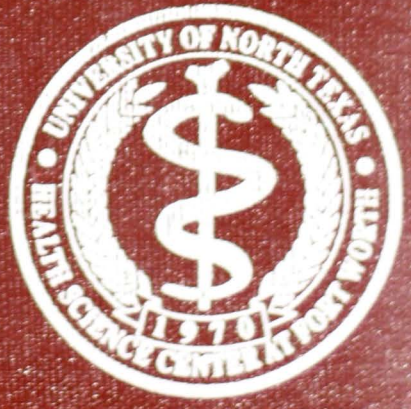
UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

Lawrence, Michael C., The Role of Calcineurin and NFAT in the Regulation of Insulin Gene Transcription. Doctor of Philosophy (Biomedical Sciences), December, 2001, 185 pp., 41 illustrations, references, 222 titles.

In an effort to understand glucose and hormone regulated insulin gene transcription elicited by increased intracellular calcium, a novel pathway has been identified. This pathway involves the calcium/calmodulin-dependent phosphatase 2B (calcineurin) and nuclear factor of activated T-cells (NFAT), which in the studies herein, have been determined to up-regulate insulin gene transcription in response to glucose and glucagon-like peptide-1 (GLP-1) in pancreatic β-cells. Three NFAT elements within the first 410 base pairs of the rat I insulin gene promoter were first identified, two of which are conserved (by presence and location) among mammals including dogs, mice, and humans. The presence of NFAT in rat insulinoma β-cells (INS-1) and rat pancreatic islets was detected by immumobotting, immunofluorescence, and RT-PCR. Electrophoretic mobility shift assays displayed NFAT-specific DNA-binding activity that could be competed with unlabelled NFAT probe when incubated with INS-1 cells or rat islet nuclear extracts and shifted with extracts pre-incubated in the presence of either anti-calcineurin or anti-NFAT antibodies. Transfection experiments with either the -410 rat I (rInsI-Luc) or the NFAT-Luc promoter-reporter showed increased promoter activity when stimulated by glucose or cell depolarization (increases intracellular calcium) and displayed a synergistically enhanced response when co-stimulated with glucose and GLP-1. The GLP-1-induced responses were mimicked by forskolin and concentration-

dependently inhibited by each of two selective but distinct protein kinase A (PKA) inhibitors, H-89 and myristoylated PKI (14-22) amide. The selective calcineurin-inhibitor FK506, as well as the chelation of intracellular Ca^{2+} by BAPTA, also abolished the effects of high glucose and GLP-1. Moreover, co-transfection experiments with a constitutively active form of calcineurin and the promoter-reporters (rInsI-Luc and NFAT-Luc) showed increased reporter activity over controls. Furthermore, two-point base pair mutations in any of the three identified NFAT sites within the rat insulin I promoter resulted in a significant ($p< 0.05$) reduction in the combined effect of glucose and GLP-1. These studies establish the presence of NFAT in insulin-secreting cells, its ability to bind to elements within the insulin gene promoter, and show that glucose and GLP-1 synergistically enhance NFAT-mediated insulin gene transcription by PKA- and calcineurin-dependent pathways in pancreatic β-cells.

THE ROLE OF CALCINEURIN AND NFAT IN THE REGULATION OF INSULIN GENE TRANSCRIPTION

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DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas
Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHOLOSOPHY

By

Michael C. Lawrence, B.A.

Fort Worth, Texas

December, 2001

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CHAPTER I

INTRODUCTION

The research described herein was performed as part of an ongoing mission to develop a better understanding of the devastating disease, diabetes mellitus. This disease is directly related to a defection or loss of pancreatic β-cells of the islets of Langerhans, which produce and release insulin in response to physiological stimuli. Data were collected to examine the global hypothesis that calcineurin and NFAT play a role in regulating insulin biosynthesis at the level of transcription. The studies are divided into two main parts: 1) the identification of calcineurin and NFAT as a calcium-responsive pathway to up-regulate the insulin gene promoter and 2) the determination of glucose and GLP-1 as potent physiological regulators of insulin gene transcription, effects which are mediated via the calcineurin/NFAT pathway. The significance of these findings has direct relevance to remedial approaches to diabetes such as the engineering of pancreatic β-cells for transplantation, the design of immunosuppressant drugs which are less invasive to β-cells, the development of pharmaceutical analogs or replacements of GLP-1 that are most conducive for therapeutic treatment of diabetes, and gene therapy methodology which targets the expression of pharmacological agents or replacement genes in β-cells. A prerequisite for discussing the current studies as they relate to

physiological processes that regulate insulin gene transcription is a brief description of the basic principles of metabolic fuel homeostasis and pancreatic islet pathophysiology, as well as key components and issues regarding insulin production. Also, a review of the currently recognized features of the calcineurin/NFAT signaling pathway and its involvement in gene transcription will be included in this introduction.

Islet Physiology

Metabolic homeostasis is critically regulated by the Islets of Langerhans of the pancreas. These tiny clusters of cells are dispersed throughout the pancreas and serve as neuroendocrine organs, which are responsible for maintaining blood glucose levels within a narrow margin of fluctuation. These groups of heterogeneous cell populations include the β-cells, α-cells, δ-cells, and PP-cells. These cells uniquely produce and secrete the peptide hormones insulin, glucagon, somatostatin, and pancreatic polypeptide, respectively. The islets comprise only approximately 1% of the total number of cells found in the pancreas and are primarily composed (65-90%) of an inner core of the insulin-secreting β-cells (1). Insulin is central to the regulation of glucose disposal, utilization, and storage. However, a fine-tuned interplay between these counter-balanced hormones, neuronal input, and circulating fuels results in the optimal regulation of carbohydrate and lipid metabolism.

A prime example of this is the effect of glucagon released from α-cells to elicit glucose from the liver, which opposes the actions of insulin in order to prevent plasma

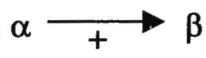
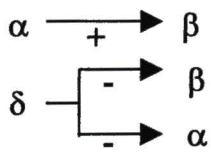
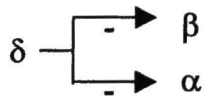
glucose levels from falling below a specific threshold concentration. In addition, the δ-cells appear to have a balancing effect on insulin and glucagon secretion from β-cells and α-cells by negative feedback inhibition (Fig 1). Furthermore, several studies indicate that the singular actions of insulin in both animals and humans cannot reproduce a normal physiological profile of glucose homeostasis. This is clearly demonstrated in diabetic patients who administer insulin for therapy and in post-transplant patients who have received β-cell grafts. Although it is accepted that a pure β-cell population can suffice to maintain blood glucose within a targeted range, a smooth modulation of the concentration-response curve and long-term metabolic control requires the functional presence of non-β-cells as well (2).

The Pathophysiology of Diabetes

Diabetes mellitus is well in need of attention for improved therapies. It is still the seventh leading cause of death in the United States based on death certificate data collected in 1996. More recently the incidence of diabetes in the U.S. has been increasing at an alarming rate, reaching an average of 2,200 new diagnosed cases per day. The epidemic appears to be widespread, as evidence indicates that the incidence of one class of diabetes (type I) has doubled over the last two decades in northern Europe (3).

There are two distinct classes of diabetes mellitus: type 1 and type 2 (4). Type 1 diabetes (insulin-dependent diabetes mellitus or IDDM) is the result of an

Figure 1. Effect of glucose on various cell types and paracrine effects within the Islets of Langerhans. Key: (+) = elevated hormonal secretion, (-) = inhibited hormonal secretion. Taken from Soria B, Andreu E, Berna G, Fuentes E, Gil A, Leon-Quinto T, Martin F, Montanya E, Nadal A, Reig JA, Ripoll C, Roche E, Sanchez-Andres JV, Segura J: Engineering pancreatic islets. *Pflugers Arch* 440:1-18, 2000

Cell Type	Glucose concentration (mM)		
	0.5	**3**	**11**
α	+	+	-
β	-	-	+
δ	-	+	+
Paracrine Interactions:			

auto-immune disorder characterized by the loss of β-cells, most often occurring in children and young adults. These patients are required to administer insulin from an external source to survive. However, these individuals rarely display a normal physiological profile of regulated plasma glucose, and life expectancy drops precipitously, depending on the age of onset of the disease. Type 1 diabetes (non-insulin-dependent diabetes mellitus or NIDDM) is the result of a metabolic disorder that is characterized by insulin resistance and defective β-cells. It is a progressive disease in which many symptoms are not obvious until the disease has inflicted irreversible physiological damage. Thus, type 2 diabetes mellitus is often referred to as the 'silent killer' and represents 90-95% of all cases of diabetes (5). Severe diseases commonly associated with diabetic patients include blindness, kidney disease, nerve disease and amputations, heart disease and stroke. Each of these diseases can be alleviated or prevented by maintaining blood glucose levels within a target range. For example, the published findings of the 10-year Diabetes Control and Complications Trial (DCCT) statistically showed that improved blood glucose control prevents the onset or delays the progression of both diabetic retinopathy and nephropathy in type 1 diabetes patients (6). In addition, The United Kingdom Prospective Diabetes Study (UKPDS), a European study completed in 1998 with a median follow-up of 8.4 years, showed that intensive control of blood glucose and blood pressure reduced the risk of life-threatening diseases including blindness, kidney disease, stroke, and heart attack in people with type 2 diabetes (7). Moreover, numerous studies alike confirm a general trend that the

frequency, duration, and degree to which individuals fluctuate from a normal physiological blood glucose profile is inversely proportional to life expectancy.

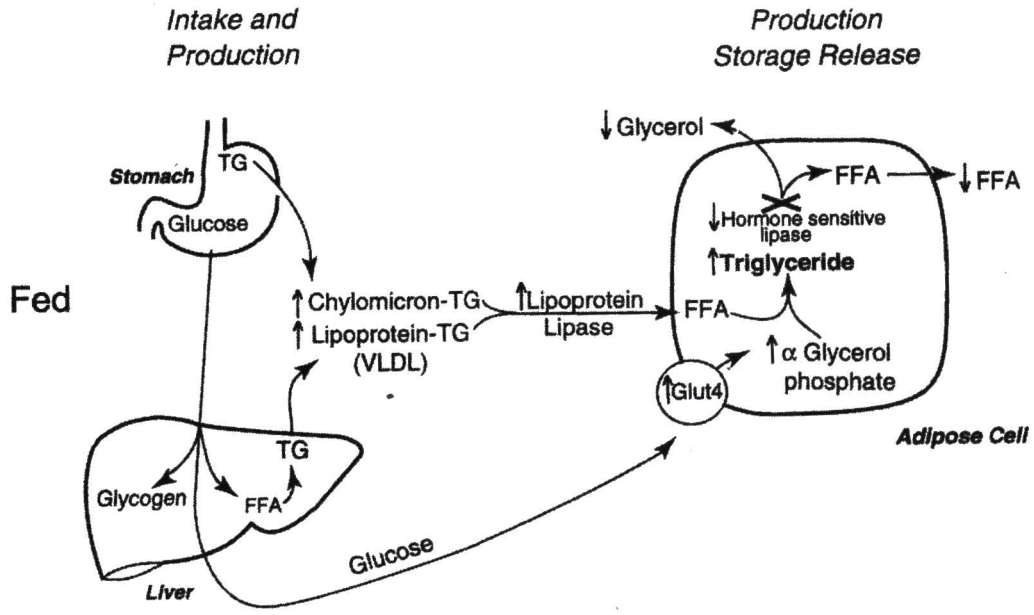
Physiological Effects of Insulin

Insulin was discovered in 1921 by Canadian researchers Frederick Grant Banting and Charles Herbert Best, who were the first to obtain insulin-enriched pancreatic extracts which could serve as replacement therapy in diabetic humans and animals. Since then, a wealth of information has come forth that describes the mechanisms of receptor-mediated action of insulin on tissues. In simple terms, insulin is the hormone that allows passage of glucose into tissues such as fat and muscle. In its absence, glucose cannot enter these tissues, and the body paradoxically starves in the fed state, although nutrients are abundant. Another note of clinical significance is that the brain is in constant need of a critical supply of glucose and is not regulated by insulin, as glucose diffuses passively into brain tissue. Without the complex, dynamic integration of hormonal signals, which balance the effect of insulin on blood glucose levels, the insulin-dependent tissues will compete with the brain for glucose in the fed state. As defined by the World Health Organization Study Group (WHO), 'normal' individuals fluctuate within a narrow range of plasma glucose (4). On the other hand, individuals who administer insulin to treat diabetes mellitus display larger fluctuations in blood glucose levels, which take a toll on the function of several targeted organ tissues over time.

Insulin facilitates entry of glucose into muscle, adipose, and several other tissues by recruiting hexose transporters to the plasma membrane. In the case of muscle tissue, the GLUT4 hexose transporter exists primarily in cytoplasmic vesicles in the absence of insulin. The binding of insulin to the insulin receptors in muscle tissue results in the rapid fusion of these vesicles to the plasma membrane, allowing the cells to efficiently uptake glucose. A few exceptions to insulin-dependent uptake of glucose by tissues include β-cells, the brain, red blood cells, and the liver, which continuously express hexose transporters in the plasma membrane. This allows the β-cells to monitor glucose levels and provide insulin on an as-needed basis. Essentially, the β-cells allow insulin-dependent tissues to access glucose while exposing the brain to minor fluctuations in plasma glucose concentrations.

A large portion of glucose absorbed from the small intestine is immediately taken up by the liver hepatocytes, which convert glucose to glycogen (Fig 2). Insulin promotes this action by two direct means. The first mechanism involves increasing glucokinase activity and inhibiting glucose-6-phosphatase activity. The resultant pool of glucose-6-phosphate is prevented from passing through the glucose transporters. This primary mechanism is referred to as the 'trapping of glucose' within the hydrophobic confines of the cell. The second means by which insulin promotes the storage of glucose in the liver is by activating enzymes that are directly involved in glycogen synthesis, such as glycogen synthase. Glycogen synthesis proceeds until the liver glycogen stores are replenished. When the liver is saturated with glycogen (approximately 5% of liver mass), the hepatocytes shunt excess glucose into pathways that lead to fatty acid synthesis

Figure 2. Glucose disposal and energy storage during the fed state. Glucose is rapidly diffused into insulin-dependent cells (via GLUT4 transporters) . Insulin promotes the conversion of glucose to glycogen and fatty acids in the liver and inhibits the breakdown of triglycerides in adipose cells. Key: TG=triglycerides, FFA=free fatty acids, VLDL=very low density lipids. Taken from Shulman GI, Barrett EJ, Sherwin, RS: In *Ellenberg and Rifkin's diabetes mellitus*, 5th ed, Port D, Sherwin RS, Eds. Stamford, Appleton and Lange, 1990, p. 1-17

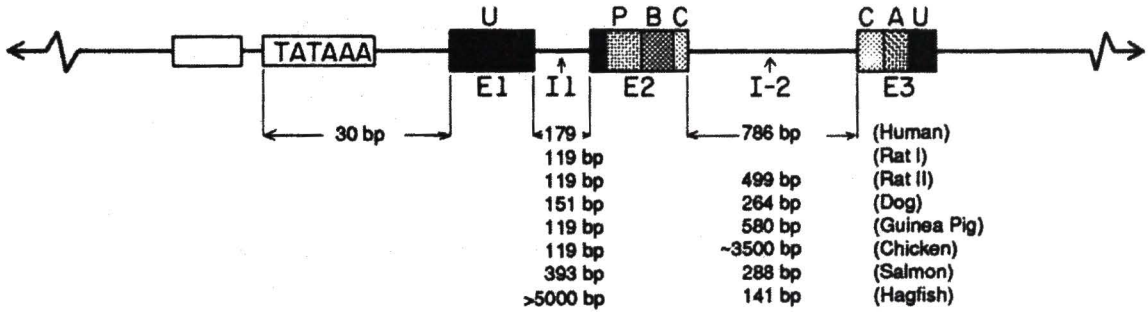

Intake and
Production
Production
Storage Release
Fed
Stomach
TG
Glucose
↑Chylomicron-TG
↑Lipoprotein-TG
(VLDL)
↑Lipoprotein
Lipase
↓Glycerol
FFA
↓FFA
↓Hormone sensitive
lipase
↑Triglyceride
FFA
↑α Glycerol
phosphate
↑Glut4
Adipose Cell
TG
Glycogen
FFA
Glucose
Liver

Insulin promotes the synthesis of fatty acids in the liver, which are transported via lipoproteins (VLDLs) to other tissues, such as adipocytes for storage. The lipoproteins are catalytically digested to release free fatty acids, which are taken up by adipose tissue. Insulin also inhibits the hormone sensitive intracellular lipase in adipocytes to inhibit the breakdown of triglycerides. Glucose taken up in response to insulin (via GLUT4) by adipocytes may be used to synthesize glycerol. The glycerol can then be used to synthesize triglycerides from the available free fatty acids. In this way, insulin has the function of promoting glucose disposal and energy storage, in addition to glucose utilization by insulin-dependent tissues.

The Production of Insulin

Insulin biosynthesis is centrally regulated by glucose. The expression of functionally mature insulin is regulated at several steps of biosynthesis, including gene transcription, post-transcription, translation, and post-translation. The first step, insulin gene transcription (restricted to pancreatic β-cells), produces insulin pre-mRNA, which represents three exon and two intron sequences from the insulin gene (8) (Fig. 3). In contrast to other mammals, rats and mice have two insulin genes, which are equally transcribed (9). However, all species display striking homology among insulin exons, and although highly variable in size, the relative length and positions of the introns (intron 1 is always shorter than intron 2) are highly conserved (8). The first exon (E1) contains a majority of the 5' untranslated region (UTR). The second exon (E2) encodes the remaining portion of the 5'-UTR, as well as the signal peptide, the insulin B-

Figure 3. Schematic diagram of the insulin gene. Arrangement of exons (E1-E3) and introns (I1 and I2) among various species. Although highly variable in size, I1 is always shorter than I2. I1 interrupts the 5' untranslated region and I2 is precisely located between the first and second nucleotides of the codon for amino acid 7 of the C-peptide. Key: U = 5' and 3' untranslated region of mRNA, P = pre-peptide, B = insulin B chain, C = C-peptide, A = insulin A chain. Taken from Docherty K, Steiner DF: In *Ellenberg and Rifkin's diabetes mellitus*, 5th ed, Port D, Sherwin RS, Eds. Stamford, Appleton and Lange, 1990, p. 29-48


U
P B C
C A U
TATAAA
E1
I1
E2
I-2
E3
30 bp
179
786 bp
(Human)
119 bp
(Rat I)
119 bp
499 bp
(Rat II)
151 bp
264 bp
(Dog)
119 bp
580 bp
(Guinea Pig)
119 bp
~3500 bp
(Chicken)
393 bp
288 bp
(Salmon)
>5000 bp
141 bp
(Hagfish)

chain, and a portion of the C-peptide. Lastly, the third exon (E3) encodes the remaining portion of the C-peptide, the insulin A-chain, and a 3'-UTR region. During RNA processing, the pre-mRNA introns are spliced to form the mature mRNA molecule, which is then transported to the cytosol (Fig 4). RNA-protein interactions have been detected within the 5'- and 3'-UTR's of the preproinsulin mRNA transcript, and these regions have been functionally determined to regulate glucose-induced insulin gene expression. For example, there are factors which interact with the 3'-UTR which stabilize preproinsulin mRNA in the presence of glucose. Additionally, the 5'-UTR sequence has been determined to be essential for preproinsulin translation in response to glucose (10).

