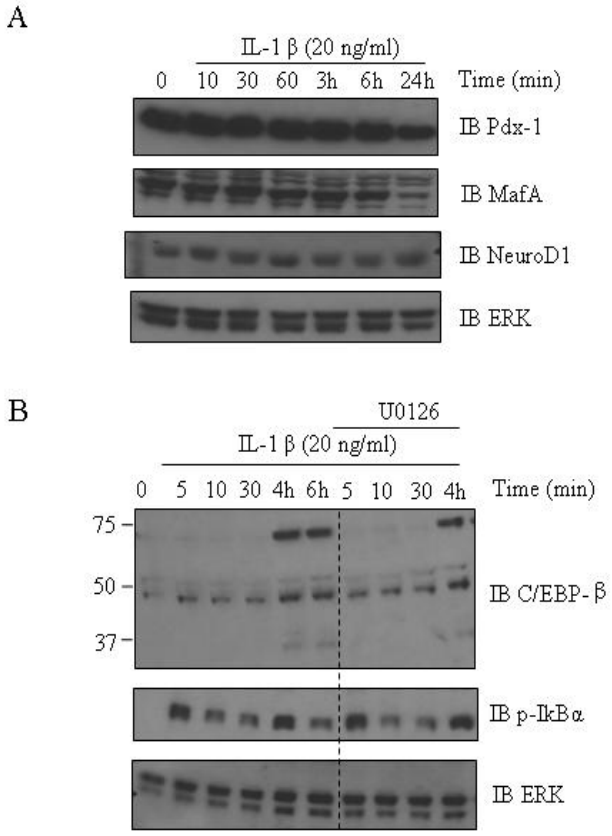


Figure 3-8. Effects of IL-1β on the expression of insulin gene transcription factors in beta cells.

A and B. INS-1 cells were stimulated with 20 ng/ml of IL-1β for different times in the presence or absence of 10 μM U0126. Cell lysates were immunoblotted with the indicated antibodies. Experiments in each panel were repeated three times.

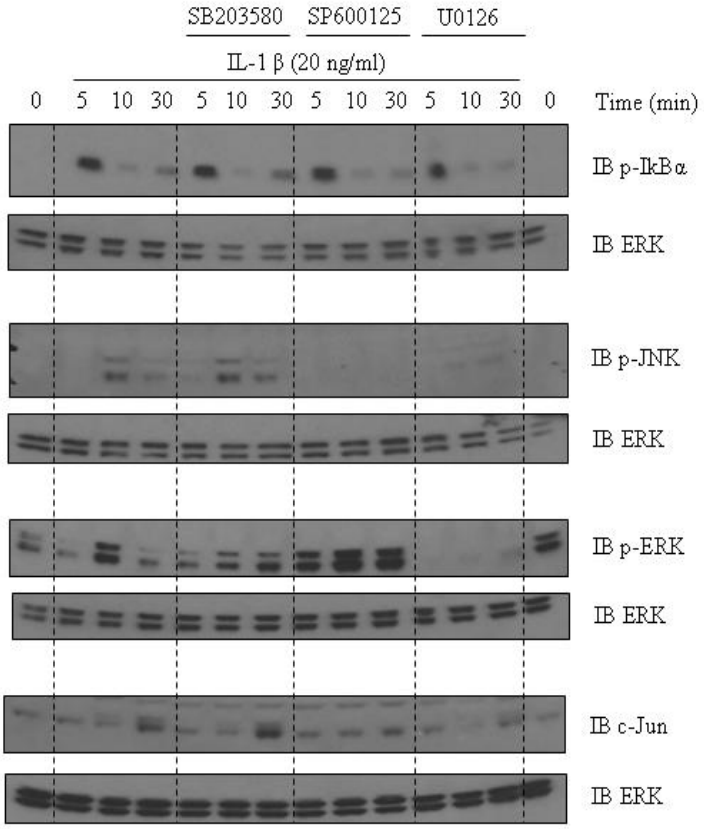


Figure 3-9. Regulation of c-Jun expression by MAP kinases in beta cells.

Min6 cells were stimulated with 20 ng/ml IL-1β for different times in the presence or absence of 10 μM U0126, 25 μM SB203580, or 20 μM SP600125. Cell lysates were immunoblotted with the indicated antibodies. Experiments in each panel were repeated three times.

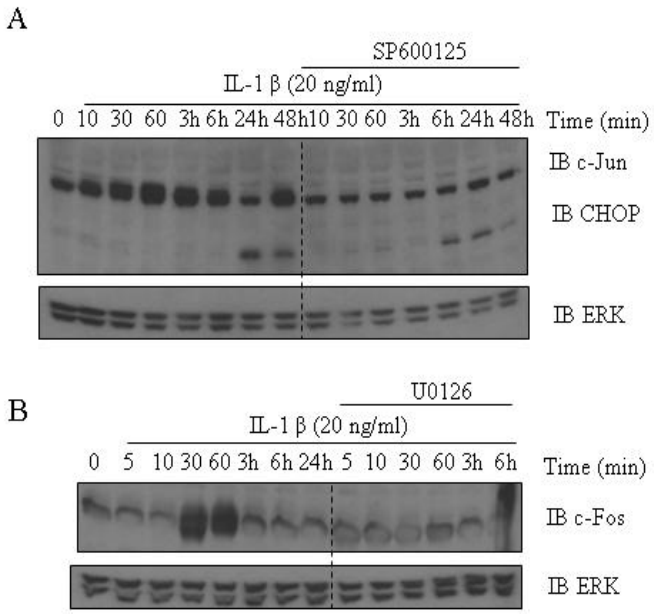


Figure 3-10. Inhibition of IL-1β-induced c-Jun and c-Fos expression by MEK or JNK inhibitor.

A and B. Min6 cells were stimulated with 20 ng/ml IL-1β for different times in the presence or absence of 10 μM U0126 or 20 μM SP600125. Cell lysates were immunoblotted with the indicated antibodies. Experiments in each panel were repeated three times.

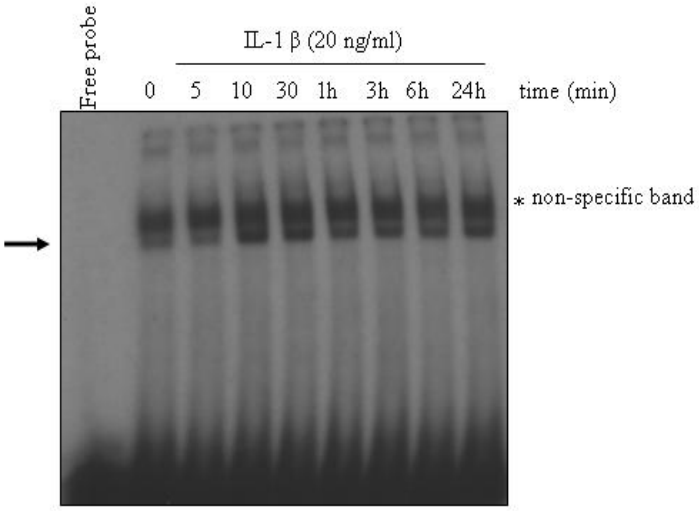


Figure 3-11. The binding of NF-κB to the Chop promoter in IL-1β-stimulated INS-1 cells.