The processing of preproinsulin is also an elaborate process (Fig 5). During translation, the 24 amino acid preproinsulin signal sequence is removed by the signal peptidase to generate proinsulin. Proinsulin forms disulfide linkages and undergoes further processing, which is influenced by glucose (Fig 6). Glucose regulates the biosynthesis of mature insulin at the level of post-translation by increasing the activity (11,12) and biosynthesis of prohomone convertases (PC2 and PC3/PC1) in parallel (13-15) (Fig 7). PC2 and PC3/PC1 are calcium-dependent endopeptidases, which catalyze the proteolytic processing of proinsulin to insulin at di-basic amino acid residues at the B-chain/C-peptide (Arg-31,Arg32) and C-peptide/A-chain (Lys-64, Arg-65) junctions (8). PC3/PC1 cleaves on the carboxyl side of Arg-31, Arg-32 and PC2 cleaves the carboxyl side of Lys-64, Arg-65. Immediately following proteolytic cleavage, the exopeptidase

Figure 4. Splicing of the preproinsulin mRNA transcript. The pre-mRNA introns are excised to form the mature preproinsulin mRNA. A 5' methylguanine cap is added, the mRNA is polyadenylated, and the mRNA is transported to the cytosol for translation. Taken from Clark AR, Docherty K: In *Insulin: molecular biology to pathology*, 1st ed, Ashcroft FM, Ashcroft S, Eds. Oxford, Oxford University Press, 1992, p. 37-63

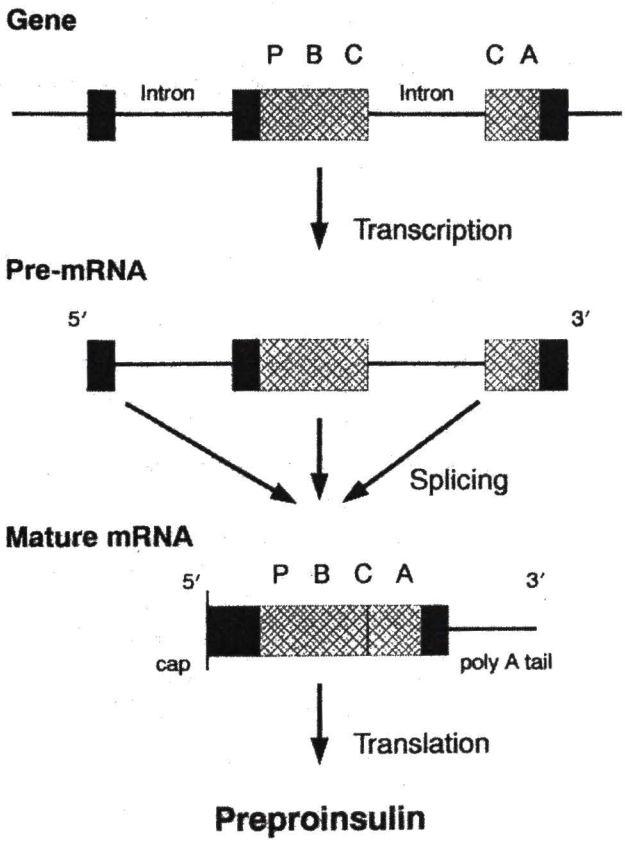

Gene
P B C
C A
Intron
Intron
Transcription
Pre-mRNA
5′
3′
Splicing
Mature mRNA
5′
P B C A
3′
cap
poly A tail
Translation
Preproinsulin

Figure 5. An illustration of preproinsulin processing. Preproinsulin is translated by ribosomes and directed to the rough endoplasmic reticulum by the signal peptide, which is cleaved to form proinsulin. Proinsulin is transferred to the Golgi networks where immature β-granules form. Within the granules, proinsulin undergoes proteolytic conversion to insulin and C-peptide. The mature granules are stored and fuse to the plasma membrane upon stimulation by glucose. Taken from Rhodes CJ: In *Diabetes mellitus: A fundamental and clinical text*, 2nd ed, LeRoith D, Taylor SI, Olefsky JM, Eds. Philadelphia, Lippincott Williams and Wilkins, 2000, p. 20-38

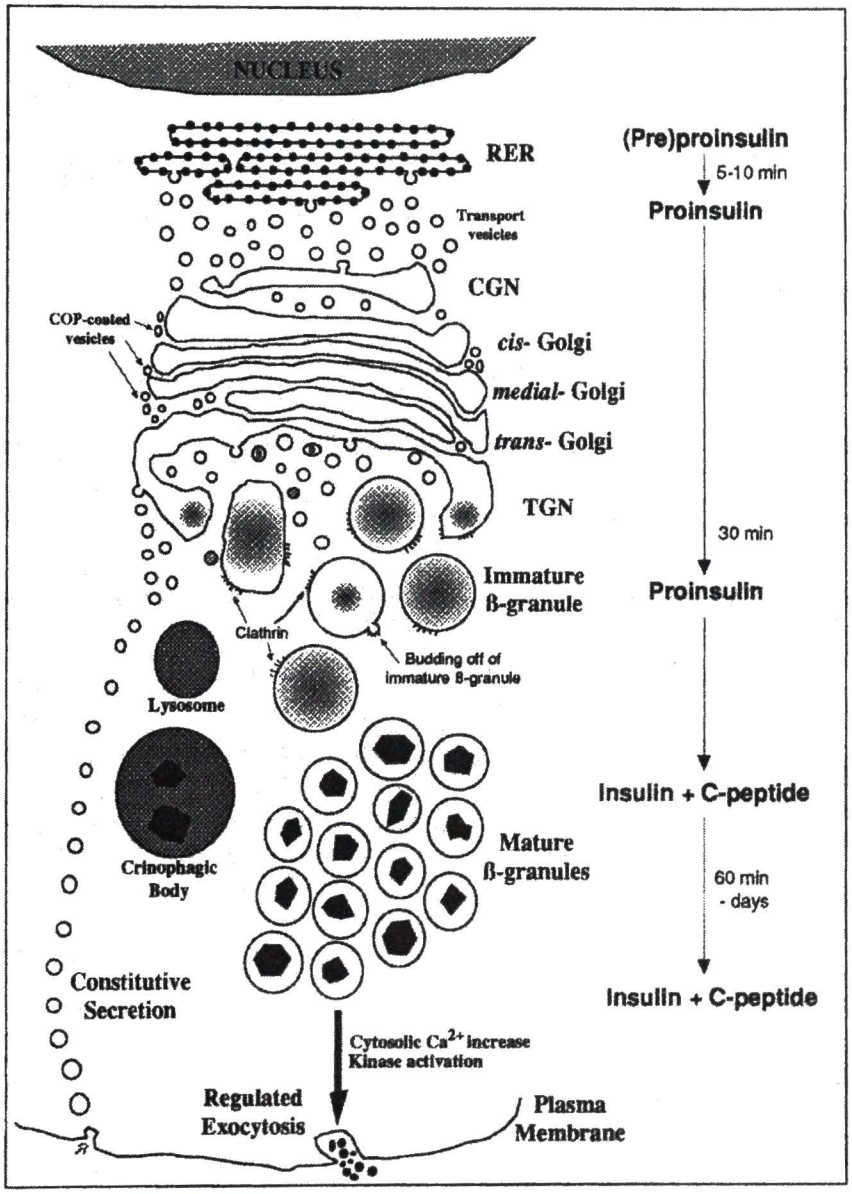

NUCLEUS
RER
Transport vesicles
CGN
COP-coated vesicles
cis- Golgi
medial- Golgi
trans- Golgi
TGN
Immature ß-granule
Clathrin
Budding off of immature ß-granule
Lysosome
Crinophagic Body
Mature ß-granules
Constitutive Secretion
Cytosolic Ca^{2+} increase
Kinase activation
Regulated Exocytosis
Plasma Membrane
(Pre)proinsulin
5-10 min
Proinsulin
30 min
Proinsulin
Insulin + C-peptide
60 min - days
Insulin + C-peptide

Figure 6. Schematic drawing of preproinsulin. The amino acid sequence of the human preproinsulin peptide is illustrated in letter-form. The preproinsulin molecule consists of the signal peptide, the insulin B-chain, the interconnecting C-peptide, and insulin A-chain as shown. Disulfide cysteine (C) linkages are also shown. The two dibasic sites are cleaved during hormone conversion and the signal peptide is cleaved upon transfer to the golgi network. Taken from Rhodes CJ: In *Diabetes mellitus: A fundamental and clinical text*, 2nd ed, LeRoith D, Taylor SI, Olefsky JM, Eds. Philadelphia, Lippincott Williams and Wilkins, 2000, p. 20-38

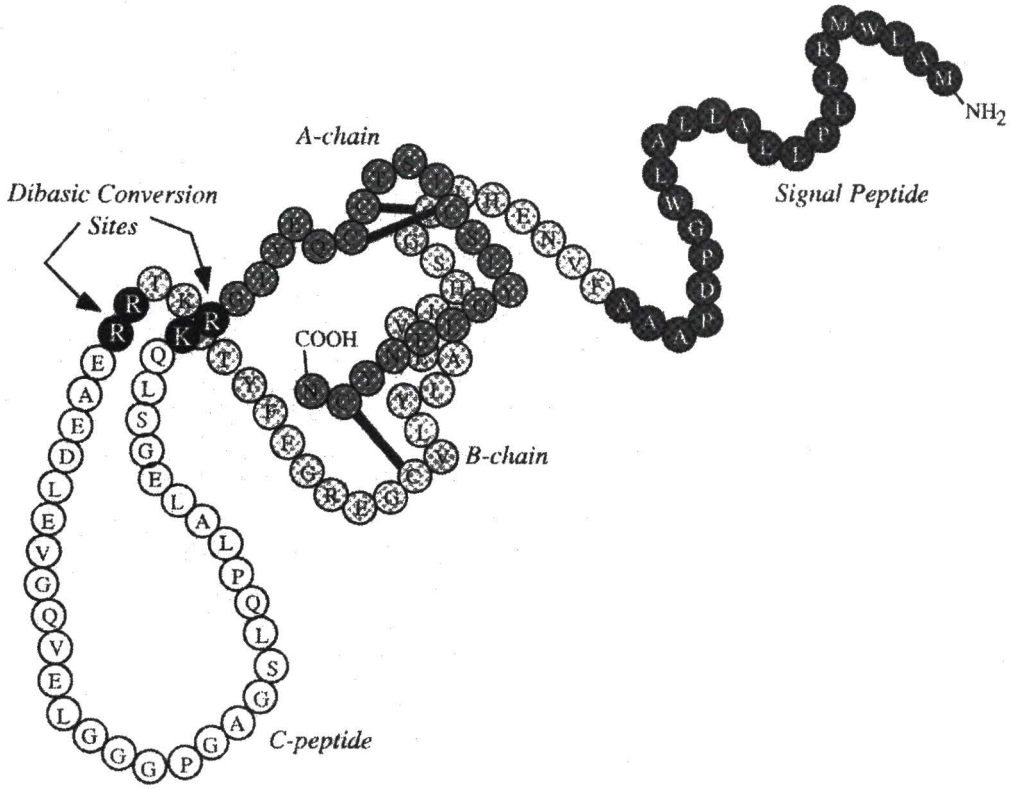
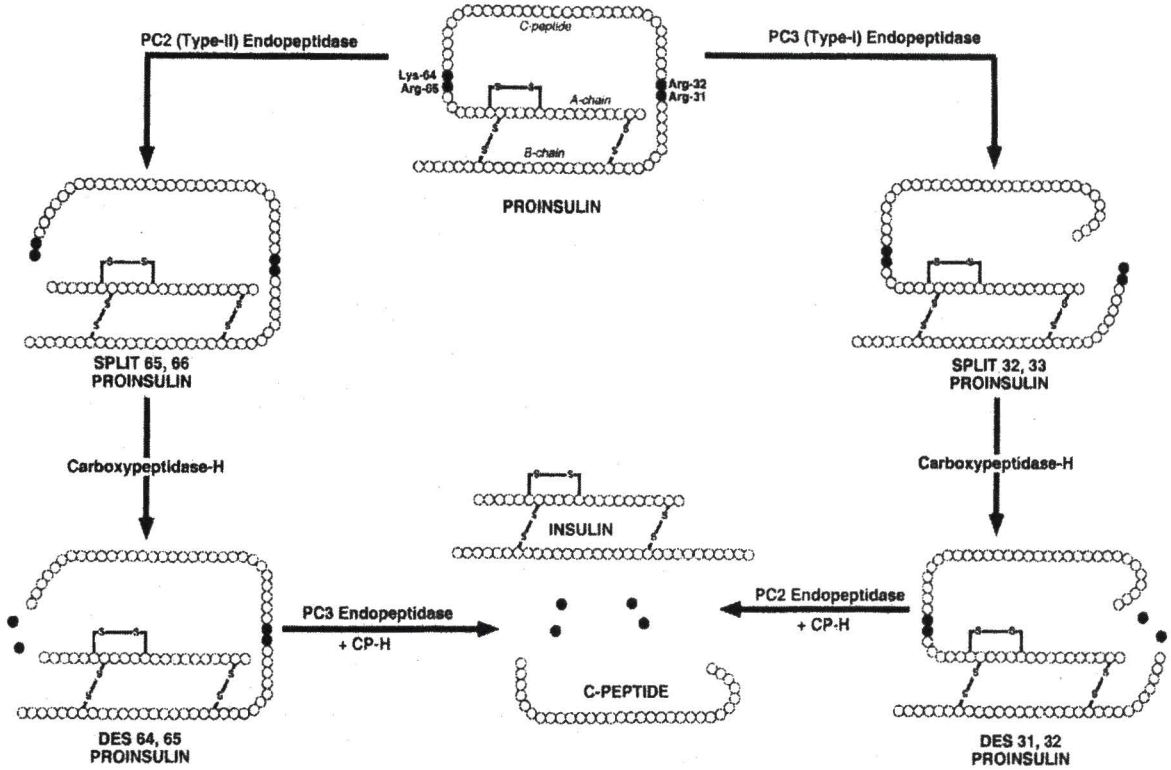

A-chain
Dibasic Conversion Sites
Signal Peptide
NH2
COOH
B-chain
C-peptide

Figure 7. Proinsulin conversion. The proinsulin molecule is cleaved at the dibasic residues (Lys-64/Arg-65 and Arg-32/Arg31) by endopeptidases (PC2 and PC3) to form intermediated split forms of proinsulin (split 65,66 and split 32,33). The dibasic residues are removed by Carboxypeptidase H to form the mature insulin molecule. Taken from Rhodes CJ: In *Diabetes mellitus: A fundamental and clinical text*, 2nd ed, LeRoith D, Taylor SI, Olefsky JM, Eds. Philadelphia, Lippincott Williams and Wilkins, 2000, p. 20-38


C-peptide
Lys-64
Arg-65
Arg-32
Arg-31
A-chain
B-chain
PROINSULIN
PC2 (Type-II) Endopeptidase
PC3 (Type-I) Endopeptidase
SPLIT 65, 66
PROINSULIN
SPLIT 32, 33
PROINSULIN
Carboxypeptidase-H
Carboxypeptidase-H
INSULIN
PC3 Endopeptidase
+ CP-H
PC2 Endopeptidase
+ CP-H
C-PEPTIDE
DES 64, 65
PROINSULIN
DES 31, 32
PROINSULIN

carboxypeptidase-H (CP-H) removes Arg and Lys from the carboxyl terminals to form the mature, functional form of insulin. Insulin is stored in secretory granules, which arereleased by the β-cell in response to elevated plasma glucose.

Mechanisms of Insulin Secretion

Most information about physiological signaling in pancreatic β-cells has been gained from insulin secretion studies. Stimulus-coupled insulin secretion is determined by levels of circulating fuels in the blood. It is well known that glucose is the primary physiological regulator of insulin release. High K_m glucose transporters (GLUT2), specific to β-cells and hepatocytes, permit facilitated diffusion of glucose across the membrane, allowing the cell to equilibrate (up to 90%) with blood glucose concentrations. Glucokinase, specific to β-cells and hepatocytes, phosphorylates glucose to create glucose-6-phosphate, thereby trapping it within the cell (Fig. 8). Because of their specialized high K_m for glucose, glucokinase and GLUT2 are often referred to as the β-cell 'sensors' of glucose metabolism.

Glucose metabolism results in an increased ATP/ADP ratio, which causes ATP-sensitive potassium channels to close. Closure of these channels results in cell depolarization, which activates voltage-dependent calcium channels (VDCC's). This action increases intracellular calcium, which is a critical component of insulin secretion (Fig. 9). Insulin secretion is biphasic, where an initial burst of stored insulin is released and followed by a steady increase in the rate of insulin secretion. The physiological

Figure 8. Glucose metabolism in pancreatic β-cells is coupled to insulin secretion. Glucose is taken up by GLUT-2 and phosphorylated by glucokinase. ATP production results from 1) the distal portion of glycolysis, 2) reduced NADH shuttles, and 3) oxidation of pyruvate by the TCA cycle. A rise in ATP/ADP ratio closes ATP-sensitive potassium channels, thereby depolarizing the cell. Cell depolarization triggers the opening of voltage-dependent calcium channels. Increased intracellular calcium correlates with insulin secretion. Taken from Newgard CB, Johnson JH: In *Diabetes mellitus: A fundamental and clinical text*, 2nd ed, LeRoith D, Taylor SI, Olefsky JM, Eds. Philadelphia, Lippincott Williams and Wilkins, 2000, p. 38-47

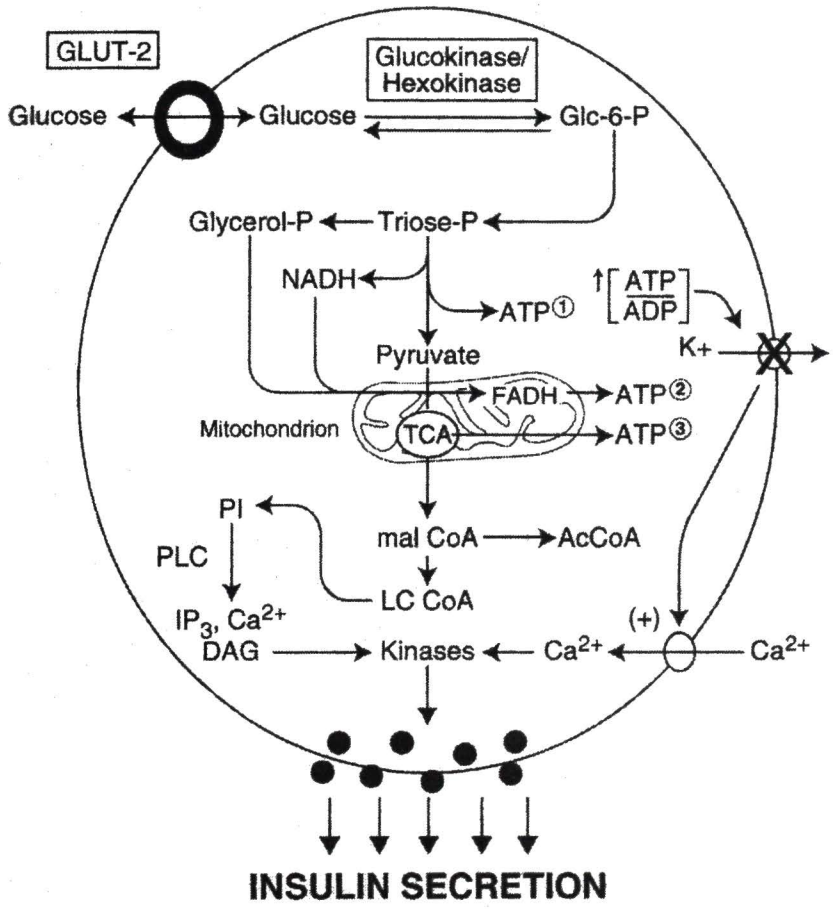
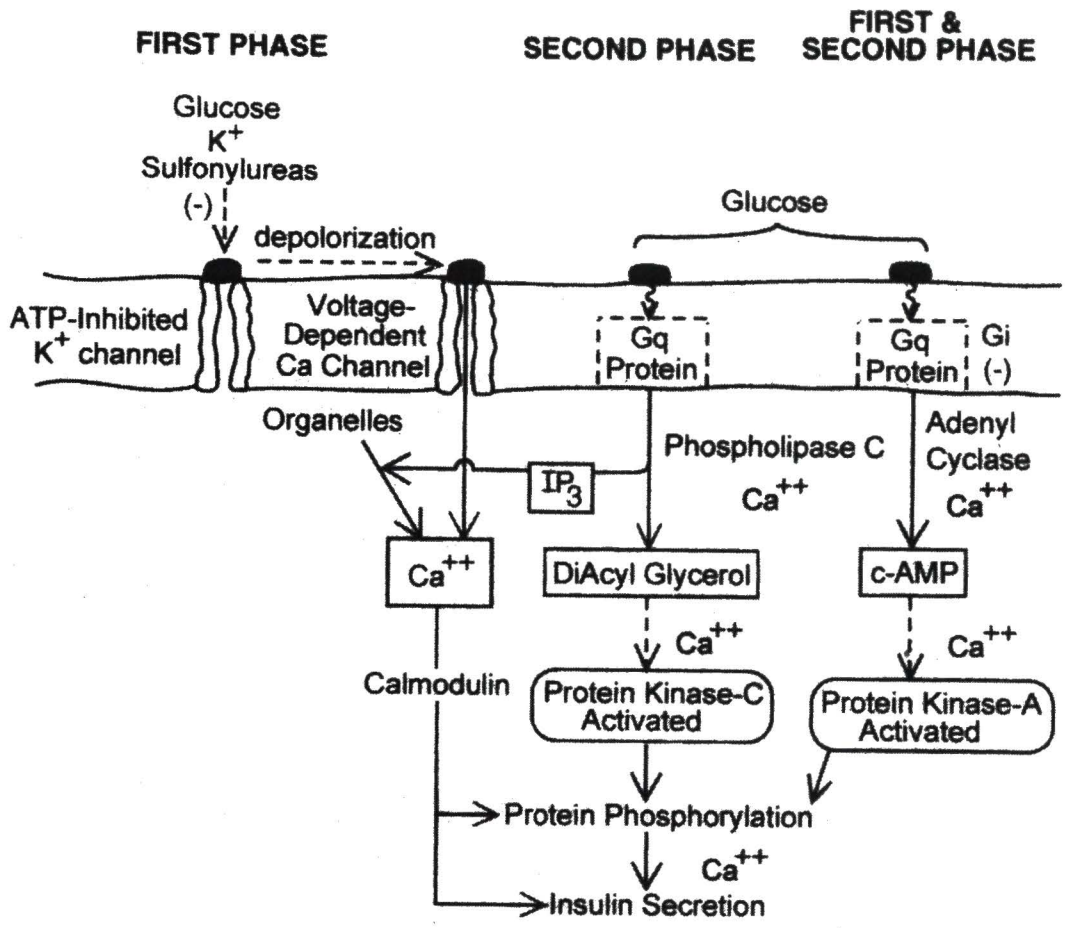