INS-1 cells were stimulated with 20 ng/ml IL-1β for different times. Cell lysates were incubated with [γ-^{32}P]ATP-labeled wild type probe containing NF-κB binding site followed by electrophoresis. The result is a representative of three experiments.

NF-κB dimer bound to the [γ-^{32}P]ATP-labeled wild type probe in control cells. As expected, IL-1β induced robust binding to the probe at 10 to 30 min of the treatment followed by a gradual reduction of DNA binding, consist with the conclusion that the activated NF-κB dimer translocated into beta-cell nuclei and bound to the CHOP promoter (Figure 3-11).

V. Discussion

Pancreatic beta cells are selectively destroyed by the immune system in type I diabetes through multiple signaling pathways (Kumar et al. 2004). Microarray studies have indicated that IL-1β regulates diverse genes associated with beta-cell apoptosis through the NF-κB signaling pathway (Cardozo et al. 2001). But the molecular basis of the effects on specific genes remains unknown. Among the NF-κB-regulated genes, I was interested in CHOP gene expression because ER stress contributes to beta-cell apoptosis in both type I and II diabetes. The regulation of the CHOP gene varies and depends on the cell type and the kind of stress. CHOP expression is increased in beta cells from both diabetic mice and humans (Yusta et al. 2006; Laybutt et al. 2007). Although CHOP-deficient mice do not display any detectable phenotype under basal conditions, beta cells isolated from CHOP-deficient mice are protected from apoptosis caused by either nitric oxide or the accumulation of a folding-defective mutant of proinsulin (Oyadomari et al. 2001; Oyadomari et al. 2002). My EMSA data demonstrated that the IL-1β-activated NF-κB dimer can bind to the NF-κB consensus site on the CHOP gene promoter. Immunoblotting also showed that IL-1β strongly activates NF-κB and increases CHOP

expression. These findings suggest that activated NF-κB may upregulate CHOP gene expression through its function as a transcription activator in IL-1β-treated beta cells. In addition, NF-κB has an indirect effect on CHOP expression. For example, ATF-4, a downstream target of NF-κB, is a transcription factor that increases expression of the CHOP gene (Pirot et al. 2007). Thus, the NF-κB signaling pathway has multiple inputs to CHOP expression in beta cells regulated by IL-1β.

In contrast to its actions in pancreatic beta cells, NF-κB has been shown to be anti-apoptotic in most cell types. It is well documented that many different types of human tumors have constitutively active NF-κB (Karin and Lin 2002). Active NF-κB could upregulate the expression of genes, which maintain cancer cell proliferation and protect them from conditions that would otherwise cause apoptosis. Defects in NF-κB result in increased susceptibility to apoptosis because NF-κB regulates anti-apoptotic genes, such as TRAF1 and TRAF2 (TNF receptor-associated factor), thereby controlling the activities of caspases that are essential in most apoptotic processes. The opposite effects of NF-κB in these different settings may due to the different activators and the involvement of different death effectors. It has been demonstrated that NF-κB activation by TNF family members leads to an anti-apoptotic effect. Whereas, the activation of NF-κB by IL-1, UV light, or chemotherapeutic agents causes pro-apoptotic effects in which the Fas ligand may be involved. Therefore, it is critical to identify the combined effects of cytokines and chemokines on the NF-κB pathway in pancreatic beta cells.

It has been reported that the JNK signaling pathway plays a key role in IL-1β-induced beta-cell apoptosis because the inhibition of JNK, by chemical inhibitors or overexpression of a JNK scaffold protein, can protect beta cells from apoptosis (Weston

and Davis 2007). My data confirmed that IL-1β activates JNK and its downstream target c-Jun in beta cells. Interestingly, JNK activation was transient, lasting only about 30 min. Immunoblotting also showed that SP600125 inhibited JNK activity and attenuated c-Jun and CHOP expression. These data indicate that the JNK pathway is involved in the regulation of IL-1β-induced CHOP expression and suggest that it occurs at least in part through c-Jun transcriptional activation in beta cells. Studies to follow will focus on how c-Jun regulates CHOP expression because the phosphorylation state of c-Jun controls its transactivation potential. By using EMSA, Pirot et al. reported that IL-1β and IFNγ stimulation induces an AP-1 complex composed of c-Jun, c-Fos, and/or JunB to bind to the CHOP promoter. In contrast, Gurzov et al. showed that JunB protects beta cells against IL-1β- and IFNγ-mediated apoptosis through the inhibition of iNOS and CHOP (Pirot et al. 2007). Thus, it is essential to determine the effect of the AP-1 complex on CHOP expression upon cytokine stimulation *in vivo* to clarify this disparity.

Besides JNK, IL-1β induced the activation of ERK1/2 and p38 as well. Both mRNA and protein levels of c-Fos, an ERK1/2 downstream target, were markedly increased by IL-1β. U0126 blocked ERK1/2 activation and c-Fos expression caused by IL-1β. Little is known about the downstream targets of p38 activation under these conditions. Interestingly, the JNK inhibitor, SP600125, increased ERK1/2 activation suggesting crosstalk between JNK and ERK1/2 pathways.

In addition, three MAPK inhibitors appear to have no effect on NF-κB activation. These data implicate that NF-κB may be parallel to the MAP kinase pathways under this specific condition.