GLUT-2
Glucokinase/
Hexokinase
Glucose
Glucose
Glc-6-P
Glycerol-P
Triose-P
NADH
ATP①
↑[ATP/ADP]
K+
Pyruvate
FADH
ATP②
Mitochondrion
TCA
ATP③
PI
PLC
mal CoA
AcCoA
LC CoA
IP3, Ca2+
DAG
Kinases
Ca2+
(+)
Ca2+
INSULIN SECRETION

Figure 9. Calcium is a central regulator of biphasic insulin secretion. Glucose metabolism (or artificially depolarizing the cell by sulfonylureas or K^+) increases intracellular calcium to elicit insulin release from pancreatic β-cells. Activation of PLC or adenylate cyclase can potentiate the level of secretion by the first phase (via PKC) or first and second phase (via PKA), respectively. Taken from Grodsky GM: In *Diabetes mellitus: A fundamental and clinical text*, 2nd ed, LeRoith D, Taylor SI, Olefsky JM, Eds. Philadelphia, Lippincott Williams and Wilkins, 2000, p. 2-11


FIRST PHASE
SECOND PHASE
FIRST &
SECOND PHASE
Glucose
K+
Sulfonylureas
(-)
depolorization
Glucose
ATP-Inhibited
K+ channel
Voltage-
Dependent
Ca Channel
Gq
Protein
Gq
Protein
Gi
(-)
Organelles
Phospholipase C
Ca++
IP3
Adenyl
Cyclase
Ca++
Ca++
DiAcyl Glycerol
c-AMP
Ca++
Ca++
Calmodulin
Protein Kinase-C
Activated
Protein Kinase-A
Activated
Protein Phosphorylation
Ca++
Insulin Secretion

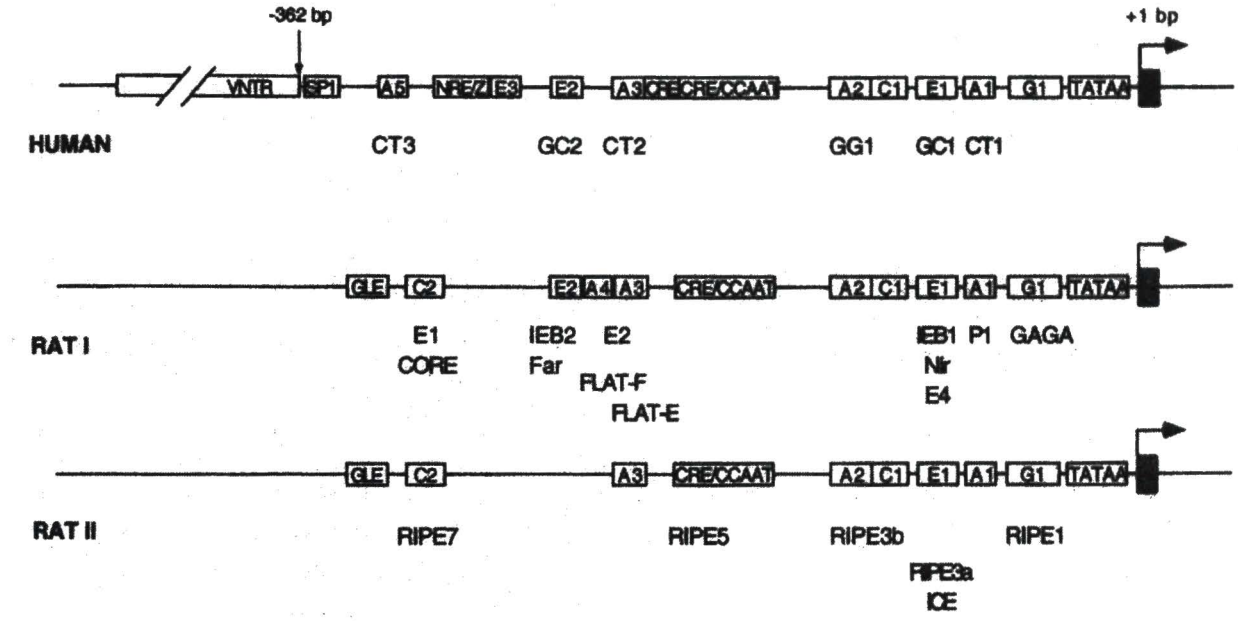
activators, acetylcholine (ACh) and glucagon-like peptide-1 (GLP-1), appear to potentiate glucose-induced biphasic insulin secretion by activating phospholipase C (PLC) and adenylate cyclase, respectively. PLC potentiates the first phase, whereas cAMP has an effect on both. Each effect is dependent on intracellular calcium, which is a component that also correlates with other aspects related to the production and release of insulin by β-cells, including the activation of calcium/calmodulin-dependent enzymes (16,17), granular fusion (18), preproinsulin prohormone conversion (12), and insulin gene transcription (19).

The Regulation of Insulin Gene Transcription

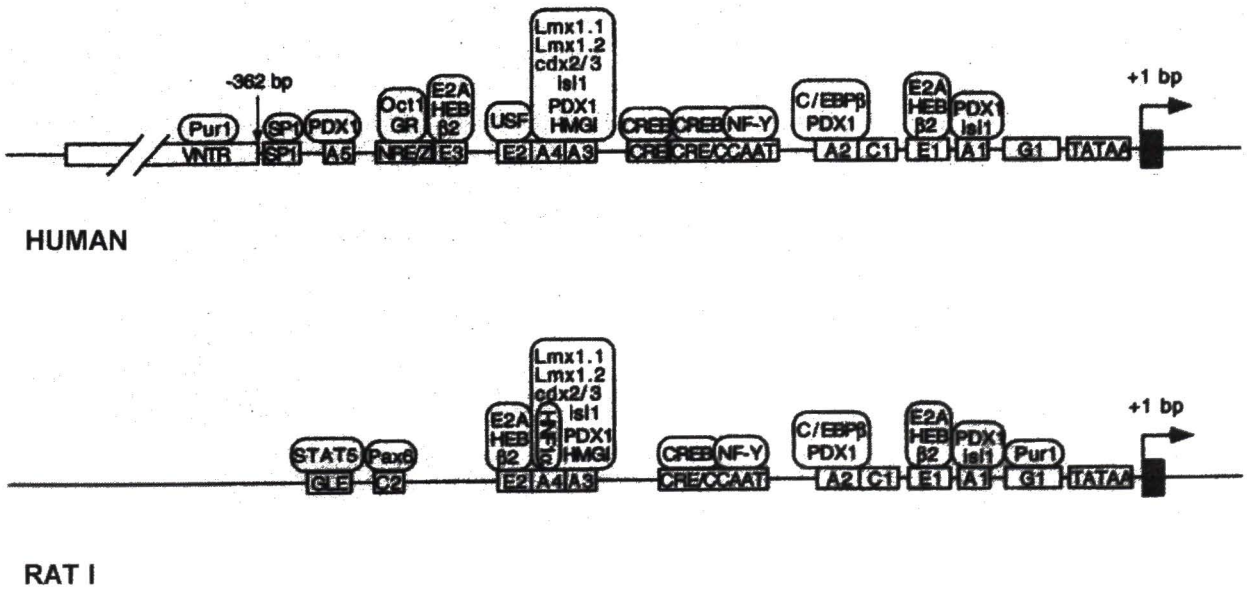
Preproinsulin gene transcription is controlled by the 5'-flanking promoter region of the insulin gene in a β-cell-specific manner. Just as in the case of the insulin gene sequence, the 5'-flanking regulatory promoter elements are highly conserved among mammals (Fig 10A). Within approximately the first 400 base pairs of this region (insulin gene promoter), several *cis*-acting elements have been identified, which specifically interact with nuclear DNA-binding *trans*-acting factors and regulate insulin promoter activity during islet development and in mature, differentiated β-cells. Cooperative interaction among the nuclear *trans*-acting DNA-binding proteins occurs in response to β-cell stimulation to regulate insulin gene promoter activity (20) and to confer β-cell-specific expression (21) (Fig. 10B).

Figure 10. Comparison of rat and human insulin gene promoters by known cis-acting elements and trans-acting factors. A) Insulin gene promoter sequence elements. Rats and humans display high homology within 400bp of the 5'-flanking insulin promoter region. The current names of the known elements are indicated in boxes, and the older names are shown below each promoter. B) Transcription factors of the insulin gene promoter. The known *cis*-acting elements of the human (top) and rat I (bottom) promoters are indicated in boxes. The cloned and characterized *trans*-acting factors are circled above. Taken from German M: In *Diabetes mellitus: A fundamental and clinical text*, 2nd ed, LeRoith D, Taylor SI, Olefsky JM, Eds. Philadelphia, Lippincott Williams and Wilkins, 2000, p. 11-19

A)


-362 bp
+1 bp
VNTR
SP1
A5
NRE/Z
E3
E2
A3
CRE
CRE/CCAAT
A2
C1
E1
A1
G1
TATAA
HUMAN
CT3
GC2
CT2
GG1
GC1
CT1
GLE
C2
E2
A4
A3
CRE/CCAAT
RAT I
E1
CORE
IEB2
Far
E2
FLAT-F
FLAT-E
IEB1
Nir
E4
P1
GAGA
RAT II
RIPE7
RIPE5
RIPE3b
RIPE1
RIPE3a
ICE

B)


-362 bp
+1 bp
Pur1
SP1
PDX1
VNTR
SP1
A5
Oct1
GR
E2A
HEB
β2
NRE/Z
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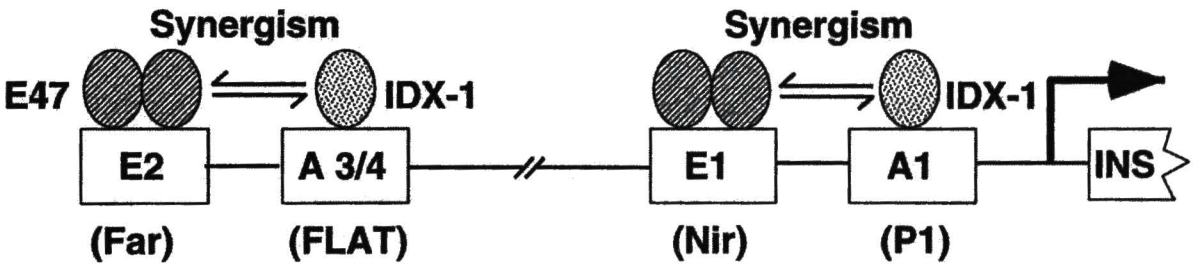
USF
Lmx1.1
Lmx1.2
cdx2/3
Isl1
PDX1
HMGI
E2
A4
A3
CRE
CRE
NF-Y
CRE/CCAAT
C/EBPβ
PDX1
A2
C1
E2A
HEB
β2
E1
PDX1
Isl1
A1
G1
TATAA
HUMAN
STAT5
Pax6
GLE
C2
Lmx1.1
Lmx1.2
cdx2/3
Isl1
PDX1
HMGI
E2A
HEB
β2
E2
A4
A3
CRE
NF-Y
CRE/CCAAT
C/EBPβ
PDX1
A2
C1
E2A
HEB
β2
E1
PDX1
Isl1
A1
Pur1
G1
TATAA
RAT I

Among the sites of regulation by these factors are the highly conserved symmetrical E/A element arrays (Nir/P1 and Far/FLAT) within the insulin gene promoter, which act in synergy to activate insulin gene transcription (22). The E elements (E boxes) are identical 8-bp motifs corresponding to positions -104 to -111 bp (Nir) and -231 to –238 (Far) of the rat I insulin gene promoter (rInsI) (23), which bind to the insulin enhancer factor 1 (IEF). IEF is an islet-specific transcription factor composed of heterodimers (E12/E47 or E47/BETA2) of the basic helix-loop-helix (bHLH) family of transcription factors (21). Mutations in either the Nir or Far elements results in a 10% reduction in insulin promoter activity, whereas a mutation in both elements completely eliminates insulin promoter activity (20). This indicates that at least one E-box is required for insulin gene transcription.

The cooperating A elements (A boxes) consist of A/T rich sequences that bind to PDX1 (formerly IDX-1/STF-1/IPF-1/IUF1/GSF), the homeodomain-containing transcription factor expressed in β-cells (24) (Fig. 11). It has been shown by mutational analysis that PDX-1 activation of the rat I insulin gene in β-cells is not only dependent on intact PDX-1-binding sites, but also on an intact adjacent IEF binding site (25). Moreover, it has been demonstrated that two modular E and A box components (Far and FLAT) do not activate reporter transcription individually, but only in their natural juxtaposed position, as they are presented in the insulin gene promoter (26). Thus, the E-boxes and adjacent A boxes act in concert to regulate insulin gene transcription.

As the rate of insulin production and release increases in response to demand, an increased rate in the transcription of preproinsulin is also required (19,27-34). Although

Figure 11. Cooperative interactions among trans-acting factors that bind to the insulin gene promoter. IEF (E12/E47 or E47/BETA2) binds to E boxes and synergizes with IDX-1 (renamed PDX-1), which binds to A boxes. Taken from Lu M, Seufert J, Habener JF: Pancreatic beta-cell-specific repression of insulin gene transcription by CCAAT/enhancer-binding protein beta: inhibitory interactions with basic helix-loop-helix transcription factor E47. *J Biol Chem* 272:28349-59, 1997


Synergism
Synergism
E47
IDX-1
IDX-1
E2
A 3/4
E1
A1
INS
(Far)
(FLAT)
(Nir)
(P1)

much is known about the expression of transcription factors in β-cells, which regulate insulin transcription, the activation of these factors in response to stimuli is less clear. Recently, however, PDX-1 has been implicated in the activation of the insulin gene promoter in response to glucose. When β-cells are exposed to stimulatory concentrations of glucose, PDX-1 is phosphorylated and translocated to the nucleus where it binds to A-boxes and mediates multiple protein-protein interactions to form a transcriptionally active enhanceosome (35) (Fig 12). The mechanisms whereby glucose metabolism drives PDX-1 phosphorylation is largely unknown, although PI3 kinase and the stress-activated protein kinase 2 (SAPK2) have been proposed to mediate the event. Also, it is largely unknown how glucose metabolism affects the cooperating IEF transcription factor, which is required for the maximal effect of PDX-1 activation of insulin gene promoter activity.

Interestingly, other homeodomain transcription factors produced by β-cells can also bind to and activate the A boxes and cooperate with transcription factors bound to the E boxes. For example, the LIM-homeodomain proteins (Lmx1.1 and Lmx1.2) can interact with IEF bound to the Far element and produce higher levels of transcriptional activity than the PDX-1-IEF combination (24) (Fig. 13). Targeted disruption of PDX-1 in mice results in the development of maturity-onset diabetes of the young (MODY) and failure of the mice to develop a pancreas beyond the initial bud stage. In spite of this, a few insulin-producing cells remain (24). In the case where PDX-1 is disrupted in mice and humans after pancreatic development, the β-cells are reduced in mass and function, but insulin is still produced. Thus, it is important to note that the β-cell likely displays redundancies in the regulation of insulin gene transcription.

Figure 12. PDX-1 activation of the insulin promoter in response to glucose.

Proposed pathways for glucose regulation of insulin gene transcription are shown. E and β2 represent bHLH factors of IEF. Question marks indicate unknown pathways or potential intersection among pathways. Taken from Ohneda K, Ee H, German M: Regulation of insulin gene transcription. *Semin Cell Dev Biol* 11:227-33, 2000

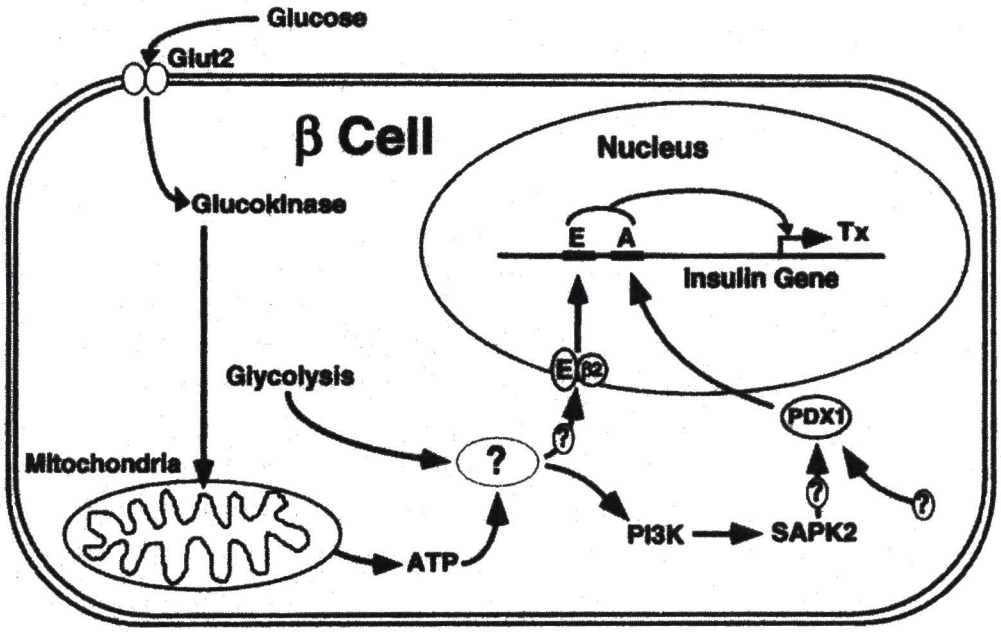
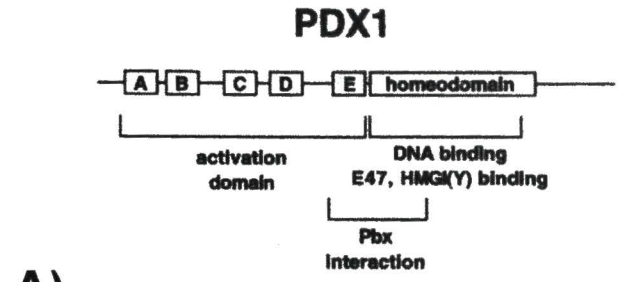
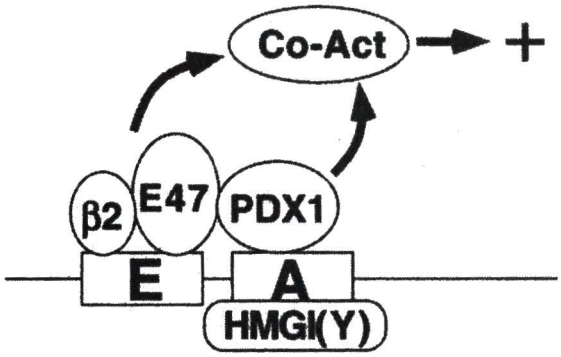