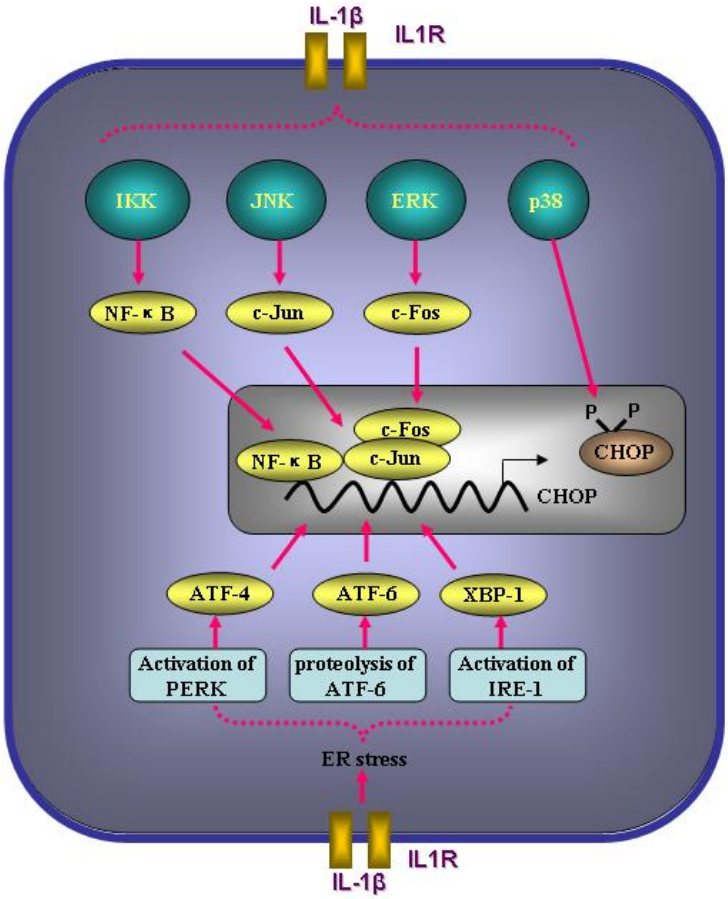


Figure 3-12. Schematic representation of signaling pathways regulating CHOP gene expression.

Chapter Four: Other Observations in Pancreatic Beta Cells

I. Localization of ERK1/2 in pancreatic beta cells.

The MAP kinases ERK1/2 are involved in multiple biological processes including cell proliferation, differentiation, survival and gene transcription. In pancreatic beta cells, ERK1/2 are activated by glucose, GLP-1, certain amino acids, free fatty acids, EGF (epidermal growth factor), and other various stimuli. Although the entire picture describing ERK1/2 function in beta cells is not complete, it has clearly been demonstrated that ERK1/2 are central components in controlling glucose-stimulated insulin secretion and progression of diabetes. Thus, characterizing the ERK1/2 profile in beta cells, including kinase activation, localization, and crosstalk with other signaling pathways, will provide great value to understand beta-cell physiology.

Localization of ERK1/2 has been intensively explored because activation of ERK1/2 in different cellular compartments results in different consequences. For example, about half of ERK1/2 binds to cytoplasmic microtubules and modulates polymerization dynamics (Drechsel et al. 1992; Reszka et al. 1995). Nuclear localization of ERK1/2 is essential to phosphorylate a group of transcription factors. Ligands and stimuli can cause ERK1/2 nuclear localization mainly through phosphorylation that activates the protein kinases. Import reconstitution experiments demonstrated that unphosphorylated ERK2 enters the nucleus through direct binding of ERK2 to nuclear pore proteins instead of a carrier-mediated process (Robinson et al. 2002). Two different nucleoporins (Nups), Nup153 and Nup214, were shown to bind to ERK2 *in vitro*.

Recently, phosphorylated ERK2 was found to enter the nucleus through another import mechanism which requires both energy and cytosolic factors (Ranganathan et al. 2006). Reconstitution experiments using permeabilized cells indicate that the export of ERK2 occurs through at least two processes. One process is Nup-mediated bidirectional transport of ERK2, which is carrier- and energy-independent. The other process is active and dependent on export carrier CRM1 (Chromosome Region Maintenance 1) (Adachi et al. 2000; Burack and Shaw 2005).

In addition, ERK1/2 binding proteins play an important role in determining their subcellular localization. For example, MEK1/2, the upstream activators of ERK1/2, have been implicated in ERK1/2 cytoplasmic retention (Rubinfeld et al. 1999). PEA-15, a 15 kDa antiapoptotic protein, was originally identified as a protein enriched in astrocytes. In import reconstitution assays, PEA-15 was shown to bind to ERK2 and sequester it in the cytoplasm (Danziger et al. 1995). Pulldown assays demonstrated that PEA-15 competes with Nups for binding to ERK2 and prevents ERK2 from entering the nucleus (Whitehurst et al. 2004). The expression of PEA-15 in fibroblasts blocks the ability of ERK1/2 to phosphorylate nuclear transcription factors, such as Elk-1. In contrast, the protein has no effect on the phosphorylation of cytoplasmic substrates, such as p90RSK. Additionally, genetic deletion of PEA-15 markedly increases ERK1/2-dependent proliferation and gene transcription. The reason is that PEA-15 contains a nuclear export sequence that mediates its capacity to anchor ERK1/2 to the cytoplasm. Thus, PEA-15 expression level modulates biological function of ERK1/2 pathway through regulating the localization of ERK1/2. Surprisingly, increased expression of PEA-15 is found to occur among a wide arrag of tissues in a group of patients with type II diabetes, but not

type I diabetes (Condorelli et al. 1998). Transgenic mice ubiquitiously overexpressing PEA-15 display reduced glucose tolerance, impaired insulin secretion, and develop diabetes on a high fat diet (Vigliotta et al. 2004). These findings indicate that localization of ERK1/2 contributes to the regulation of glucose response in pancreatic beta cells.

Shih and colleagues showed that a dramatic increase in activated ERK1/2 was observed especially in the nucleus of INS-1 cells exposed to high glucose plus forskolin (Khoo and Cobb 1997). To gain insight into the dynamics of ERK1/2 distribution in beta cells under various conditions and the importance of PEA-15 on ERK1/2 localization, I performed indirect immunofluorescence staining in cultured beta cells.

Material and Methods

Immunofluorescence. Min6 cells were plated onto cover slips in 12-well dishes. After 48 h, cells were starved for 2 h in KRBH buffer containing 2 mM glucose and 0.1% bovine serum albumin before stimulation with 30 mM glucose for different times. Cells were fixed and permeabilized as described in chapter 2. An anti-phospho-ERK1/2 antibody was incubated with cells overnight at 4 °C. After washing, cells were incubated with secondary antibody (1:5000) and DAPI (1:10000) for 1 h. The cells were washed 3 times with the blocking solution and 1 time with PBS before mounting for analysis by fluorescence microscopy.

Results

Glucose increases ERK1/2 activation in nuclei of Min6 cells.

Phosphorylated ERK1/2 were found mainly in the cytoplasm of Min6 cells cultured in 25 mM glucose plus FBS (Figure 4-1). Starvation with KRBH buffer containing 2 mM glucose in the absence of FBS for two hours did not markedly alter the amount and localization of active ERK1/2. Stimulation with 30 mM glucose for one minute after starvation increased the amount of activated ERK1/2 and altered the cytosolic localization to nuclei. However, active ERK1/2 were exported from nuclei to cytoplasm by three minutes of stimulation and were largely cytoplasmic within 15 min.

To confirm these results, subcellular fractionation and immunoblot will be performed. I also planned to determine the effect of PEA-15 on ERK1/2 localization in beta cells under various conditions. Unfortunately, the commercially available anti-PEA-15 antibody was not sufficiently sensitive for this analysis.

Lawrence et al. demonstrated that ERK1/2 are essential in controlling the activity of many insulin gene transcription factors that are glucose responsive (Lawrence et al. 2005). Thus, regulation of subcellular localization of ERK1/2 is one of the strategies to control their biological outcomes. PEA-15 expression level or its interaction with ERK1/2 may be critical in glucose-induced insulin secrection.

II. Pdx-1 phosphorylation in pancreatic beta cells.

Pdx-1, a homeodomain-containing protein, plays an essential role in pancreatic development, beta-cell differentiation, and maintenance of mature beta-cell function through regulation of certain beta-cell genes, such as insulin, GLUT2 and glucokinase.

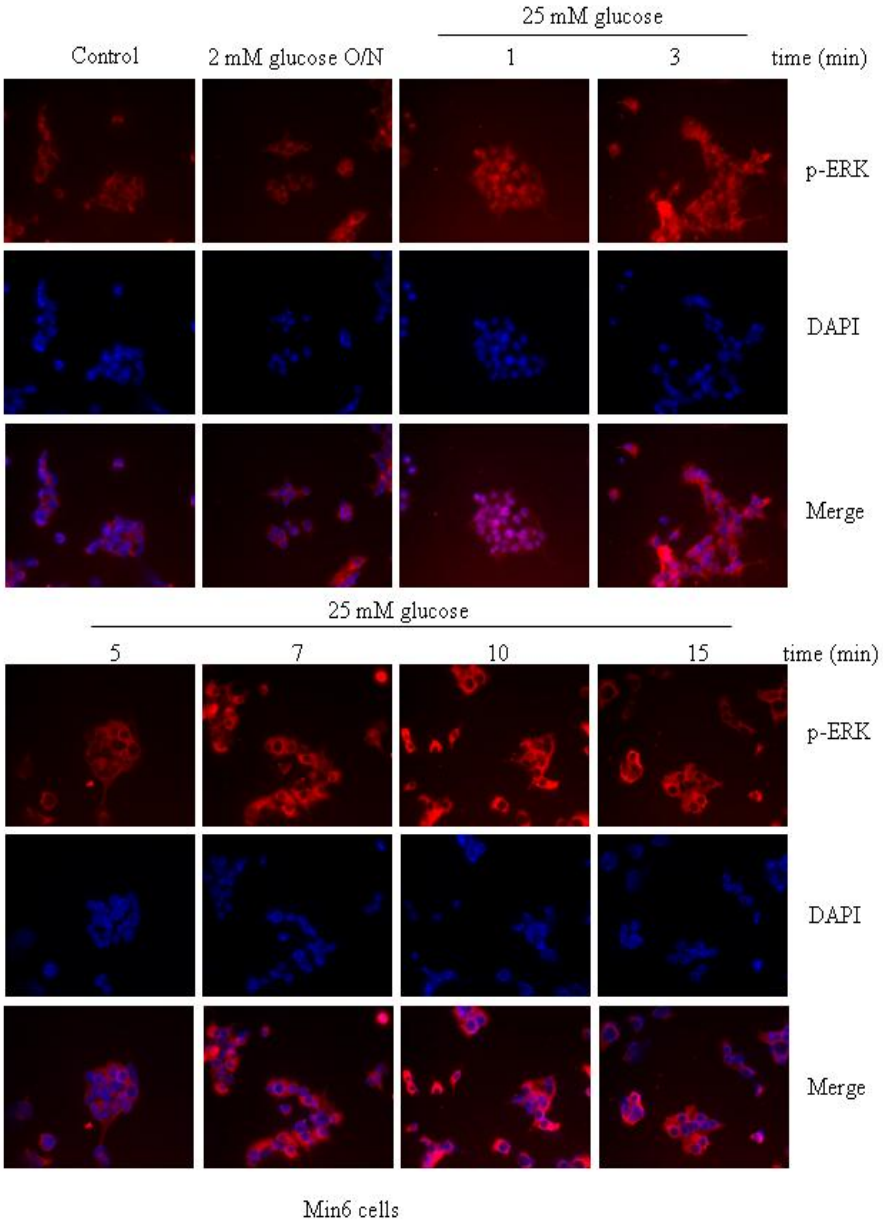


Figure 4-1. Translocation of active ERK1/2 in nuclei in Min6 cells after glucose stimulation.

Min 6 cells were starved with 2 mM glucose overnight followed by stimulation with 25 mM glucose for 1, 3, 5, 7, 10 and 15 min. Localization of active-ERK was detected by immunofluoresence staining.

Pdx-1 is expressed in precursors of the endocrine and exocrine compartments of the pancreas and becomes restricted to beta and delta cells in the mature pancreas. Targeted disruption of Pdx-1 in pancreatic beta cells leads to diabetes, whereas reduced expression impairs insulin expression and secretion (Kaneto et al. 2008). Although the role of Pdx-1 in beta-cell function has already been determined, the regulation of Pdx-1 is not clearly understood.

Phosphorylation is one of the post-translational modifications that regulates Pdx-1 activity in beta cells. It has been suggested that glucose increases Pdx-1 binding to the insulin gene promoter in part through activation of PI3K. And glucose-stimulated translocation of Pdx-1 from the cytoplasm to the nucleus is dependent on Pdx-1 phosphorylation. It was also reported that oxidative stress-induced JNK activation reduces Pdx-1 DNA binding to the insulin promoter and enhances Pdx-1 translocation from the nucleus to the cytoplasm (Kaneto et al. 2008). Shih and colleagues showed that in *in vitro* kinase assays, Pdx-1 could be phosphorylated by ERK2 and JNK1. Mutation of Ser61 and 66 suggested that these sites may be modified although results are ambiguous (Khoo et al. 2003). Boucher et al. demonstrated that Pdx-1 could be phosphorylated by GSK3β on serine 61 and/or serine 66, which may induce the degradation of Pdx-1 through the proteasome (Boucher et al. 2006). Oxidative stress can increase Pdx-1 Ser 61 and/or Ser 66 phosphorylation leading to elevated degradation rate. Thus, the aim of my project was to identify Pdx-1 phosphorylation sites by ERK1/2 and their biological significance in beta cells.