Glucose
Glut2
β Cell
Nucleus
Glucokinase
E
A
Tx
Insulin Gene
Glycolysis
E
β2
PDX1
Mitochondria
?
P13K
SAPK2
ATP

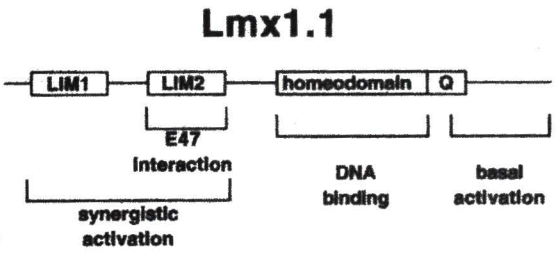
Figure 13. Activation of transcription by PDX-1 and Lmx1.1. Both PDX-1 and Lmx1.1 can bind to A boxes of the insulin gene promoter to activate transcription by distinct mechanisms. A) and C) An outline of the functional domains of the homeodomain transcription factors PDX-1 and Lmx1.1, respectively. B) PDX-1 interacts with the IEF bHLH dimer (β2/E47) to enhance transcription in cooperation with unidentified co-activators. The high mobility group protein, HMG I(Y), increases the synergistic transcriptional activity of PDX-bHLH. D) The second LIM domain (L2) of Lmx1 activates the second activation domain (AD2) of the E2A proteins. In addition, the HLH of the bHLH dimer releases an allosteric inhibition of the Lmx1 activation domain (Act) to further increase transcription. Taken from German M: In *Diabetes mellitus: A fundamental and clinical text*, 2nd ed, LeRoith D, Taylor SI, Olefsky JM, Eds. Philadelphia, Lippincott Williams and Wilkins, 2000, p. 11-19


PDX1
A
B
C
D
homeodomain
activation domain
DNA binding
E47, HMGI(Y) binding
Pbx interaction

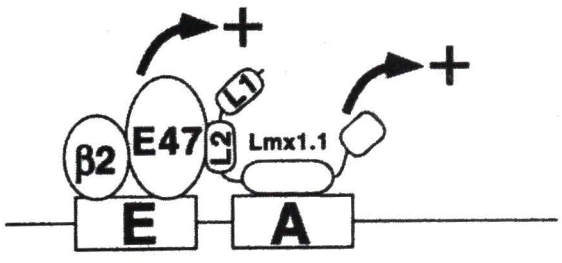
A)


Co-Act
+
β2
E47
PDX1
E
A
HMGI(Y)

B)


Lmx1.1
LIM1
LIM2
homeodomain
Q
E47 interaction
synergistic activation
DNA binding
basal activation

C)


+
+
L1
L2
β2
E47
Lmx1.1
E
A

D)

A class of inhibitors which globally inhibit insulin production in β-cells include the pharmacological immunosuppressants, FK506 and cyclosporin A (CsA). It has recently been observed that FK506 and CsA potently inhibit insulin gene transcription (16). These inhibitors, therefore, disrupt key components involved in the regulation of the insulin gene promoter. Several features regarding the targeted effects of FK506 and CsA on cytokine gene transcription have been revealed. These known characteristics of the drugs provide insight to understanding critical pathways that are common to both cytokine and insulin gene transcription. The mechanisms of inhibition of insulin gene transcription by FK506 are examined in studies presented in this dissertation.

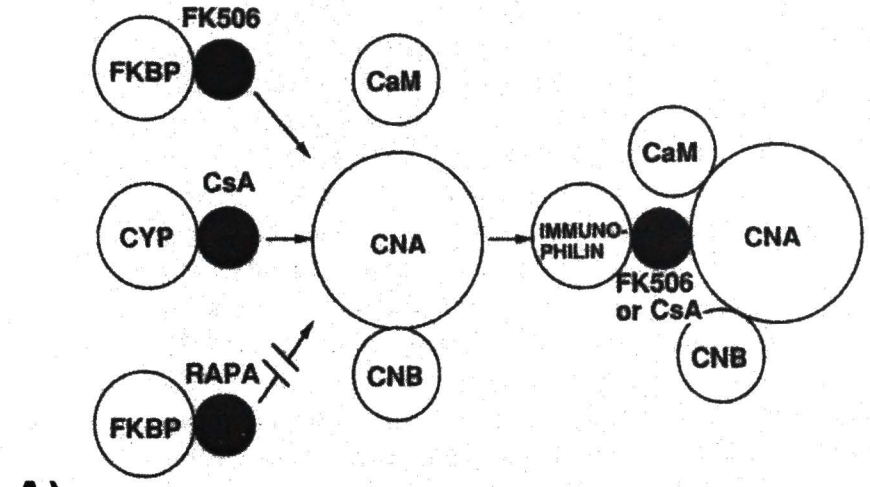
Cellular and Molecular Actions of Immunosuppressants: FK506 and CsA

The immunosuppressant drugs FK506 and CsA are widely used in organ transplantation to reduce tissue rejection and also have been used to treat autoimmune disorders such as psoriasis, uveitis, rheumatoid arthritis, type 1 diabetes mellitus, primary biliary cirrhosis, aplastic anemia, lichen planus, and severe allergic asthma (36,37). CsA is an undecapeptide and FK506 is a macrolide. Although structurally diverse, these two classes of immunosuppressants have been shown to selectively inhibit the class 2B calcium/calmodulin-dependent protein phosphatase, calcineurin, by similar mechanisms (37-41). The inhibition of calcineurin activity is indirect, requiring intracellular receptors commonly referred to as immunophilins (40). FK506-binding proteins (FKBPs) and cyclophilins bind FK506 and cyclosporin A, respectively, to form complexes that, in turn, bind and inhibit calcineurin. The FKBP-FK506 complex is 100-fold more potent than the

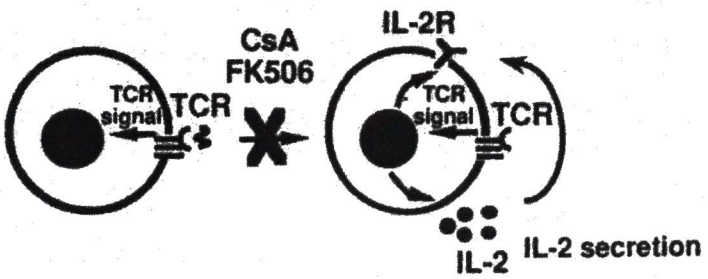
cyclophilin-CsA complex and for this reason has largely replaced CsA in the practice of organ transplantation (36).

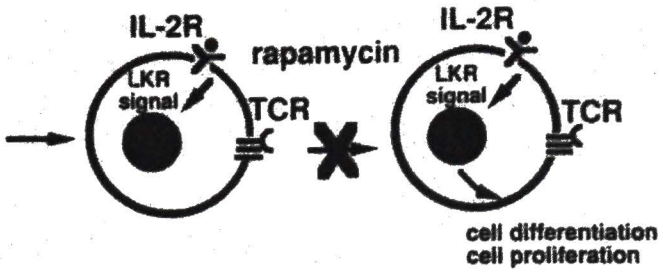
The immunophilins are ubiquitously expressed and have multiple family members as well (42). Several species of the FKBPs have been identified, including 12, 12.6, 1.6, 13, 25r, 38, 51 and 52. These members have different binding affinities for FK506, and hence different potencies in inhibiting calcineurin. FKBP12 and 12.6 have been found to be the most potent of immunophilins when bound to FK506 to inhibit calcineurin *in vitro* (42). Collectively, these observations suggest that there is a potentially large variability in the effects that these immunosuppressant drugs have on specific cellular and tissue functions, not to mention the likely variations in dose-response concentrations among different tissues. The immunophilins are generally believed to be unrelated to the cellular function of calcineurin, wheras the FK506-binding proteins, FKBP12.6 and FKBP12 have been found to be associated with ryanodine (RyR) and inositol 1,4,5-trisphosphate receptors (IP3R), respectively, which regulate calcium mobilization (43-45). FKBP12, one of the most abundant and conserved proteins in biology, appears to have a functional role in 1) 'anchoring' calcineurin to IP3R and 2) physically regulating calcium flux through the IP3R channel. Recent reports show that FKBP12 binds to specific residues on IP3R, a leucyl-prolyl dipeptide epitope that is structurally similar to FK506. It has been suggested that by mimicking this site, FK506 disrupts the ability of calcineurin to modulate IP3R (46). It has also been indicated that FK506 disrupts the association of FKBP12 with IP3R, preventing it from efficiently regulating the mobilization of intracellular calcium. Curiously, the closely related structural analogue of FK506,

Figure 14. Effects of FK506, CsA, and rapamycin on immune-system cells. A) FK506-FKBP and CsA-cyclophilin complexes bind with high affinity to calcineurin, whereas the RAPA-FKBP comlex does not. Key: CaM=calmodulin, CNA=calcineurin A, CNB=calcineurin B, CYP=cyclophilin, RAPA= rapamycin, FKBP=FK506 binding protein. B) FK506 and CsA inhibit the transcription of cytokines (such as IL-2) and related surface receptors (such as IL-2R) in T-cells, thus blocking the cytokine production. In contrast, rapamycin does not inhibit calcineurin activity or impair IL-2 or IL-2R expression, but blocks the response of T-cells to cytokines for cell differentiation and proliferation. Key: TCR=T-cell receptor, LKR=lymphokine receptor. Taken from Thomson AW, Starzl TE: FK 506 and autoimmune disease: perspective and prospects. *Autoimmunity* 12:303-13, 1992


FK506
FKBP
CaM
CsA
CYP
CNA
CaM
IMMUNO-
PHILIN
CNA
FK506
or CsA
CNB
RAPA
FKBP
CNB

A)

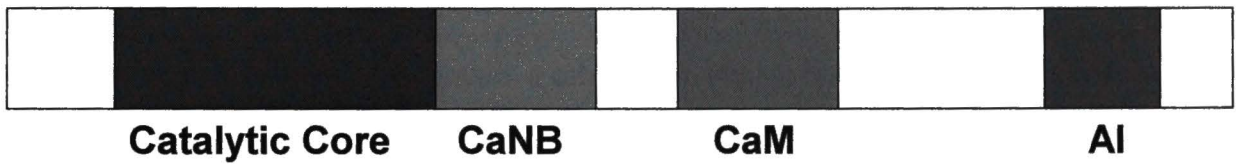

CsA
FK506
IL-2R
TCR
signal
TCR
TCR
signal
TCR
IL-2
IL-2 secretion


IL-2R
rapamycin
IL-2R
LKR
signal
TCR
LKR
signal
TCR
cell differentiation
cell proliferation

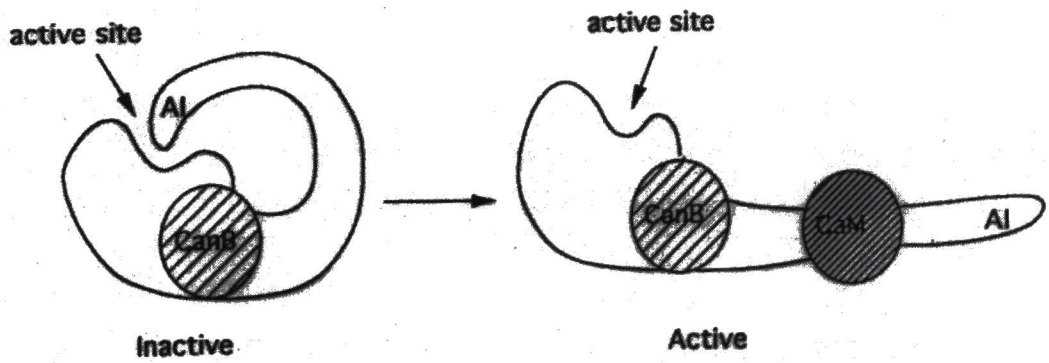
B)

Figure 15. Components of the calcineurin (PP2B) holoenzyme. A) Schematic representation of the calcineurin A subunit (CaNA). The primary structure is illustrated with known domains/binding-sites indicated below. B) Cartoon depicting the activation of calcineurin. Calcium binds to the low affinity sites of CaNB, which induces a conformational change in the CaNA/CaNB interaction to expose the CaM-binding site. When CaM binds to CaNA, it displaces the AI portion of CaNA from the active site to produce a catalytically active enzyme. Key: CaNB=calcineurin B, CaM=calmodulin, AI=autoinhibitory domain.

A)


Catalytic Core
CaNB
CaM
AI

B)


active site
AI
CaNB
Inactive
active site
CaNB
CaM
AI
Active

rapamycin, also forms immunocomplexes with the FKBP's which do not inhibit calcineurin, but suppress the immune system by a distinct cellular mechanisim involving the inhibition of T-cell proliferation (36) (Fig 14).

Effect of FK506 and CsA on Calcineurin

Calcineurin is a heterodimer, which is composed of two core subunits. The A subunit consists of an autoinhibitory domain, a calmodulin (CaM) binding domain, a catalytic domain, and the B subunit binding domain (47) (Fig. 15A). Several isoforms of the catalytic subunit have been identified, indicating that the enzyme may have distinctphosphoprotein substrate specificities or regulatory properties. The transcripts from identified species range from 0.8 kb and 1.8kb in the testis, up to 3.6 and 4.0 kb in the brain and skeletal muscle. It also has been shown that at least three distinct mammalian calcineurin genes can undergo alternative splicing to give rise to additional molecular variants (47).

Calcineurin B serves as a regulatory subunit, but also contributes to the conformation of the active site. Structurally homologous to calmodulin, the B subunit amino acid sequence contains four EF-hand calcium-binding domains (48). However, the B subunit does not substitute for calmodulin to activate phosphatase activity, and calmodulin cannot substitute for the B subunit to produce an active holoenzyme (39). Recent studies indicate that the A and B subunit of calcineurin form the catalytic pocket, which the N-terminal autoinhibitory portion of the A subunit occupies to inhibit the

phosphatase activity (37) (Fig 15B). When calcium binds to the low-affinity sites of calcineurin B, it affects the conformational interaction between the A and B subunits. This results in the exposure of the calmodulin-binding domain. In support of this, limited proteolysis experiments show that the calmodulin-binding domain is completely protected against proteolytic attack when the low affinity sites of calcineurin B are not occupied (49). A sustained increase in intracellular calcium increases the affinity of calmodulin for the A subunit, which physically displaces the autoinhibitory portion of the enzyme. This has been confirmed by studies involving the use of a synthetic autoinhibitory peptide, which can act *in trans* to inhibit the phosphatase activity (37). Truncation of this autoinhibitory domain produces a constitutively active holoenzyme. Similarly, further truncation of the calmodulin domain, which is downstream of the autoinhibitory domain, results in the irreversible activation (constitutive form) of calcium-independent calcineurin phosphatase activity (48). Many investigators have taken advantage of this property of calcineurin by the use of cDNA-truncated mutants to study the effect of constitutively active calcineurin on cellular functions in various transfected cells.

The inhibition of calcineurin by FK506 and CsA in immune-system cells inhibits the expression of several cytokines and immunologically-related surface receptors including IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, CD40 ligand, Fas ligand, granulocyte-macrophage colony stimulating factor (GM-CSF), interferon gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) (50-52). Calcineurin is a widely distributed class of protein phosphatases with isoforms present in various tissues (48,53).

The brain is noted to have an exceptionally high amount of calcineurin expression, with at least 20 fold more than most other tissues (54). These findings have prompted efforts to determine how the use of FK506 and CsA successfully prevent tissue rejection in tissue transplantations, but also adversely affect organ systems at the cellular and molecular level. Significant side effects in post-transplant patients in addition to increased incidence of type II diabetes mellitus are well documented, including neurotoxicity and nephrotoxicity (55,56). On the other hand, these drugs have also been valuable tools in the investigation of the role that calcineurin plays in complex schemes of calcium/calmodulin-dependent regulation in a wide variety of cellular functions. Calcineurin has been found to play a role in neural and non-neural calcium-regulated signaling events, including those involved in muscle glycogen metabolism, ion channel regulation, sperm motility (47), lymphocyte activation and proliferation (37), and insulin secretion and production (16,57). Discovering differences in the regulation of the global calcium-sensitive calcineurin phosphatase to target tissue-specific functions may contribute to the design of pharmaceutical agents, which target the immune system in a less invasive manner.

Despite the challenge of overcoming the weakness of these effective immunosuppressants as aids in transplantation and immune disorders, the drugs have served as helpful tools in deciphering calcium signaling pathways involving calcineurin. Of notable importance is the role of calcineurin in the regulation of transcription and calcium channels and the various effects that it has on the particular function of the tissue. In pancreatic β-cells, calcineurin has been found to inhibit the first phase of

insulin secretion, possibly by inactivating calcium influx. On the other hand, it has been found to enhance the transcription of insulin by permitting the entry of transcription factors into the nucleus. Cell signaling cascades appear to be quite complex, involving many players and pathways that overlap. The highly selective calcineurin-targeting drugs FK506 and CsA provide a useful instrument for examining the involvement of calcineurin in any particular process under investigation, and it is likely that other roles in other tissues will be discovered by this means. The experiments and results presented herein, describe the role of calcineurin in insulin gene transcription.

Nuclear Factor of Activated T-cells (NFAT): Regulation by Calcineurin

It is well documented that NFAT is a downstream target of calcineurin in the regulation of lymphokine gene expression (58-60). The NFAT family proteins are responsible for cytokine gene transcription in activated T-cells, B cells, mast cells, and natural killer cells. The transcription of any of these gene products is markedly reduced in the presence of the immunosuppressant drugs FK506 and cyclosporin A. NFAT family members consist of two major regions of sequence homology, which include the DNA-binding domain (DBD) and NFAT homology region (NHR) (Fig 16). The NFAT DBD is highly conserved and appears to be closely related to the Rel family protein DNA-binding domains (51) (Fig 17). The NHR domain consists of nine conserved motifs, two of which define calcineurin binding domains (61). Upon activation by a rise in intracellular calcium, calcineurin dephosphorylates a series of serine residues within the NHR domain, which results in the exposure of the nuclear localization sequence

Figure 16. Schematic representation of the NFAT family members. The primary structures of NFAT are aligned to depict regions of homology among members. The DNA-binding domain (DBD) is the region of highest homology within the NFAT family. A second region of homology is the NFAT homology region (NHR), which contains 9 conserved motifs, two of which are calcineurin-binding sites. For protein isoforms, identical shading patterns represent identical sequences. The numbers represent amino acid lengths of human isoforms, except for NFAT1A, which was cloned from mouse. Taken From Rao A, Luo C, Hogan PG: Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol* 15:707-47, 1997