Material and Methods

Materials and immunoblotting. Maintenance and lysis of Min6 and HEK293 cells were as described in Chapter 2. Lysis buffer used in phosphatase 2A (PP2A) experiments does not contain any phosphotase inhibitors. Min6 cells at about 80% confluence were incubated overnight in KRBH buffer containing 2 mM glucose and 0.1% bovine serum albumin before stimulation with 30 mM glucose. U0126, SB203580, SP600125 and LY294002, purchased from LC Laboratories (Woburn, MA), were added 30 min before stimulation. The cDNA encoding hamster Pdx-1 was obtained from Dr. Michael German (UCSF). Site-directed mutagenesis was done with the QuikChange kit (Stratagene) according to the manufacturer's instructions. All constructs and mutants were confirmed by sequencing. PP2A purified as described (Kremmer et al. 1997) in reaction buffer (20 mM MOPS (4-morpholinepropanesulfonic acid) pH 7, 0.5 mg/ml BSA, 1 mM DTT (L-Dithiothreitol)) was added to cell lysates and incubated at 30 ^{o}C for 1 h. Aliquots of cell extracts containing 20 μg of protein were subjected to SDS/polyacrylamide gel electrophoresis. Immunoblotting was done as described in chapter 2.

Results

To investigate glucose-induced Pdx-1 phosphorylation in beta cells, Min6 cells were starved with 2 mM glucose overnight followed by stimulation with 30 mM glucose for 5, 10 and 15 min. Pdx-1 migrated as several bands in Min6 or INS-1 cells with high or low glucose. To determine if these bands were due to heterogeneous phosphorylation,

I treated Min6 lysates with PP2A. The majority of bands collapsed into one predominant species, indicating that the bands that were lost were phosphorylated species (Figure 4-2B). The lack of effect of glucose may either reflect the possibility that Pdx-1 phosphorylation is not glucose sensitive or that Pdx-1 remains modified even in very low glucose. Neither MAP kinase nor PI3K inhibitors blocked the appearance of the Pdx-1 phosphorylated bands in Min6 cells stimulated with 30 mM glucose for 15 min.

Mass spectrometry of recombinant Pdx-1 produced in bacteria phosphorylated by purified active ERK2 indicated that Ser61, Ser66, Ser29 and Ser268 were phosphorylated and thus are potential ERK2 phosphorylation sites (Figure 4-2A). I made mutations of S61A, S66A, S29A, S268A, S29, 61A, and S29, 268A by using site-directed mutagenesis followed by transfection in both Min6 and HEK293 cells as indicated. It appears that Ser29 and Ser61 were phosphorylated *in vivo* (Figure 4-2C, D). Phosphorylation of Pdx-1 is complicated because many kinases may phosphorylate Pdx-1 and each site may be phosphorylated by different kinases.

It has been reported that Pdx-1 could be sumoylated in pancreatic beta cells. The reduction of SUMO-1 by RNA interference reduces nuclear localized Pdx-1 (Kishi et al. 2003). Based on the amino-acid sequence of Pdx-1, there is a SUMO modification consensus motif (WK202KE) in the homeodomain. However, I did not detect sumoylated Pdx-1 by immunoblotting and no difference between wild type and K202R Pdx-1 was observed. K202R Pdx-1 migrated in the same pattern of multiple species as did the wild type protein (Figure 4-2D).

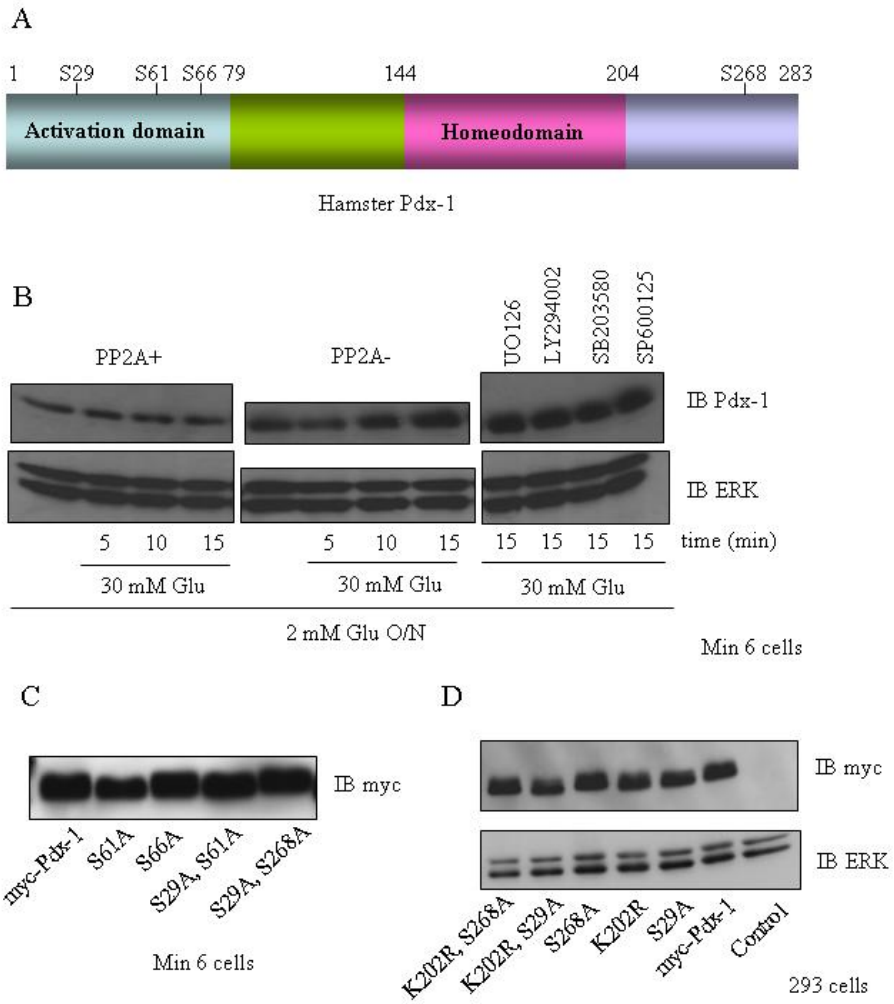


Figure 4-2. Pdx-1 phosphorylation.
A. Diagram of the domain structure of Pdx-1. B. Min 6 cells were starved with 2 mM glucose overnight followed by stimulation with 30 mM glucose for 5, 10 and 15 min in the presence or absence of 10 μM U0126, 20 μM SP600125, 25 μM SB203580, or 1 μM LY294002. Lysates treated with or without PP2A were immunoblotted with anti-Pdx-1 and anti-ERK antibodies. C and D. Lysates from Min6 or HEK293 cells transfected with the indicated plasmids were immunoblotted with anti-myc and anti-ERK antibodies.

III. ERK pathway and glucose-induced histone modifications on the insulin gene promoter.

Transcriptional regulation of gene expression has been studied extensively. In the past two decades, it has become clear that chromatin is not only a structure to allow compaction of DNA within the nucleus, but is also involved in the regulation of a number of cellular processes, such as gene transcription and DNA replication. Epigenetic regulation of gene expression controls the accessibility of promoters to the transcription machinery, thereby leading to repression or activation of gene expression.

The basic unit of chromatin is the nucleosome which is wrapped around by 146 bp of DNA. Each nucleosome contains a histone octamer that includes two heterodimers of histones H2A and H2B and a tetramer of histones H3 and H4. Every histone contains a structured domain and an N-terminal 25-40 amino-acid tail. A variety of post-translational modifications have been identified on both histone tails and globular domains, such as methylation of arginines, methylation, acetylation, ubiquitination, ADP-ribosylation, and sumoylation of lysines, and phosphorylation of serines and threonines. Different patterns of modifications have been linked to different outcomes with respect to gene expression regulation. Euchromatin modifications often associated with active transcription include acetylation of histone 3 and histone 4 (H3, H4), and di- or tri-methylation of H3 lysine 4. Heterochromatin modifications are thought to indicate inactive transcription and include H3 lysine 9 methylation and H3 lysine 27 methylation. Modifications may occur within the promoter region, and at the 5’ or 3’ ends of the open reading frame. All are precisely regulated (Strahl and Allis 2000). Several possible

mechanisms by which chromatin modifications regulate gene expression have been proposed. First, chromatin modifications could change the net charge of nucleosomes except for methylation, which may alter the interaction between DNA and histones. Second, histone modification patterns could be recognized by other proteins, which may result in recruitment of additional activities to decide the outcome of modifications.

Histone acetylation occurs at specific lysines on four histones. The modifications are catalyzed by histone acetyltransferases (HATs) through the transfer of the acetyl group from acetyl-coenzyme A to the ε-amine of the target lysine. Histone acetylation can be reversed by enzymes, histone deacetylases (HDACs). The balance between HATs and HDACs controls the extent of histone acetylation. It is widely accepted that histone acetylation can alter the interaction between DNA and histones, thereby leading to a more open chromatin architecture. Many transcriptional coactivators, such as CREB binding protein (CBP/p300) and p300/CBP-associated factor (PCAF) have intrinsic HAT activity (Eberharter and Becker 2002). Mirmira group have reported that H3 is hyperacetylated relative to H4 at the proximal insulin promoter in beta cells, which is probably mediated by p300 (Chakrabarti et al. 2003).

Histone phosphorylation often occurs at serine or threonine residues mediated by different kinases. For example, H2A is phosphorylated through a DNA-damage signaling pathway. Phosphorylation of H2B is catalyzed by Mst1 (mammalian Ste20-like kinase). Phosphorylation at H3 Ser 10 and H3 Ser 28 during mitosis is catalyzed by the Aurora kinases. Other kinases in the ribosomal S6 kinase / mitogen- and stress-activated protein kinase (MSK/RSK) family have been shown to phosphorylate H3 Ser 10 during gene expression (Cheung et al. 2000). Many studies suggest that MAP kinase pathway

activation elicits H3 phosphorylation and/or acetylation. For example, activation of ERK1/2 *in vitro* significantly increases H3 phosphorylation in hippocampal area CA1 neurons (Chwang et al. 2006). Sharrocks recently demonstrated that MAP kinase-mediated phosphorylation of Elk-1 enhances the histone acetylase activity of p300 at the c-fos promoter, which causes changes in the histone acetylation state. This allows the recruitment of the additional transcription factor NFI (nuclear factor I) to the promoter (O'Donnell et al. 2008). Our previous data showed that the activation of ERK1/2 is a key step in glucose-induced insulin gene transcription. However, little is known about the role of ERK1/2 in regulation of histone modifications at the insulin promoter. I initiated studies to determine histone modifications, especially phosphorylation and acetylation, at the insulin promoter induced by glucose and the role of ERK1/2 in this process.

Material and Methods

Chromatin immunoprecipitation assay. Min6 cells (in 10 cm dishes) were treated with different concentrations of glucose in the presence or absence of U0126 as indicated in legends. After treatment, Min6 cells were crosslinked with formaldehyde at a final concentration of 1% for 8 min at room temperature with gentle agitation. Reactions were terminated by addition of 0.125 M glycine for 5 min. Cells were washed twice with cold PBS containing 0.2 mg/ml PMSF and scraped in 0.5 ml PBS followed by centrifugation at 5000 rpm for 5 min at 4 ^{o}C. Pellets were resuspended in 0.3 ml SDS-lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 10 μg/ml aprotinin, 5 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.2 mg/ml PMSF) and incubated on ice for 10 min.

0.2 ml dilution buffer (0.01% SDS, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 1.1% Triton X-100, 167 mM NaCl, 10 µg/ml aprotinin, 5 µg/ml pepstatin A, 5 µg/ml leupeptin, 0.2 mg/ml PMSF) was added into the lysates. Lysates were sonicated using a Sonic Dismembrator 500 (Fisher Scientific) to shear crosslinked chromatin into 500 to 1000 bp pieces followed by centrifugation at 14000 rpm for 10 min. The concentration of disrupted chromatin was measured by its absorbance at 260 nm (Ultrospec 2000 UV/Visible spectrophotometer). 0.06 mg chromatin was used for each immunoprecipitation reaction and 5% of the reaction was used as the input. 5 µg of each antibody was added to the lysates with rotation overnight at 4 ^{o}C, followed by incubation with 20 µl protein A-agarose beads and 10 µg salmon sperm DNA for 2 h at 4 ^{o}C. Extensive washing was performed: one time with low salt wash buffer for 5 min (0.1% SDS, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 1% Triton X-100, 150 mM NaCl), one time with high salt wash buffer for 5 min (0.1% SDS, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 1% Triton X-100, 500 mM NaCl), one time with LiCl wash buffer for 5 min (1 mM EDTA, 10 mM Tris-HCl, pH 8.1, 1% NP-40, 1% deoxycholate, 0.25 M LiCl), and twice with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). To elute the bound chromatin and proteins, precipitates were rotated for 15 min at room temperature twice in 0.25 ml freshly made elution buffer (1% SDS, 0.1 M $NaHCO_3$). To reverse crosslinks, 20 µl of 5 M NaCl was added and incubated at 65 ^{o}C for 4 h. 10 µl of 0.5 M EDTA, 200 µl Tris-HCl pH6.5, and 2 µl 10 mg/ml proteinase K were then added and incubated for 1 h at 55 ^{o}C followed by extraction with phenol/chloroform/isoamyl alcohol. DNA was precipitated with 10 µg glycogen, 50 µl 3 M Na-acetate, and 1 ml EtOH at -80 ^{o}C for 30 min followed by centrifugation at 14,000 rpm for 30 min. Precipitated DNA was washed

and dissolved in water and analyzed by PCR. The primers to amplify mouse insulin I gene promoter region (-9 to -295 bp) are forward 5'-GCCAAAGATGAAGAAGGT-CTCACC, reverse 5'-CTTTGGACTATAAAGCTGGTGGGC.