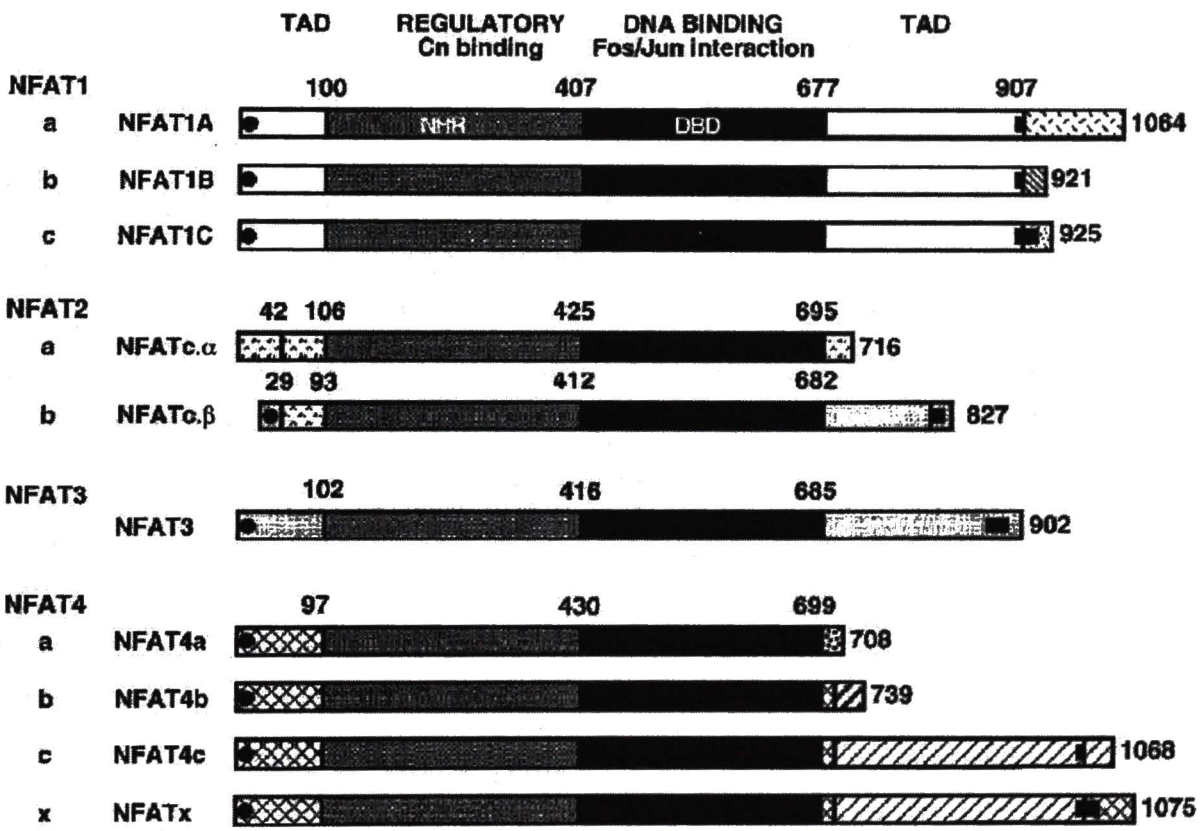

TAD
REGULATORY
Cn binding
DNA BINDING
Fos/Jun interaction
TAD
NFAT1
100
407
677
907
a
NFAT1A
NHR
DBD
1064
b
NFAT1B
921
c
NFAT1C
925
NFAT2
42
106
425
695
a
NFATc.α
716
29
93
412
682
b
NFATc.β
827
NFAT3
102
416
685
NFAT3
902
NFAT4
97
430
699
a
NFAT4a
708
b
NFAT4b
739
c
NFAT4c
1068
x
NFATx
1075

Figure 17. Schematic representation of the NFAT homology region (NHR). There are 9 conserved motifs within the NHR. The two calcineurin-binding sites, CnBP-A and CnBP-B, are located within the outermost motifs. Amino acid sequences of certain key elements are indicated in parentheses or expansion brackets. Key: SRR=serine-rich region, SP motif=serine-proline motif, NLS=nuclear localization sequence. Taken from Park S, Uesugi M, Verdine GL: A second calcineurin binding site on the NFAT regulatory domain. *Proc Natl Acad Sci U S A* 97:7130-5, 2000

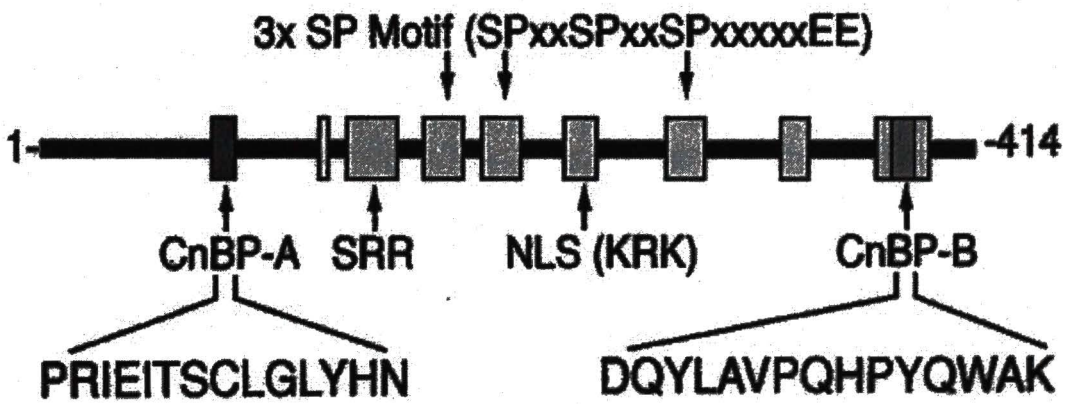
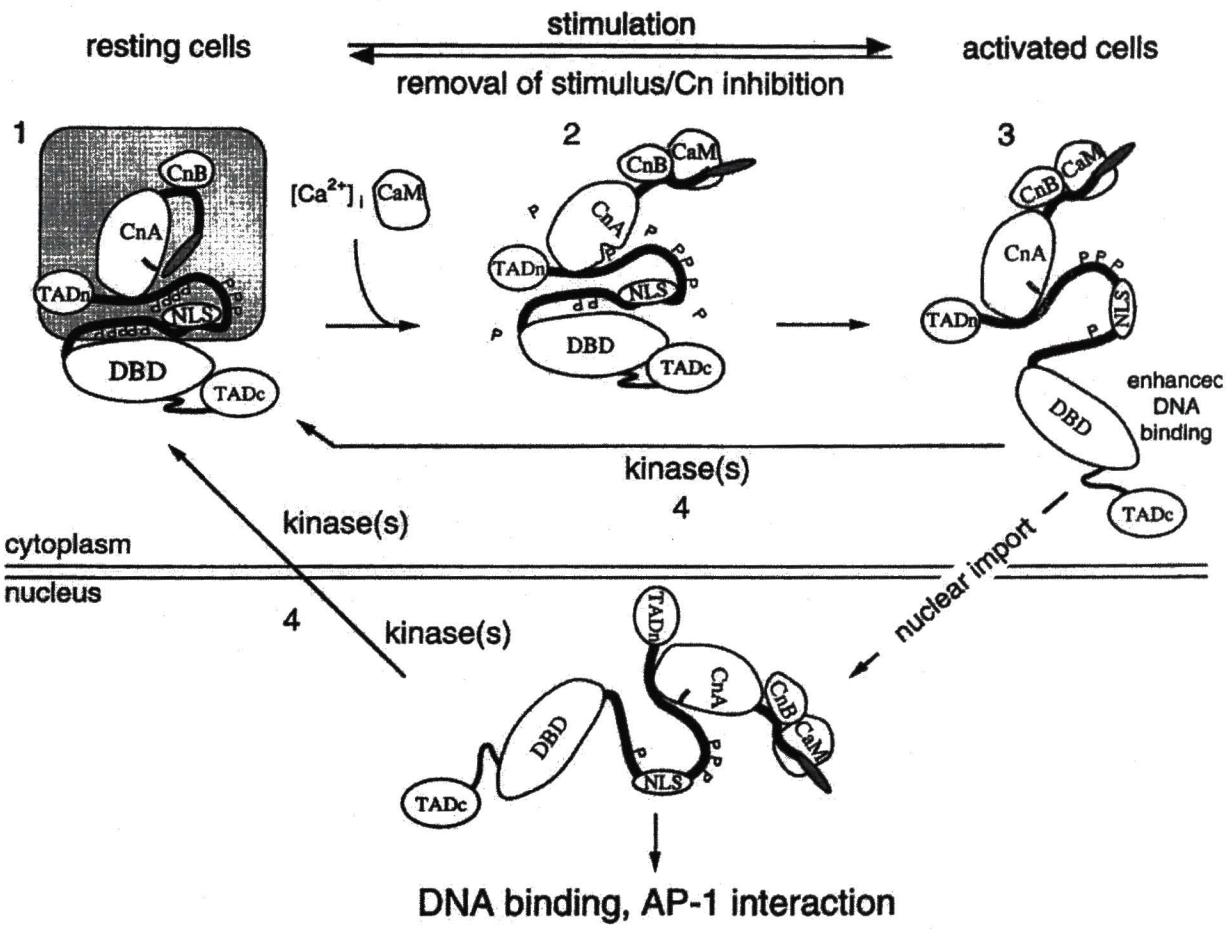

3x SP Motif (SPxxSPxxSPxxxxxEE)
1-
-414
CnBP-A
SRR
NLS (KRK)
CnBP-B
PRIEITSCLGLYHN
DQYLAVPQHPYQWAK

Figure 18. Activation of the NFAT-calcineurin complex. The NFAT-calcineurin complex is retained in the cytoplasm with other proteins (shaded) in resting cells. The highly phosphorylated NHR masks or inhibits the NLS for nuclear import. Upon stimulation of increased intracellular calcium, calcineurin dephosphorylates NFAT to activate the NLS, which is responsible for the transport of NFAT to the nucleus. Counteracting kinases balance the effect of calcineurin on NFAT in a fine-tuned regulatory manner. Taken From Rao A, Luo C, Hogan PG: Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol* 15:707-47, 1997


resting cells
stimulation
removal of stimulus/Cn inhibition
activated cells
1
2
3
CnB
CnA
CaM
TADn
NLS
DBD
TADc
[Ca2+]i
enhancec
DNA
binding
kinase(s)
4
cytoplasm
nucleus
nuclear import
DNA binding, AP-1 interaction

(NLS) (Fig 18). NFAT is then translocated to the nucleus where it binds to the NFAT consensus sites ([T/A]GGAAA[A/N][A/T/C]N), where N = any base, and activates gene transcription via N- and C- terminal transactivation domains (TAD's) (51).

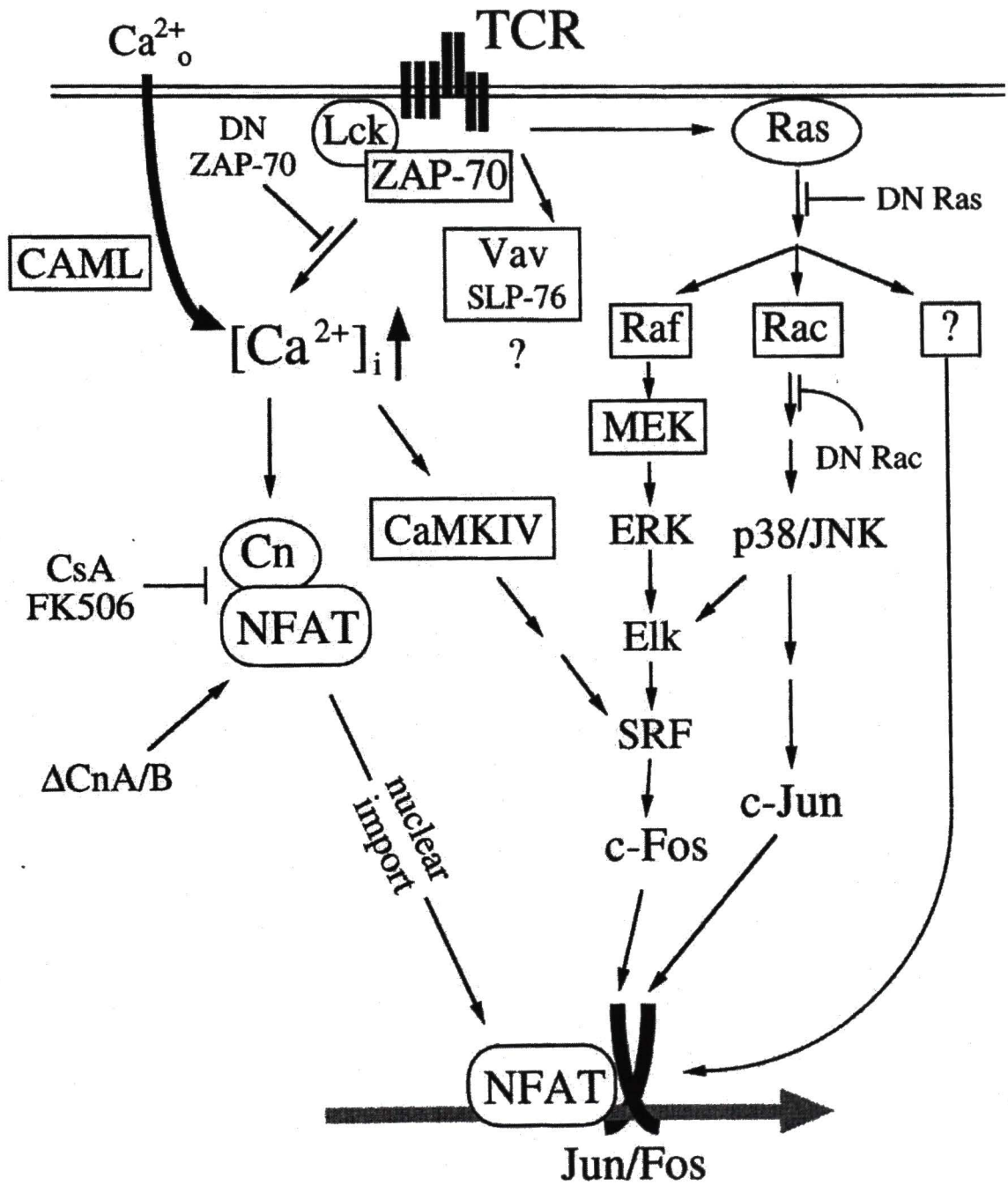
Studies employing FK506 and CsA have led to the elucidation of the function of NFAT in gene transcription. FK506 and CsA inhibit NFAT-dependent reporter gene transcription as well as the nuclear detection of NFAT DNA-binding activity byelectrophoretic mobility shift assays. This has also been demonstrated by the use of a green fluorescent protein fused to NFAT4 (GFP-NFAT4) expressed in BHK fibroblasts. GFP-NFAT4 is exclusively cytoplasmic when the cell is in the resting state. Upon stimulation by calcium ionophore, the protein can be visualized in the nucleus within 10 minutes (58). The effect is blocked upon addition of FK506 or CsA. In addition, the overexpression of a constitutively active form of calcineurin in U2OS cells has been shown to partition between the cytoplasm and nucleus, whereas the co-expression of this mutant with NFAT4 results in the accumulation of the mutant calcineurin in the nucleus (58). Treatment of these cells with 100 nM cyclosporin A for 30 minutes results in a complete relocation of both the mutant calcineurin and NFAT4 from the nucleus to the cytoplasm.

It has been previously accepted that calcineurin is a cytoplasmic protein phosphatase. However, studies have indicated that calcineurin may function in the nucleus as well. For example, with specific reference to NFAT4, it has been reported that calcineurin functions in the unmasking of nuclear-localization signals (NLSs) encoded in the NFAT primary protein sequence and that calcineurin is transported to the nucleus by

its mere association with NFAT. Calcineurin was shown to compete with the c-Jun amino terminal kinase (JNK), which is involved in the attenuation of calcium signaling in the nucleus (58,62). NFAT4 also has a nuclear export sequence (NES), which is not masked by phosphorylation. Thus, it appears that calcineurin activity is counter-balanced by kinase activity to determine the compartmentalization of NFAT based on the phosphorylation state of the NFAT NLS and NES (Fig 18). Cytokine transcription parallels that of other induced genes in that it is controlled by regulatory kinases, which are themselves regulated at the level of nuclear import. The latest discoveries concerning the calcineurin phosphatase involvement in transcriptional control complement this general notion, as kinase activity would likely be balanced by phosphatase activity in the nucleus.

Although the regulation of gene transcription by calcineurin and NFAT is activated primarily by calcium-mobilizing stimuli, it is well documented that NFAT interacts with other transcription factors bound to adjacent cis-acting elements, which are activated by additional stimuli. Such is the case of T-cell receptor-mediated cytokine gene transcription, where receptors involving the activation of tyrosine kinases (Lck and ZAP-70) induce calcium- and Ras- activated pathways. These pathways converge to produce a complete response (51) (Fig 19). This has been demonstrated by the use of calcium ionophores and phorbol 12-myristate 13-acetate (PMA), which increase intracellular calcium and activate protein kinase C (PKC) and Ras, respectively. Although FK506 and CsA classically block cytokine transcription, it was found that the calcineurin/NFAT pathway is not sufficient for transcriptional activation alone (51).

Figure 19. Schematic of T-cell receptor-mediated activation of cytokine gene transcription. Two factors are required to induce downstream converging signal pathways to activate transcription: calcium and Ras, which activate NFAT and AP1, respectively. Calcium activates calcineurin and CaMKIV. Calcineurin activates NFAT and CaMKIV feeds into the Raf/MEK/ERK pathway to activate c-Fos. Ras activates Raf and Rac to provide the AP-1 (Fos/Jun) component of the NFAT/AP-1 cooperative interaction. Key: CaMKIV= calcium/calmodulin-dependent protein kinase IV, CAML=calcium-signal modulating cyclophilin ligand, Cn=calcineurin, ΔCn=constitutively active calcineurin lacking the autoinhibitory domain, JNK=c-Jun N-terminal kinase, DN=dominant negative, SRF=serum response factor. Taken from Rao A, Luo C, Hogan PG: Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol* 15:707-47, 1997


Ca^{2+}_o
TCR
DN ZAP-70
Lck
ZAP-70
Ras
DN Ras
CAML
Vav SLP-76
?
$[Ca^{2+}]_i$
Raf
Rac
?
MEK
DN Rac
CaMKIV
ERK
p38/JNK
CsA FK506
Cn
NFAT
Elk
SRF
ΔCnA/B
nuclear import
c-Jun
c-Fos
NFAT
Jun/Fos

PMA, which synergistically activates cytokine gene transcription in the presence of calcium ionophore, is also ineffective as a single stimulant. Moreover, the overexpression of a constitutively active form of calcineurin in the presence of Ras activates a Raf/MEK/ERK pathway to activate c-Fos and a Rac/p38/JNK pathway to activate c-Jun, which together provide the AP-1 (bZIP) transcriptional component required for NFAT-mediated IL-2 gene expression (51).

Several of these neighboring NFAT/AP-1 elements have been identified, which act in concert to stimulate gene transcription of cytokines and related surface receptors. Promoters, which contain these NFAT/AP-1 modules include IL-2, IL-4, IL-5, TNF-α, IFN-γ, GM-CSF, CD40L, and CTLA-4 (51). Other NFAT/bZIP as well as NFAT/non-bZIP interactions have been documented. An ATF-2/Jun heterodimer complexes with NFAT to activate the TNF-α promoter in B cells and T cells (63,64). The proto-oncogene, c-Maf (also a bZIP transcription factor), cooperates with NFAT1 to activate the IL-4 promoter (51). NFAT3 interacts with the zinc finger transcription factor GATA-4 to activate genes related to cardiac hypertrophy (65). In addition, several studies indicate that myocyte enhancer-binding factor (MEF-2) family synergizes with NFAT to regulate genes involved in muscle morphology in myocytes (66,67). MEF-2/NFAT complexes have also been shown to activate gene transcription required for apoptosis in T-cells (68). Therefore, evidence suggests that NFAT often requires trans-acting partners in the regulation of gene transcription.

Within the four recognized NFAT family members (NFAT1-4), several isoforms have been identified (51) (Fig. 16). In addition, two novel NFAT-related forms (NFAT5 and NFATC5) have recently been cloned, which have added complexity to the nomenclature, such that the HUGO Nomenclature Committee had to revise the NFAT family classifications (Fig 20). Each NFAT transcript undergoes RNA splicing to produce multiple variants. These spliced variants may represent one mechanism whereby the calcineurin/NFAT pathway dictates functional specificity within tissue even though several isoforms may be expressed in one cell type. The variable regions between the NFAT members likely determine interactions, which form on promoter DNA between trans-acting elements and transcriptional co-activators. There also lies the possibility that the isoforms are sub-localized (in addition to cytosolic/nuclear partitioning) as protein complexes within cellular compartments to restrict activation to specific transducing signals. Thus, it is not surprising that an increasing number of functions of NFAT in gene regulation outside the immune system have been identified. Most recently, NFAT isoforms have been identified in brain and also skeletal, heart, and smooth muscle. The studies presented herein this dissertation identify a novel function of NFAT in pancreatic β-cells.

Figure 20. The current nomenclature for NFAT published by the [Human Genome Organisation (HUGO) Nomenclature Committee] (HGNC). The HGNC had to revise the classifications to include NFAT5. The scheme was not well accepted primarily because of a general disagreement as to whether the new NFAT-related proteins should be included. Therefore, the NFAT community was asked to vote on proposed NFAT nomenclatures to reduce confusion within the literature and databases, and the results were accepted and published by the HGNC. Taken from HGNC Family Nomenclature [article online], 2000. Available from http://www.gene.ucl.ac.uk/nomenclature/genefamily/NFAT/NFAT.shtml. Accessed 17 August 2001

Current Approved Symbol	Literature Aliases	Chromosome loc (human)	Sequence accessions
NFATC1	NFAT2, NF-ATC, NFATc	18q	U08015
NFATC2	NFAT1, NF-ATP, NFATp	20q13.2-q13.3	U43341/2
NFATC3	NFAT4, NFATX	16q13-q24	L41067, U14510
NFATC4	NFAT3		L41066
NFATC5			
NFAT5	TonE-BP, KIAA0827		AF089824, AB020634, AF134870

Hypothesis: Calcineurin and NFAT up-regulate insulin gene transcription in pancreatic β-cells.