Anti-acetyl-H3 (K9, 14), acetyl-H4 (K5, 8, 12, 16), and phospho-H3 (S10) antibodies were purchased from Upstate Biotech (Lake Placid, NY).

Results

To investigate histone modifications at the insulin gene promoter under various conditions, I performed CHIP assays in Min6 cells. Both histone 3 and 4 acetylation occurred at the insulin promoter when Min6 cells cultured in 25 mM glucose were starved with 2 mM glucose for 2 h. Treatment with 30 mM glucose for 15 min strongly stimulated histone 3 but not 4 acetylation (H3K9 and H3K14). The MEK inhibitor U0126 blocked high glucose-induced H3 acetylation but had no effect on H4 acetylation (Figure 4-3A). Similar results were observed when Min6 cells were cultured in 5.5 mM glucose (Figure 4-3B). In addition, H3 phosphorylation on Ser 10 at the insulin promoter occurred when Min6 cells were treated with low or high glucose (Figure 4-3C).

These data suggested that histone modifications are involved in the regulation of insulin expression, and that activation of ERK1/2 may directly or indirectly control the process (Figure 4-4). Lawrence et al. recently reported that glucose stimulation causes the binding of ERK1/2 and other ERK pathway modulators to insulin gene promoter. ERK1/2 could phosphorylate insulin gene transcription factors, modify histone proteins, and/or recruit other proteins essential for transcription initiation and/or elongation.

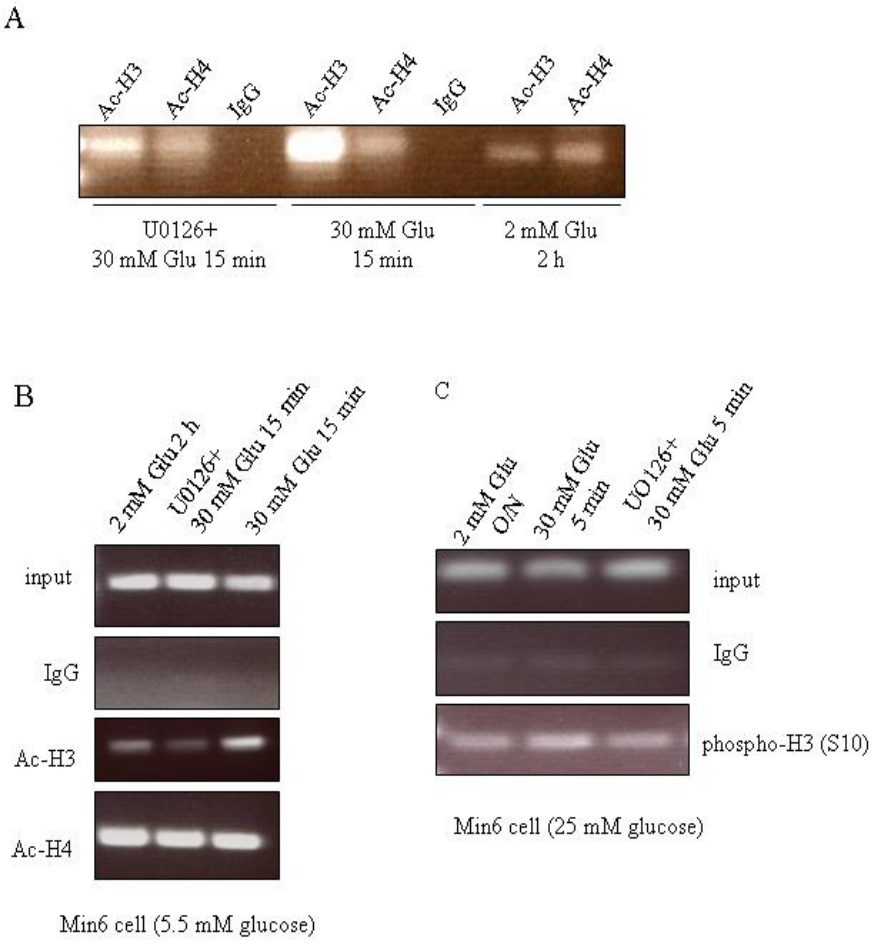


Figure 4-3. Histone modifications on the insulin gene promoter.
A - C. Min6 cells cultured in 5.5 or 25 mM glucose were starved with 2 mM glucose for 15 min or overnight followed by stimulation with 30 mM glucose for 5 or 15 min in the presence or absence of 10 μM U0126. Histone 3 and 4 acetylation and phosphorylation on the insulin I gene promoter (-9 to -295 bp) were detected by chromatin immunoprecipitation assays.

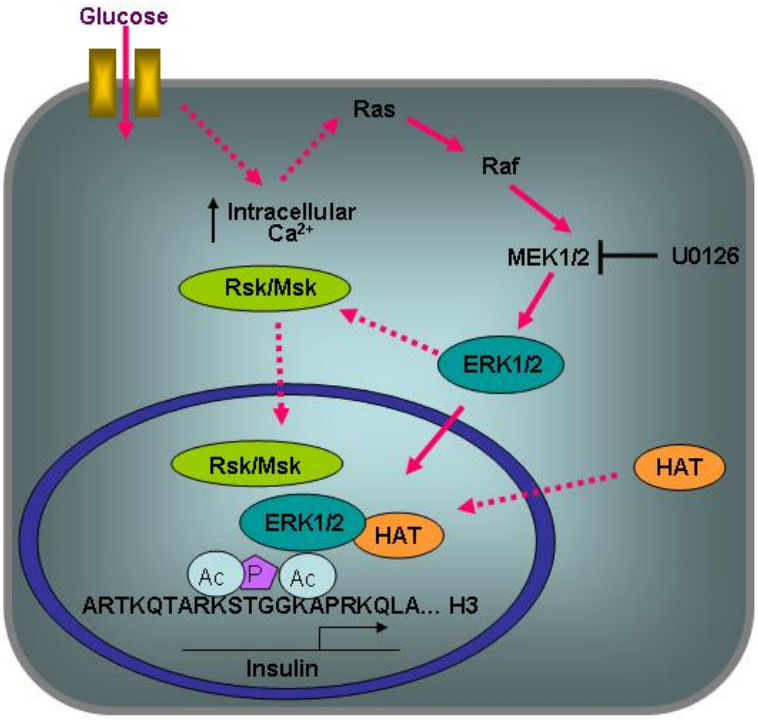


Figure 4-4. Diagram of histone 3 modifications on the insulin gene promoter.

Chapter Five: Conclusions and Future Directions.

The rapid increase in the incidence of diabetes has increased the urgency of understanding pancreatic beta-cell function. In my studies, the major goal has been to elucidate the regulation of insulin gene expression and the mechanisms of immune-mediated pancreatic beta-cell destruction in type I diabetes. The first part of my thesis focuses on the sumoylation of the insulin gene transcription factor MafA in beta cells. Transcriptional regulation of insulin expression is a key homeostatic step in controlling insulin levels in the circulation. MafA is a beta-cell specific and glucose-responsive insulin transactivator. Post-translational modifications modulate its transactivating activity. In cultured beta cells, both endogenous and exogenous MafA can be modified by SUMO. Lysine 32 is the major sumoylation site. Oxidative stress and starvation of beta cells with low glucose increased MafA sumoylation. Sumoylated MafA results in reduced transcriptional activity towards insulin gene promoter and increased suppression of CHOP gene promoter. However, the underlying mechanism remains to be determined because MafA sumoylation has no apparent effect on its nuclear localization, DNA binding, and protein stability.

The second part of my thesis focuses on mechanisms of IL-1β-induced CHOP gene expression in pancreatic beta cells. During the onset of type I diabetes, proinflammatory cytokines, such as IL-1β, TNF-α and IFNγ, released by invasive immune cells, cause ER stress and eventually lead to beta-cell apoptosis. CHOP is a marker of ER stress. Exposure of beta cells to IL-1β promotes CHOP gene expression at least in part through the activation of NF-κB and JNK pathways. Activated NF-κB dimer by IL-1β could bind

to the NF-κB consensus site on the CHOP promoter suggesting that CHOP gene is directly regulated by NF-κB. IL-1β could also dramatically increase c-Jun and c-Fos mRNA and protein. c-Jun and c-Fos may form an AP-1 complex and bind to the AP-1 site on the CHOP promoter. In addition, ERK1/2 and p38 signaling pathways were activated by IL-1β as well. But the extent of and sites of crosstalk among these pathways is largely unknown in this context.

Based on these results, several issues remain to be solved. First, sumoylation regulates the transcriptional activity of MafA in beta cells. But the underlying mechanism remains unknown because sumoylation seems to have no apparent effect on MafA localization, DNA binding, or stability. It is possible that MafA sumoylation might recruit other cofactors to transcriptional complexes, which in turn could affect MafA transcriptional activity or the activity of a larger transcriptional complex. Second, both sumoylation and phosphorylation are critical in regulating MafA transactivating potential on the insulin gene promoter. It would be interesting to investigate the relationship between them. A well documented example of crosstalk between these modifications comes from the ternary complex factor Elk-1. ERK1/2 activation leads to both Elk-1 phosphorylation and desumoylation. Phosphorylation is a switch to reverse the repressive activity of sumoylation to activation (Yang et al. 2003). Other studies also reported that phosphorylation may control the adjacent sumoylation in heat-shock factors (HSFs) and myocyte enhancer factor 2 (MEF2) (Hietakangas et al. 2006). The interaction between these modifications may explain the dynamic regulation of MafA under various conditions.

IL-1β can activate NF-κB and MAP kinase pathways in pancreatic beta cells. Several issues are still in question. First, which factors transduce the signal from IL-1β receptors to these pathways? Regulation of upstream factors may be important in controlling IL-1β-induced beta-cell death. Second, it is known that IL-1β activates JNK and its downstream target c-Jun in beta cells. C-Jun could also be phosphorylated by other kinases. Therefore, one next step is to determine the phosphorylation state of c-Jun and how phosphorylation affects its ability to affect the CHOP promoter upon IL-1β stimulation. It would also be valuable to investigate which JNK isoform functions as the major kinase for IL-1β-induced c-Jun phosphorylation. In the initial studies, JNK isoforms were suggested to function redundantly because either JNK1 or JNK2 deficient mice could survive, but the double knockout mice were lethal due to the defective neural tube morphogenesis (Kuan et al. 1999; Sabapathy et al. 1999). Further analyses of these JNK-deficient mice indicated that JNK isoforms function differently. Furthermore, JNK1-null mice gained less weight on a high-fat diet when compared with wild-type or JNK2-deficient mice (Hirosumi et al. 2002). And JNK1-null mice showed lower blood glucose and insulin levels, suggesting that JNK1 could protect animals from the development of obesity-induced insulin resistance. In addition, *jnk2-/-* mice reduced the invasive and destructive insulinitis of the pancreas because CD4+ T cells in these mice produced less IFNγ but more interleukin-4 and interleukin-5 (Jaeschke et al. 2005). These findings suggest the critical roles of JNK isoforms in mediating immune responses in pancreas.

In addition, both genetic and pharmacological studies indicate that JNK activation has a proapoptotic effect in IL-1β-treated beta cells. Although active JNK phosphorylates

a group of proteins including c-Jun, the nature of most of IL-1β-induced and JNK-regulated genes in pancreatic beta cells remains to be identified. To address this issue, microarray analyses may provide information about genes whose expression is changed in pancreatic beta-cell lines or sorted human beta cells exposed to IL-1β. It would be interesting to determine the genes which are involved in many aspects of the progression of diabetes, such as ER stress, islet inflammation, and insulin sensitivity. Further confirmation of JNK downstream targets is required to understand the role of JNK signaling pathway in the pathogenesis of diabetes.

As mentioned before, exposure of beta cells to IL-1β causes ER stress, which ultimately leads to an apoptotic process. Apoptosis is achieved through intrinsic or mitochondrial and extrinsic or death receptor-mediated pathways and caspases are the major components of the cell suicide machinery. Although deletion of the CHOP gene has been indicated to protect pancreatic beta cells from apoptosis, resulting in delayed diabetes onset, the mechanism by which CHOP causes beta-cell apoptosis remains undefined. For example, what are the targets regulated by CHOP in beta-cell apoptotic process? Which caspases are affected by CHOP? Furthermore, the combination of other proinflammatory cytokines, such as TNF-α and IFNγ exacerbates beta-cell destruction. The plural effects may mimic the actual environment of beta cells in the development of diabetes. IFNγ exhibits the deleterious effect mainly through the activation of JAK (Janus kinases) and STAT signaling pathways. TNF-α binds to TNF receptors and initiates certain pathways, such as NF-κB, MAP kinase pathways and death signaling. But it remains unknown how these signals crosstalk with each other? What downstream targets

are activated upon cytokine stimulation? These further investigations will shed light on the pathogenesis of diabetes.

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This dissertation was typed by the author.