Based on the initial observations that FK506 induces post-transplant diabetes and reduces insulin gene expression in pancreatic β-cells, it was proposed that calcineurin is a critical component of insulin gene transcription. Because NFAT is a known substrate for calcineurin and NFAT consensus motifs are present within the insulin promoter, it was hypothesized that calcineurin up-regulates insulin gene transcription via NFAT. Moreover, since glucose and GLP-1 can increase intracellular calcium in β-cells, the effects of glucose/GLP-1 on insulin promoter activity in light of this mechanism were investigated as well.

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CHAPTER II

EXPERIMENTAL METHODOLOGY

Cell Transfections and Promoter-reporter Assays

In general, information gained from reporter assays has been limited due to difficulties in transfecting pancreatic β-cells. Pancreatic islets, for example, contain a dense core of β-cells, surrounded by a layer of α-cells. Questions have been raised concerning the efficacy of traditional transfection methodology in penetrating the islet (1) and the authenticity of results attained by transfecting a multi-population of cells (i.e. α-cells, β-cells, δ-cells, pp-cells) when specifically studying β-cells (2,3). Cell sorting using a fluorescence-activated cell sorter (FACS) has been used to separate pancreatic β-cells from non-β-cells, but the number of cells attainable by this methodology has been primarily useful for single cell recordings in patch-clamping techniques, as opposed to transfection techniques, which typically require at least several hundreds of thousands of cells per transfection. Moreover, it has been shown that dispersed islets respond differently to stimuli than intact islets. On the other hand, insulinoma cell lines, such as the clonal β-cell line INS-1 represent a pure population of β-cells, but there are questions regarding the physiological authenticity of these cells as well. INS-1 cells are also difficult to transfect by traditional methods, as seen by comparing transfection efficiencies to other cells (such as 293 cells) and fold increases in promoter-reporter

assays in response to similar conditions to data obtained by northern analysis (4,5). Furthermore, over several passages, these cells become relatively glucose-insensitive in that they no longer produce and secrete insulin in response to glucose (the major characteristic which defines pancreatic β-cells). Thus, the experimental methodology was developed to overcome two major problems to optimize promoter-reporter assays in transfected β-cells: 1) to increase the effectiveness of transient transfections and 2) to restore and maintain glucose-responsiveness of INS-1 cells.

The first issue was resolved by using the non-liposomal Fugene (Roche, Indianapolis, IN) transfection reagent. $CaPO_4$ yielded no detectable reporter activity in transfected INS-1 cells. Although the liposomal-based LipofectAMINE (GIBCO, Carlsbad, CA) reagent produced acceptable transfection efficiencies, the Fugene reagent often produced reporter activities 2-3 fold higher (Fig. 1). The second issue was addressed by equilibrating INS-1 cells at low glucose (2mM) conditions to restore glucose responsiveness in these cells, which are typically cultured and passaged in stimulatory glucose conditions (11mM). This was based on the observation that prolonged exposure of β-cells to supra-physiological glucose conditions results in the down-regulation of insulin expression. Increased responsiveness of INS-1 cells was first observed by northern analysis by equilibrating the cells overnight before stimulating them for 6 hours prior to harvesting the total cellular RNA (Fig. 2). However, it was also noticed that the viability of INS-1 cells was compromised when cultured for extended periods (beyond 30 hrs) in 2mM glucose. A protocol for INS-1 transfections was devised

Figure 1. Data representative of INS-1 cells transfected with lipofectamine as compared to Fugene. INS-1 cells were transfected with 2μg of PSV-CAT using either Lipofectamine (white) or Fugene (grey) methods and harvested after 36 hours. CAT expression was determined by ELISA. Each bar represents one independent transfected well (60-mm) containing approximately $1x10^6$ INS-1 cells.

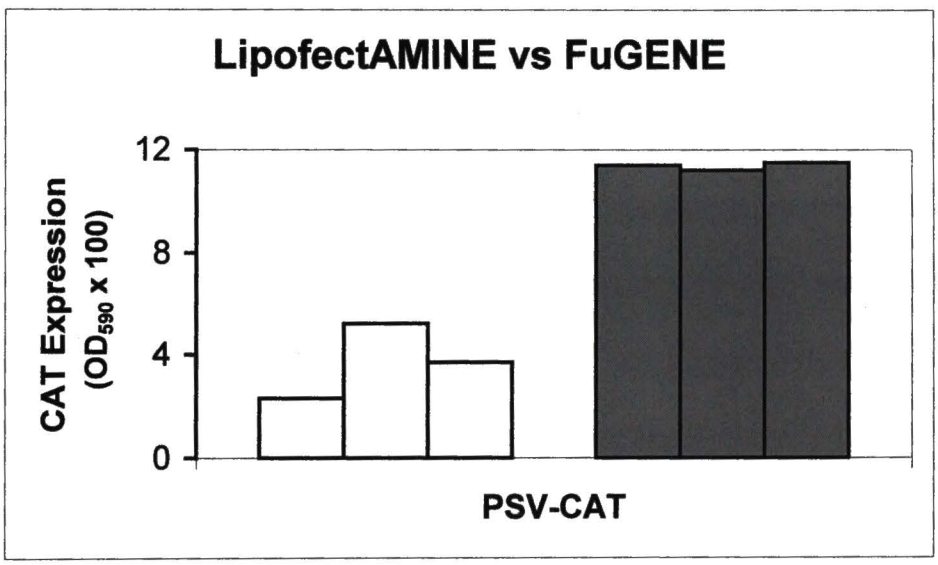
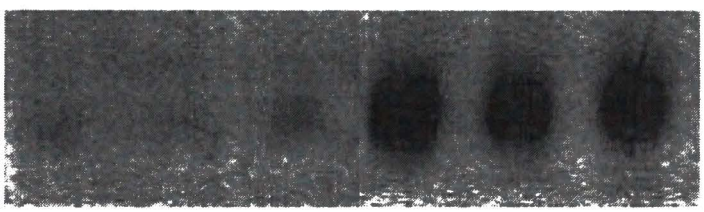

LipofectAMINE vs FuGENE
CAT Expression
(OD590 x 100)
12
8
4
0
PSV-CAT

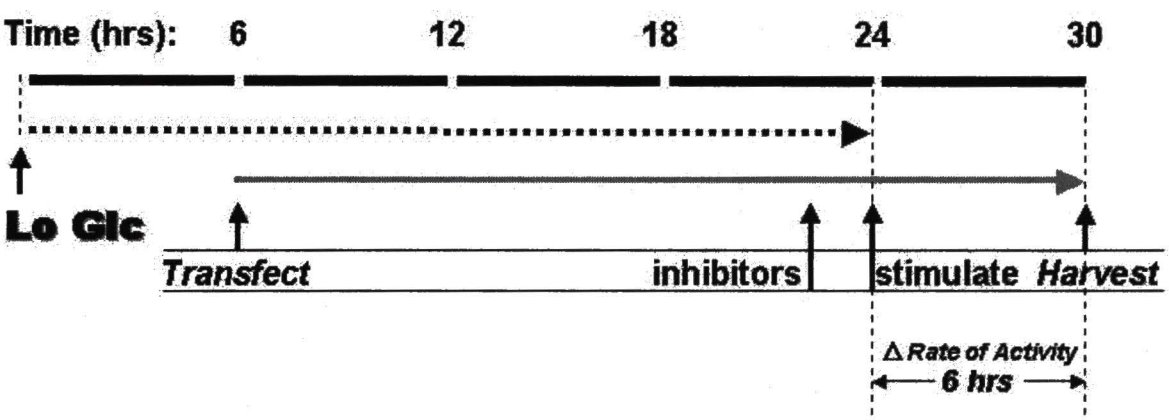
Figure 2. Northern analysis of insulin mRNA produced by INS-1. INS-1 cells were exposed to 2mM glucose overnight, then stimulated with 20mM glucose for 6 hours. Total RNA was isolated and run on a formaldahyde-based agarose gel, then blotted onto nitrocellulose filter and hybridized with end-labeled anti-fluorescein-HRP probe (400-bp insulin gene fragment). Detection was performed by CDP-star reagent (NEN). Each lane represents one independent treated well (60-mm) containing approximately $1x10^6$ INS-1 cells.

Insulin mRNA expression



2mM Glc **20mM Glc**

Figure 3. Time course of INS-1 transfection. INS-1 cells are incubated in 2mM glucose for 6 hrs prior to transfection. The cells are stimulated 18 hrs after transfection, thereby allowing a 24 hr period of equilibration. Inhibitors are added 2hrs prior to the stimulation. The INS-1 cells are harvested and assayed 6 hrs after stimulation.


Time (hrs):
6
12
18
24
30
Lo Glc
Transfect
inhibitors
stimulate
Harvest
Δ Rate of Activity
6 hrs

accordingly, such that the control cells (sustained in 2mM) were harvested within 30 hours (Fig. 3). The combined effect of these key procedures resulted in increased stimulatory responses as well as increased sensitivity in the detection of differences in insulin gene promoter activity among conditions presented to β-cells as compared to previous reports.

Four Primer Site-directed Mutagenesis

In order to study the contribution of individual NFAT elements in the regulation of insulin gene transcription, two-point base pair mutations were made within the core sequences (GGAAA) of the rat I insulin promoter 1-3NFAT consensus sites. This was achieved by a method of four primer site-directed mutatgenesis, modified from the Sarkar and Sommer 'megaprimer' method (also called three primer site-directed mutagenesis) (6). The procedure was performed by using two mutagenic primers that overlap (by complementarity) the desired mutant sequence and two primers that flank the coding region and hybridize to the vector DNA (Fig 4). Based on the idea that double-stranded DNA segments can be extended as primers in PCR reactions (7), the mutatagenic primers (sense and anti-sense) were paired with the left-hand (LH) and right-hand (RH) vector primers as separate reactions to produce overlapping 'halves', which could then be mixed and extended to generate a full-length mutated DNA product (Fig 5). The successfully mutated rat I promoter fragments (1-3NFATm) were cloned into the luciferase-reporter PGL2 control vector (Promega, Madison, WI) and verified by DNA sequencing.

Figure 4. Primers designed for four primer site-directed mutagenesis of the NFAT elements within the –410 rat I insulin gene promoter. The left-hand and right-hand primers are located within the vector (outside the coding region) and the complementary mutagenic primers (sense and anti-sense) contain 2-base pair point mutations (GA to TC) within the GGAAA core of the NFAT consensus site. The arches represent mismatched base pairs. Key: LH=left-hand, RH=right-hand, S=sense, AS=anti-sense.

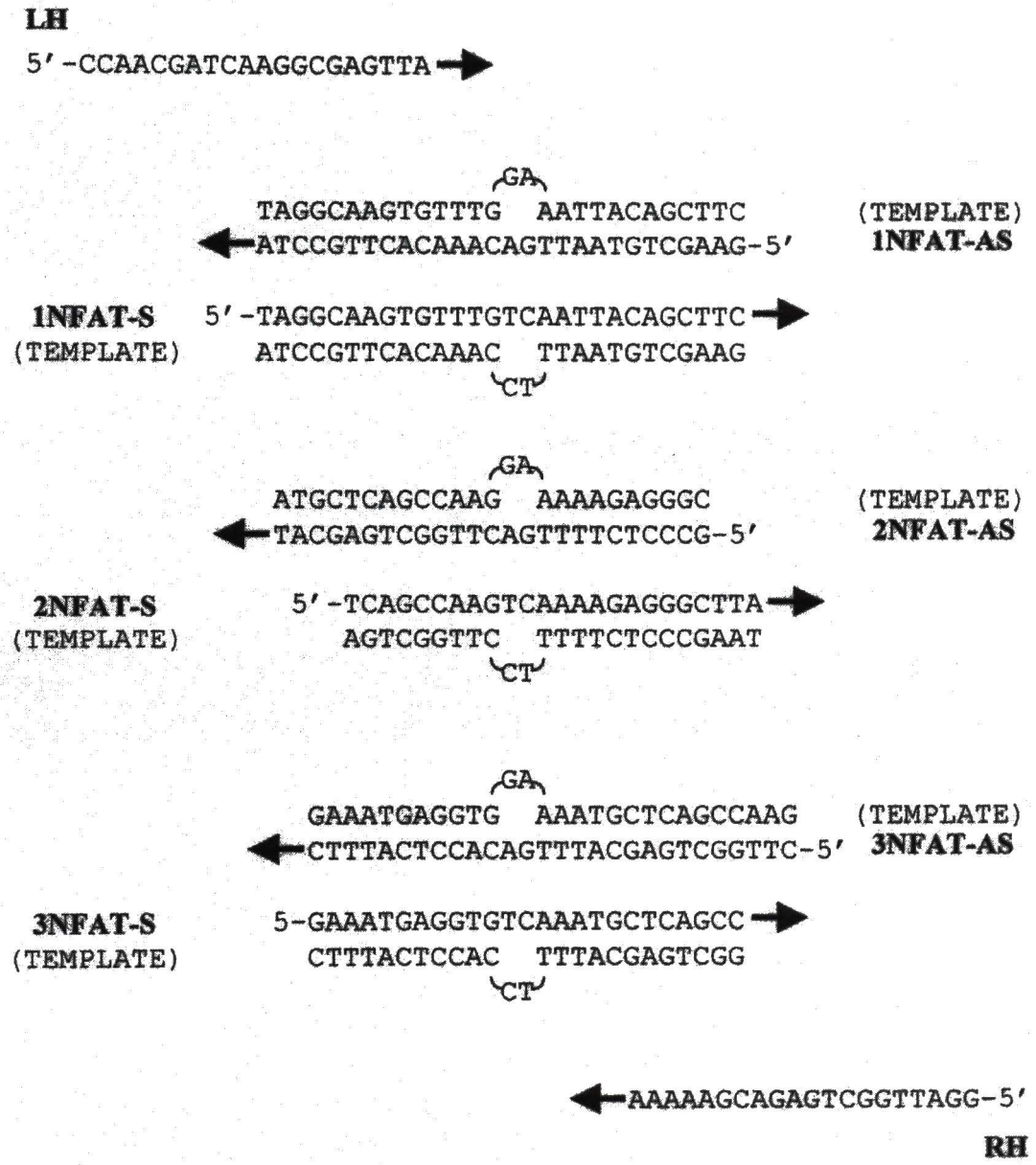
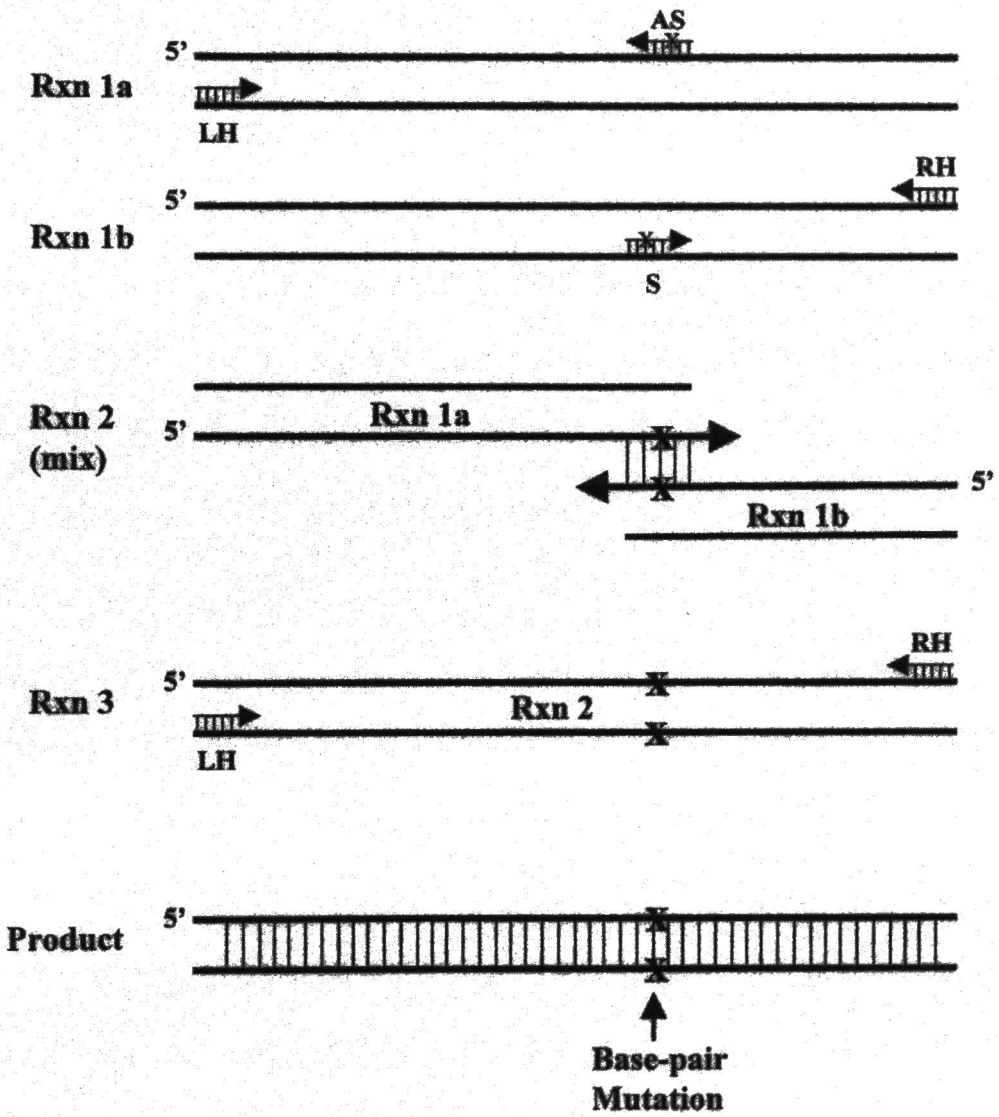

LH
5′-CCAACGATCAAGGCGAGTTA
GA
TAGGCAAGTGTTTG AATTACAGCTTC (TEMPLATE)
ATCCGTTCACAAACAGTTAATGTCGAAG-5′ 1NFAT-AS
1NFAT-S 5′-TAGGCAAGTGTTTGTCAATTACAGCTTC
(TEMPLATE) ATCCGTTCACAAAC TTAATGTCGAAG
CT
GA
ATGCTCAGCCAAG AAAAGAGGGC (TEMPLATE)
TACGAGTCGGTTCAGTTTTCTCCCG-5′ 2NFAT-AS
2NFAT-S 5′-TCAGCCAAGTCAAAAGAGGGCTTA
(TEMPLATE) AGTCGGTTC TTTTCTCCCGAAT
CT
GA
GAAATGAGGTG AAATGCTCAGCCAAG (TEMPLATE)
CTTTACTCCACAGTTTACGAGTCGGTTC-5′ 3NFAT-AS
3NFAT-S 5-GAAATGAGGTGTCAAATGCTCAGCC
(TEMPLATE) CTTTACTCCAC TTTACGAGTCGG
CT
AAAAAGCAGAGTCGGTTAGG-5′
RH

Figure 5. Schematic representation of the four primer site-directed mutagenesis. Two separate reactions (Rxn 1a and 1b) produce each half of the mutated gene fragment by base-pair mismatches (marked by an X) within the mutagenic primers (sense and anti-sense). The products are mixed and subjected to an extension reaction to form a full-length mutagenized DNA molecule (Rxn 2). The left-hand and right-hand primers are then used to amplify the final products (Rxn 3). Key: RH=right-hand primers, LH=left-hand primers, S=sense, AS=anti-sense.


AS
5'
Rxn 1a
LH
RH
5'
Rxn 1b
S
Rxn 2
(mix)
5'
Rxn 1a
5'
Rxn 1b
RH
5'
Rxn 3
Rxn 2
LH
5'
Product
Base-pair
Mutation

Electrophoretic Mobility Shift Assays

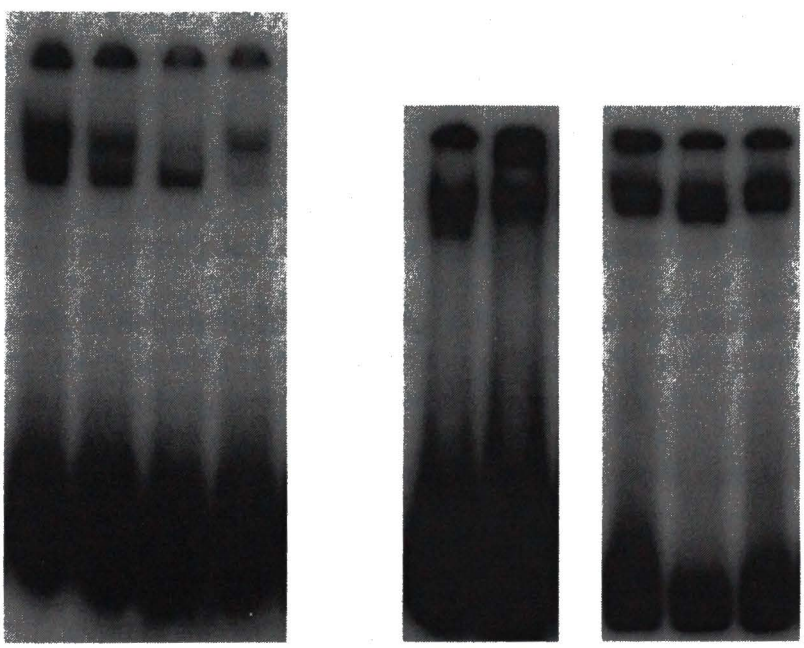
The detection of DNA-binding complexes to DNA probes by electrophoretic mobility shift assays (EMSA's) is a relatively standard procedure. There are issues, however, regarding the technique employed to separate nuclear extracts from cytosolic extracts. The most common technique involves the swelling of cells in a hypotonic buffer to make the cytosolic membrane susceptible to gentle mechanical or detergent lysis, whilst leaving the nuclear envelope intact. The conditions used are dependent on the cell type. For example, Nonidet P-40 (NP-40) detergent concentrations producing acceptable yields of intact nuclei vary among HeLa (0.3%), 70Z/3 (0.1%), and Jurkat cells (0.075%) (8). Moreover, the murine pre-B cell line 70Z/3, considered a 'fragile' cell line, does not produce consistent preparations of intact nuclei in these conditions, such that an elaborate alternative treatment (iso-osmotic/NP-40) is employed (9). In contrast, pancreatic β-cells appear to be a 'tough-skinned' cell type (they are resistant to collagenase, transfection reagents, and detergents), requiring at least 1.0 % NP-40 to completely disrupt the cell membrane. However, partial lyses of both the cyotosolic and nuclear membranes occurs at ~0.6 % when mechanically (vortex) agitated. Thus, a precise protocol was designed based on these observations to achieve consistent preparations of intact INS-1 nuclei. The protocol employs the use of 0.625% NP-40 in a hypotonic lysis buffer and an exact 15 second high vortex. This technique optimizes the separation of nuclear and cytosolic cellular extracts. It should be noted that this technique, regardless of the cell type, does not yield pure nuclear/cytosolic extracts. However, it is suitable for most techniques involving EMSA's. Higher purities require

additional purification procedures, such as ultracentrifugation of intact nuclei through a sucrose cushion to remove cytosolic contaminants.

The other issue regarding EMSA's pertains to the detection of a single transcription factor, which binds to elements that are often embedded or overlap with other *cis*-acting elements. Although single-point mutations within a consensus motif often affect the functionality of *cis*-acting elements within the context of a promoter, they do not always affect their DNA-binding activity. In addition, interactions independent of DNA-binding can occur with the transcription factor of interest. Furthermore, non-specific DNA-binding can occur on DNA, which can alter the binding pattern of a DNA probe in EMSA's. Each of these properties of DNA-protein interactions posed a challenge in confirming the identity of NFAT-DNA binding activity. Using the technique described in the following chapter, we were able to detect two NFAT DNA-binding complexes formed on an NFAT probe sequence based on the 2NFAT element of the rat I insulin gene promoter. The complexes were differentially competed with unlabeled probe, which was dependent on whether the unlabeled probe was added before or after the labeled probe (Fig. 6A). The complex which could not be completely competed-out in either condition was deemed to be a non-selective (but not necessarily unrelated) DNA-binding complex. To confirm the presence of NFAT, an NFAT DNA-binding complex super-shifted with the addition of anti-NFAT antibody, but not by the addition of non-immunized rabbit serum (control) (Fig. 6B). Interestingly, the addition of an anti-calcineurin antibody consistently resulted in a DNA-binding complex, which migrated slightly lower than the NFAT-DNA binding complex (Fig. 6C). This was

presumably the effect of blocking calcineurin binding by the antibody to produce a 'sub-shift', thus indicating that calcineurin is also present in the complex as well. These data were the first demonstration that NFAT DNA-binding activity existed in pancreatic β-cells and that an NFAT DNA-binding complex was capable of forming on the rat I insulin gene promoter.

Figure 6. Electrophoretic mobility shift assays (EMSA) for NFAT DNA-binding activity in INS-1 nuclear extracts. A) Lanes 1-3: increasing amounts (2X, 20X, 200X cold probe added after labeled probe. Lane 4: 2X cold probe added before labeled probe. B) Lane 5: non-immunized rabbit serum added. Lane 6: Anti-NFAT antibody added. C) Lanes 7 and 9: No antibody added. Lane 10: Anti-calcineurin antibody added.


A
B
C
Lane:
1 2 3 4
5 6
7 8 9

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5. **Hammonds P, Schofield PN, Ashcroft SJ:** Glucose regulates preproinsulin messenger RNA levels in a clonal cell line of simian virus 40-transformed B cells. *FEBS Lett* 213:149-54, 1987

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8. **Beg AA, Finco TS, Nantermet PV, Baldwin AS Jr:** Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. *Mol Cell Biol* 13:3301-10, 1993

9. **Dyer RB, Herzog NK:** Isolation of intact nuclei for nuclear extract preparation from a fragile B-lymphocyte cell line. *Biotechniques* 19:192-5, 1995

CHAPTER III

REGULATION OF INSULIN GENE TRANSCRIPTION BY A Ca^{2+}-RESPONSIVE PATHWAY INVOLVING CALCINEURIN AND NFAT

PREFACE

The insulin gene promoter has been extensively studied, and several *cis*-acting elements have been identified, as well as *trans*-acting factors, which bind to these elements and regulate insulin gene transcription. A large portion of the findings regarding islet transcription factors has been produced in the context of islet development. Less is known about regulation of insulin promoter activity during cell signaling. The lack of responsiveness in β-cell lines due to culture conditions and challenges in transfecting harvested pancreatic islets has hindered interpretations of the response of the insulin gene promoter to physiological stimuli. Perhaps the best understood transcription factor, which responds to glucose in β-cells is PDX-1. Upon exposure of β-cells to high glucose conditions after an adequate refractory period in basal conditions, PDX-1 is phosphorylated, translocated to the nucleus, binds to A box elements and transactivates the insulin gene promoter. However, the precise signaling pathways, which activate PDX-1 are not known, and PDX-1 alone is not sufficient to up-regulate promoter activity. It is therefore necessary to determine signaling mechanisms

arising from glucose that activate insulin gene transcription and to identify factors, which are involved in the process.

A few observations gave rise to the following experiments. The first observation concerned reports indicating that the immunosuppressant drug, FK506, gave rise to a 10-30% incidence of post-transplant diabetes. Further characterization of this drug indicated that FK506 specifically inhibited the calcium/calmodulin-dependent class 2B phosphatase, calcineurin. This was linked to the finding that calcineurin activated NFAT to induce cytokine gene transcription. Lastly, our group and others (Redmon,et al) observed that FK506 inhibited insulin production at the level of insulin gene transcription. The well-known model of glucose-induced intracellular calcium flux in pancreatic β-cells in the context of insulin release, along with these observations, made calcineurin and NFAT excellent candidates for the modulation of insulin gene transcription in β-cells in the presence of glucose.

SUMMARY

Immunosuppressants such as FK506 (tacrolimus), whose primary cellular target is calcineurin, decrease β-cell insulin content and preproinsulin mRNA expression. This study offers an explanation for this effect by establishing that calcineurin is an important regulator of insulin gene expression through the activation of a transcription factor, nuclear factor of activated T-cells (NFAT). Three putative NFAT binding sites were located within the proximal region of the rat insulin I (Rins-1) gene promoter (-410 to +1 bp). NFAT expression in the β-cell was confirmed by immunoblot and immunocytochemistry. NFAT DNA-binding activity was detected in INS-1 and islet nuclear extracts by electrophoretic mobility shift assays. Activation of the insulin gene promoter by glucose or elevated extracellular K^+ (to depolarize the β-cell) was totally prevented by FK506 (5-10 μM). K^+-induced promoter activation was suppressed (>65%) by a two-base pair mutation of a single NFAT binding site in -410 Rins-1. Both stimulants also activated a minimal promoter-reporter construct containing tandem NFAT consensus sequences. The effects of FK506 on K^+-induced NFAT reporter or insulin gene promoter activity were not mimicked by rapamycin, indicating specificity towards calcineurin. These findings suggest that the activation of calcineurin by β-cell secretagogues that elevate cytosolic Ca^{2+} plays a fundamental role in maintenance of insulin gene expression via the activation of NFAT.

INTRODUCTION

Post-transplant diabetes mellitus is among the most serious adverse effects of immunosuppressive therapy using FK506 (tacrolimus) and is manifested by hyperglycemia, insulin resistance and the appearance of islet cell antibodies (1). The incidence of diabetes in recipients of kidney transplants has been reported to be as high as 20% (2). The onset of diabetes may be even more widespread since tacrolimus is not only established for primary immunosuppression in liver and kidney transplantation, but also considered for therapies following solid organ transplantation of heart, lung, and pancreas (1).

The diabetogenic action of FK506 is not understood, but a direct effect on β-cell function is invoked. The chronic administration of FK506 *in vivo* results in a marked but reversible reduction in insulin content of endocrine islets (3). This precedes morphological changes that are partially characterized by a loss of dense core secretory granules (4). Similar results are observed with the use of another immunosuppressant, cyclosporin A (CsA), although often to a lesser extent. FK506, or the structural analog L-683,590, also reduces insulin content and ultimately insulin secretion in isolated islets or cultured β-cells *in vitro* (5,6). Since FK506 does not acutely affect insulin secretion (7), these effects are presumed to be explained by a reduced capacity to synthesize insulin. At the molecular level, FK506 has been shown to reduce β-cell preproinsulin mRNA expression (3,5,6) and dampen glucose activation of the insulin promoter (6). It

is reasoned, therefore, that a primary effect of FK-506 is to interfere with the transcriptional activation of the insulin gene.

This mechanistic scenario is similar to the activated T-cell where the immunosuppressant properties of FK506 and CsA are accounted for by their common action to inhibit the Ca^{2+}/calmodulin-dependent phosphatase 2B, calcineurin (8-10). Both compounds target calcineurin via their interaction with immunophilins, FK506 binding proteins (FKBP's) and cyclophilin for FK506 and CsA respectively. Calcineurin is critically required for the induced expression of cytokine genes necessary for the initiation and coordination of an immune response (10). Under normal conditions, the action of calcineurin in the cell cytosol results in the dephosphorylation (on multiple serines) of NFAT (nuclear factor of activated T-cells)(11,12). The resultant exposure of a nuclear localization sequence (NLS) promotes the rapid translocation of NFAT to the nucleus (13) where it binds, generally in cooperation with other *trans*-acting factors such as fos/jun components of AP-1 (14,15), to *cis*-elements located in the promoters of several cytokine genes (9,16). To date, most of the known therapeutic and toxic effects of FK506 and cyclosporin A are attributable to the inhibition of calcineurin (10).

Calcineurin is widely distributed among tissues (17), and several reports have documented its expression in islet cells of the endocrine pancreas (3,6,7,18). It is now apparent that NFAT expression is also diverse, and it is detected in non-immune tissues and cell types such as skeletal muscle, heart, neurons, adipocytes and the pancreas (12,19). The current study was therefore initiated to assess the involvement of NFAT in β-cell gene expression. The evidence generated suggests that calcineurin, via NFAT, is

an important regulator of insulin gene transcription and that the disruption of this pathway may contribute to the diabetogenic effects of FK506.

EXPERIMENTAL PROCEDURES

Cell Culture

INS-1 and Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 2-mercaptoethanol, streptomycin (100 μg/ml) and penicillin (100 U/ml) at 37 °C under an atmosphere of 95% air/ 5% CO_2.

Plasmids and Mutagenesis

A vector construct (pSYNT) harboring the promoter region (-410 to +1) of the rat insulin 1 gene (-410 Rins-1)(20) was kindly provided by Dr. M. German, CA. The -410 Rins-1 fragment was amplified by PCR with primers incorporating a 5'- XhoI linker and directionally cloned into the pGL2-Basic luciferase promoter-reporter mammalian expression vector (Promega, WI). The resultant construct is designated pGL2-Rins-1. Mutagenesis of the second NFAT site within the insulin gene promoter (2NFAT) (Fig. 1) was achieved by a four-primer mutagenesis method designed to create two point mutations and thus the disruption of the core NFAT sequence (5'-GGAAA to 5'-TCAAA). The PCR fragment was cloned into the pCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA) and sequenced for verification of the site-directed point mutations. The mutated fragment was then amplified to incorporate 5'- XhoI linker and cloned into pGL2-Basic as described above. The expression vector, pSRα-CaNΔCaM-AI, harboring

constitutively active calcineurin A (CaN-A) in which the calmodulin binding and autoinhibitory domains were deleted (21), was a generous gift from Dr. Stephen O'Keefe, Merck Research Laboratories, NJ. For calcineurin over-expression experiments, a control vector was generated by re-ligation of pSRα following restriction enzyme digestion to eliminate the CaN-A insert. The NFAT-luciferase (NFAT-Luc) reporter plasmid was a generous gift of Dr. Gerald Crabtree, Stanford, CA.

Isolation of Islets

Pancreati were isolated from male Wistar rats by collagenase P (Boehringer Mannheim, IN) digestion followed by centrifugation on a discontinuous Ficoll gradient. Islets were cultured in CMRL–1066 containing 5.5 mM glucose and supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/ml streptomycin and 100 μg/ml penicillin overnight at 24°C under an atmosphere of 95% air/5% CO_2. Immediately before experimentation, the islets were incubated at 37°C for a minimum of 60 min.

Western blots

Cell extracts from INS-1, pancreatic islets, and Jurkat cells were prepared by lysis in Laemmli buffer. Samples were boiled for 5 min and loaded (30 μg protein per lane) on an SDS/6% polyacrylamide gel. The proteins were electro-transferred to a nitrocellulose membrane (Osmonics, MA) and blotted with affinity-purified NFATp antibody (a gift from Dr. Karen L. Leach, Pharmacia & Upjohn, MI). Washes were done in PBS with

0.1% polyoxyethylene sorbitan monolaurate (Tween-20). The enhanced chemiluminescence system (ECL) was used as the method of detection by a secondary goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (Amersham Pharmacia, NJ).

Immunocytochemistry

Rat pancreati were excised from Wistar rats and fixed on ice for 4-6 hours by immersion in PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 100 mM Na_2HPO_4, pH 7.2) supplemented with 4% paraformaldehyde. Following overnight equilibration at 4°C in PBS containing 30% sucrose, the pancreati were embedded in tissue freezing medium (OCT compound) and cryosectioned (~70 nm) as previously described (22). On the day of immunocytochemistry, frozen pancreatic sections were rehydrated and permeabilized with PBS containing 0.2% Triton X-100 and blocked with PBS containing 4% BSA and 5% serum from the host animal species in which the secondary antibody was raised. Incubations with primary antibodies anti-NFAT796 (a generous gift of Dr. Nancy Rice, ABL-Basic Research Program, MD) or anti-insulin (1:200 dilution) (Linco Research Inc., MO) were continued overnight at 4 °C and followed by incubation with fluorochrome-conjugated secondary antibodies (1:200) for 1 h at 37 °C. All washes were done in PBS containing 0.1% Triton X-100. Visualization of slides was conducted on a Nikon Microphot FXA microscope.

Electrophoretic Mobility Shift Assays (EMSA).

Complementary oligonucleotides (5'-ATGAGGTGGAAAATGCTCAG) containing a -410 Rins-1 NFAT consensus site (2NFAT) were synthesized (Genosys, MO), hybridized, and end-labeled by T4 polynucleotide kinase (Amersham Pharmacia, NJ) in the presence of [γ-^{32}P]-ATP. INS-1 cells (~4 X 10^6 cells) were lysed in 400 µl of buffer A (10 mM Tris (pH 8.0), 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM NaF, 0.6% NP-40, 1 mM DTT, 0.5 PMSF, and 10 mg/ml leupeptin). Nuclear pellets were spun down and resuspended in 50 µl buffer B (10 mM Tris (pH 8.0), 1.5 mM $MgCl_2$, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM DTT, 0.5 mM PMSF, and 10 mg/ml leupeptin) to harvest extracts. Equal amounts of nuclear extract (20 µg) were incubated for 30 min with double-stranded ^{32}P-labeled NFAT probe (20,000 c.p.m.) in reaction buffer (10 mM Tris, pH 8.0, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 6% glycerol). Increasing amounts (2-, 20-, or 200-fold after; 2- or 20- fold prior) excess of cold probe were added to competition reactions either 15 min before or after the labeled probe. Anti-NFAT antibody was added 15 min after labeled probe in super-shift experiments. The reactions were subjected to electrophoresis on 6% polyacrylamide gels and bands were detected using a Packard Instant Imager Electronic Autoradiography System (Packard, CT).

Cell Transfections and Reporter Assays

INS-1 cells were cultured in 12-well plates in RPMI medium as described, then brought to 2 mM glucose 6 h prior to transfection. INS-1 cell transfection was achieved using

FuGene-6 (Roche Molecular Biochemicals, IN) according to the manufacturer's directions. All cells were co-transfected with a control vector (pSV-CAT) for the normalization of transfection efficiency. Eighteen hours after transfection, the cells were stimulated with either 11 mM glucose or 30 mM KCl. In the latter case, the cell incubations (post 18 h) were performed using a modified Krebs Ringer Bicarbonate (KRB) medium (25 mM Hepes, pH 7.4, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$) with 0.1% bovine serum albumin; a 30 mM KCl isotonic KRB solution was generated by adjusting the relative concentrations of KCl and NaCl to 30 mM and 90 mM, respectively. For inhibitor studies, FK506 (1, 5, 10 μM) or rapamycin (1, 5, 10 μM) was added to the media 2 h prior to cell stimulation. The cells were harvested 24 h after transfection by lysis in Repoter Lysis Buffer, (Promega, WI). Following brief centrifugation (~16,000 xg, 5 min) to remove cell debris, the supernatant was assayed for luciferase activity based on Luciferase Assay System (Promega, WI) using a TD-20/20 bioluminometer (Turner Designs) or chloramphenicol acetyltransferase (CAT) activity by the CAT-ELISA method (Roche Molecular Biochemicals, IN).

Statistical Analysis

Statistical significance was calculated by one-tailed *t*-test.

RESULTS

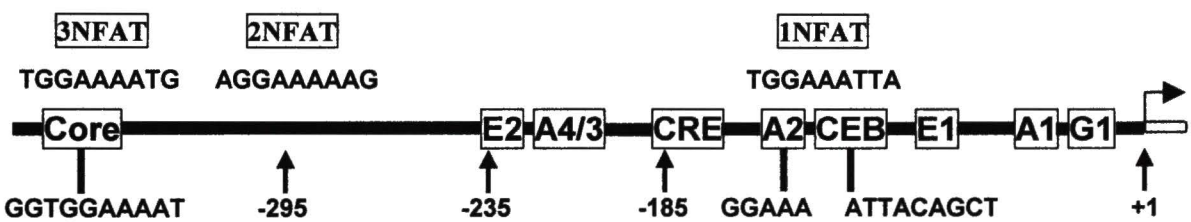
Identification of NFAT consensus sites within the insulin gene promoter

Examination of the first 410 bp of the rat I insulin gene promoter (-410 Rins-1), a region that controls >90% of the transcriptional regulation of preproinsulin gene (23), revealed the presence of three putative NFAT binding sequences (consensus (T/A)GGAAA(A/N)(A/T/C), where N= any base) (Fig. 1). These sequences are located at positions -139 to -131 (1NFAT), -299 to -291 (2NFAT) and -308 to -316 (3NFAT) on Rins-1 relative to the transcription start site (+1). Two of these binding sequences (1NFAT and 3NFAT) are positionally conserved in other mammalian insulin gene promoters, such as in human, mouse and dog. The insulin gene promoter resembles other known NFAT-dependent promoters in that it displays in common a multiplicity of NFAT-binding sites (12), implying that higher-order interactions among NFAT-containing complexes are required for effective transcription.

NFAT is expressed in pancreatic β-cells

Three independent pieces of evidence were acquired to support a role of NFAT in β-cell function. First, the expression of NFAT in β-cells was ascertained based on immunochemical analyses in rat pancreatic slices. Using an antibody raised against a peptide common to all known NFAT family members, NFAT immunoreactivity was primarily associated with islets of Langerhans with minimal reactivity in surrounding

Figure 1. Multiple NFAT consensus sequences are present within the insulin gene promoter. Shown is the promoter region (-410bp to +1) for the rat insulin 1 (-410 Rins-1) gene. The boxes represent select sequence elements previously identified as regulatory sites. The three conceptualized NFAT sites (1-3NFAT) are indicated above the promoter along with the DNA sequence conforming to the NFAT consensus motif.


3NFAT
2NFAT
1NFAT
TGGAAAATG
AGGAAAAAG
TGGAAATTA
Core
E2
A4/3
CRE
A2
CEB
E1
A1
G1
GGTGGAAAAT
-295
-235
-185
GGAAA
ATTACAGCT
+1

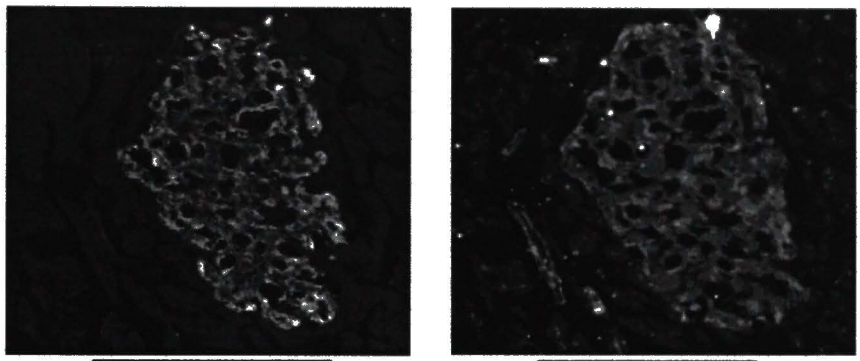
exocrine tissue (Fig. 2A). Within the islet, the cell association was similar to islets stained using anti-insulin as primary antibody, supporting its association with the β-cell. By immunoblot, NFAT was identified in isolated rat islets, as well as in the cultured clonal β-cell line (INS-1) (Fig. 2B). Furthermore, by electrophoretic mobility shift assay (EMSA), nuclear extracts of islets and INS-1 cells displayed specific binding activity towards DNA probes harboring NFAT consensus sequences from the rat I insulin gene promoter. The NFAT DNA-binding complex super-shifts in the presence of anti-NFAT antibody (Fig. 2C), which is representative of what is exhibited from EMSA's of all three identified Rins-1 promoter NFAT sites (2NFAT is shown). Although two complexes were routinely resolved, only the upper band was found to represent a specific binding event based on its competition by excess NFAT DNA probe. These observations confirm a functional expression of NFAT in the β-cell.

Glucose and potassium- induced insulin gene transcription

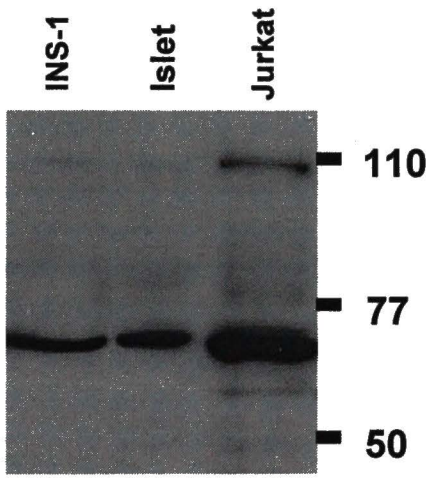
In order to permit an assessment of the involvement of calcineurin/NFAT signaling in insulin gene transcription, INS-1 β-cells were transfected with an insulin promoter-reporter construct (pGL2-Rins-1). The activity of this promoter was initially monitored in cells stimulated by glucose (11 mM) or by high extracellular concentrations (30 mM) of K^+, conditions reasoned to induce the elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and the activation of calcineurin (24). Stimulatory concentrations of either glucose (11 mM) or K^+ (30 mM) induced a similar (approximately seven-fold) elevation in reporter enzyme activity (luciferase) within 6 h of stimulation relative to basal conditions (2 mM

Figure 2. NFAT is expressed in pancreatic cells. *Panel A: Immunocytochemistry.* Cryosections of a rat pancreas double-stained with anti-insulin (left) and anti-NFAT796 (right) antibodies. Secondary Cy-2 and TX-red antibodies were used for detection, respectively. *Panel B: Western Analysis.* Whole cell extracts from INS-1, pancreatic islets, and Jurkat cells were probed with the anti-NFATp antibody. *Panel C: Electrophoretic mobility shift assay (EMSA).* An NFAT probe (2NFAT) from the rat insulin-1 promoter was used to detect NFAT-DNA binding activity in INS-1 and pancreatic islet cells. The NFAT-DNA binding complex was competed with excess non-labeled probe (2X, 20X or 200X) incubated before (pre) or after (post) the addition of the radiolabeled NFAT probe of the insulin gene promoter. The lower band was not competed by the unlabeled probe, indicative of non-selective binding. The NFAT-DNA binding complex was super-shifted (complex indicated by arrow) in the presence of anti-NFAT796 antibody.

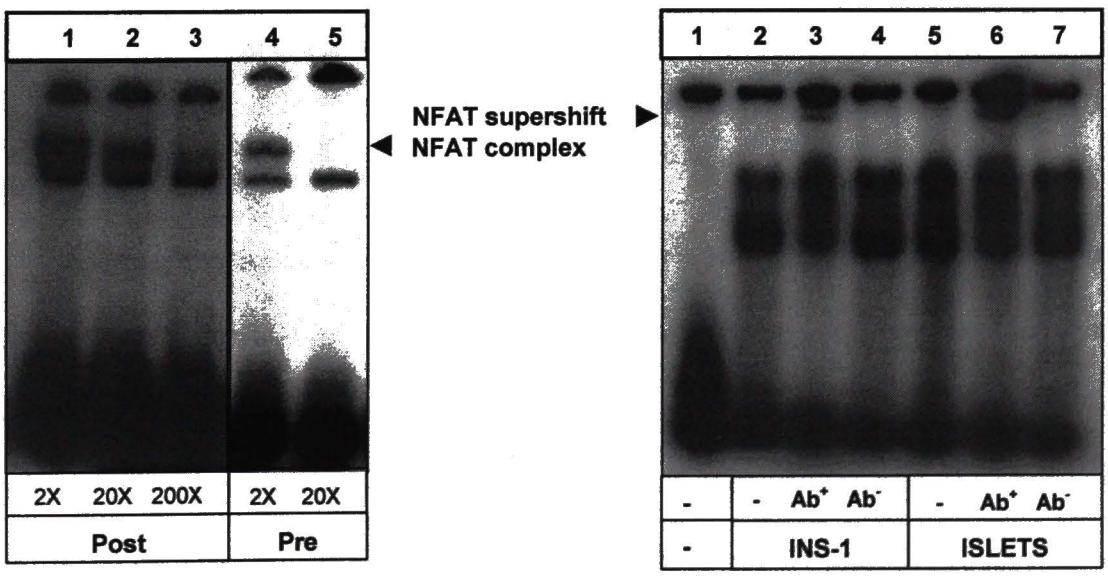
A)


Anti-Insulin
Anti-NFAT

B)


INS-1
Islet
Jurkat
110
77
50

C)


1 2 3 4 5
2X 20X 200X 2X 20X
Post
Pre
NFAT supershift
NFAT complex
1 2 3 4 5 6 7
- - Ab+ Ab- - Ab+ Ab-
- INS-1 ISLETS

glucose, 5 mM K^+) (Fig. 3A). Since high K^+ induces cell depolarization without the influence of glucose metabolism, these observations support the conclusion that an elevation in $[Ca^{2+}]_i$ is capable of enhancing insulin gene promoter activity.

Calcineurin is required for up-regulating insulin gene promoter activity

Significantly, the effect of glucose or K^+ to enhance insulin gene promoter activity was antagonized by the presence of FK506, a selective inhibitor of calcineurin (Fig. 3). FK506 dose-dependently inhibited insulin promoter activity; complete inhibition was observed at a concentration of 5-10 μM FK506 (Fig. 3B). This effect appeared to be specific since it had no effect on the expression of a control vector (pSV-CAT) co-transfected with pGL2-Rins-1. Furthermore, K^+-induced insulin promoter activity was not inhibited by rapamycin (Fig. 3C), an analog of FK506 which binds the same intracellular receptor as FK506 (FKBP-12) but does not affect calcineurin activity (25,26). In contrast, rapamycin inhibited insulin promoter activity driven by glucose nearly as efficiently as FK506. This latter observation suggests other signaling mechanisms independent of calcineurin are also necessary for glucose activation of insulin gene transcription.

To confirm that K^+-induced insulin promoter activity results from the effect of increased $[Ca^{2+}]_i$, cells were treated with intracellular calcium inhibitors, verapamil and BAPTA, which blocked the effect of high K^+ on insulin gene reporter activity (Fig. 4A). A direct effect of calcineurin to modulate insulin gene transcription was demonstrated in INS-1 cells over-expressing a constitutively active form of calcineurin A (CaNΔCaM-AI)

Figure 3. Glucose (Glc)- and K^+-induced insulin gene transcription is inhibited by FK506. INS-1 cells were co-transfected with pGL2-rIns-1/pSV-CAT and then incubated in basal (2 mM glucose) or stimulatory (11 mM glucose or 30 mM K^+) media for 6 h. Luciferase activity in cell lysates was normalized with respect to CAT activity and expressed as fold over basal. FK506 (5 μM, *panel A*; 0-10 μM, *panel B*) or control vehicle (DMSO) was added 2 h before cell stimulation. *Panel C: K^+-induced insulin gene transcription is insensitive to rapamycin.* INS-1 cells transfected with pGL2-Rins-1 were incubated in increasing concentrations of rapamycin (0-10 μM) 2h prior stimulation by 11 mM glucose (Glc) or 30 mM KCl (K^+). Data are expressed as a fold-increase in luciferase activity (normalized to CAT activity) over controls in the presence of 2 mM glucose. Data are means ± S.E for 3 or more independent determinations.

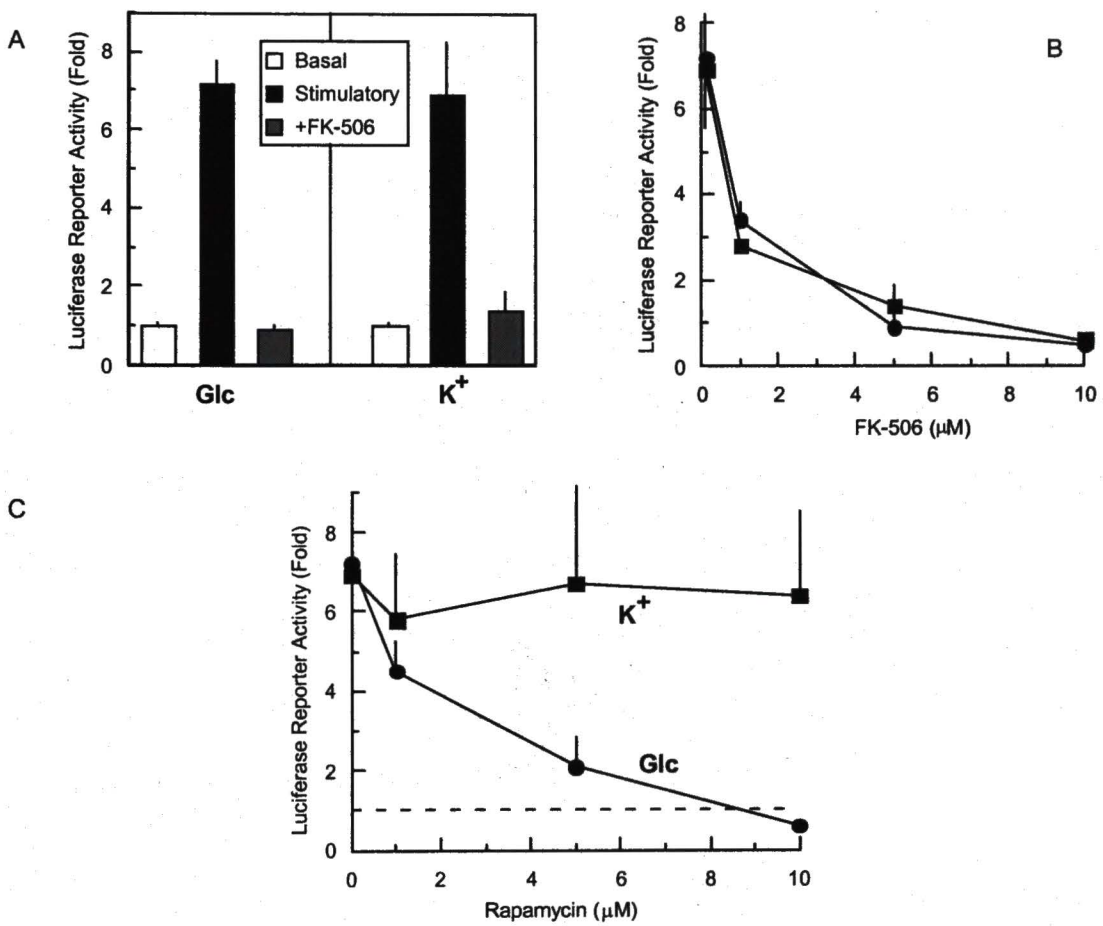
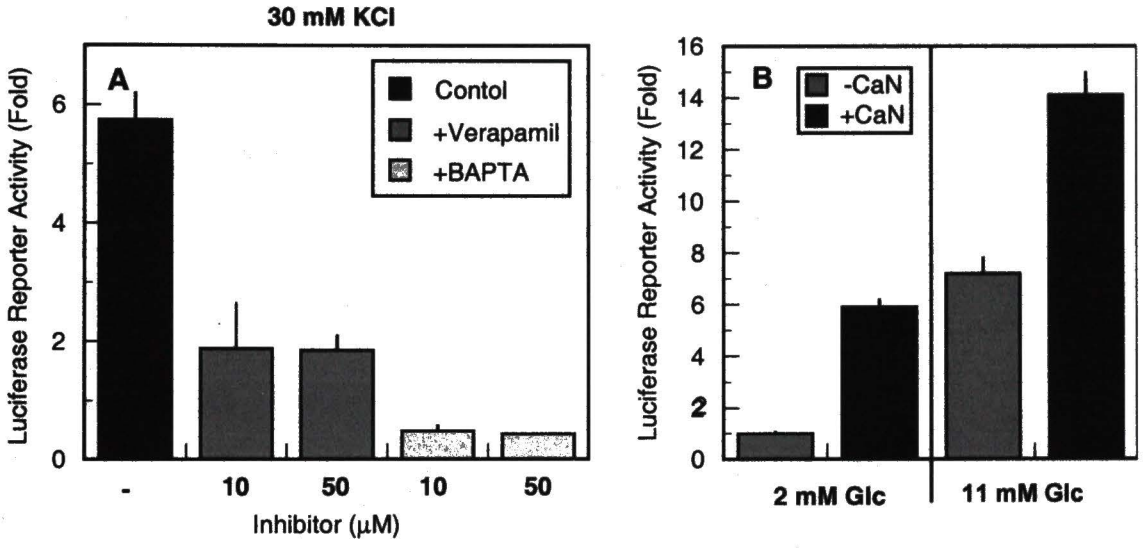

A
Luciferase Reporter Activity (Fold)
Basal
Stimulatory
+FK-506
Glc
K+
B
Luciferase Reporter Activity (Fold)
FK-506 (μM)
C
Luciferase Reporter Activity (Fold)
K+
Glc
Rapamycin (μM)

Figure 4. Calcium and calcineurin can modulate insulin gene transcription. *Panel A:* INS-1 cells were treated with intracellular calcium inhibitors verapamil or BAPTA, then stimulated with 30 mM K^+ for 6 h. *Panel B:* INS-1 cells were co-transfected with pGL2-Rins-1 and constitutively active calcineurin A (pSRα–CaNΔCaM-AI) or empty vector. Data are expressed as a fold-increase in luciferase activity (normalized to CAT activity) over controls in the presence of 2 mM glucose. Data are means ± S.E for 3 or more independent determinations.

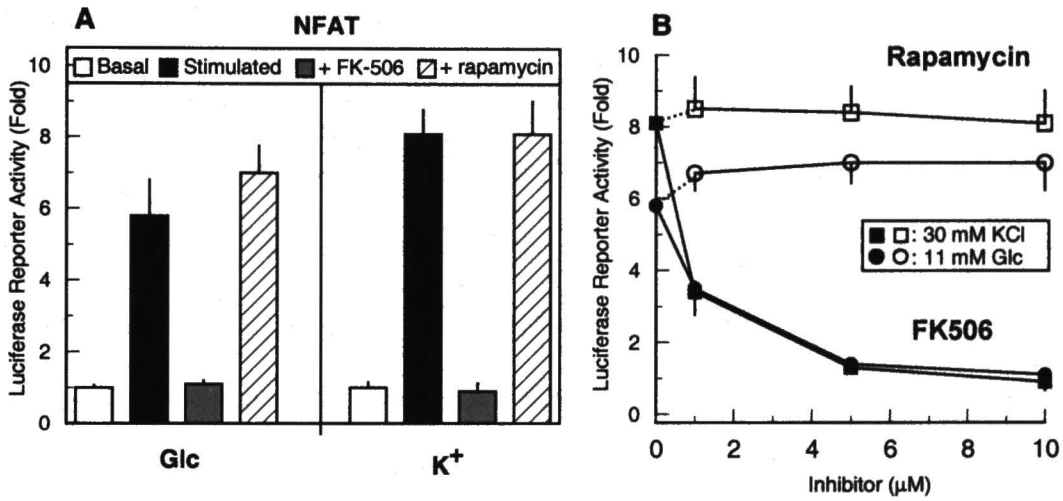

30 mM KCl
A
Contol
+Verapamil
+BAPTA
Luciferase Reporter Activity (Fold)
0
2
4
6
-
10
50
10
50
Inhibitor (μM)
B
-CaN
+CaN
Luciferase Reporter Activity (Fold)
0
2
4
6
8
10
12
14
16
2 mM Glc
11 mM Glc

lacking the autoinhibitory domain and a functional calmodulin binding domain. In cells co-transfected with pGL2-Rins-1 and constitutive calcineurin (CaNΔCaM-AI) in basal glucose concentrations, reporter activity was increased 6-fold relative to cells transfected with the control vector (no calcineurin). This stimulation approximated reporter activation achieved in the presence of 30 mM K^+ (Fig. 4B *cf.* Fig. 3A). This effect was further heightened by the addition of 11 mM glucose, which enhanced reporter activities 14-fold over those observed under basal conditions (Fig. 4A). These observations demonstrate that calcineurin can directly up-regulate insulin gene transcription and enhance the ability of glucose to modulate the activity of the insulin promoter.

Activation of NFAT in β-cells

In order to evaluate whether glucose and cell depolarization by K^+ activate NFAT in β-cells, INS-1 cells were transfected with an NFAT-reporter construct (NFAT-Luc) in which multiple NFAT-consensus sites were inserted upstream to a minimal promoter (IL-2) (8). Stimulatory concentrations of glucose (11 mM) and K^+ (30 mM) increased NFAT-Luc reporter activity by 6-fold and 8-fold over basal conditions, respectively (Fig. 5). In both cases, reporter activity was completely blocked by 5 μM FK506 as observed in cells transfected with pGL2-Rins-1 (*cf.* Fig. 3C). In contrast, rapamycin up to a concentration 10 μM had no significant effect on NFAT-mediated transcription induced by either glucose or K^+. Thus, insulin secretagogues activate NFAT in pancreatic β-cells by a calcineurin-dependent mechanism.

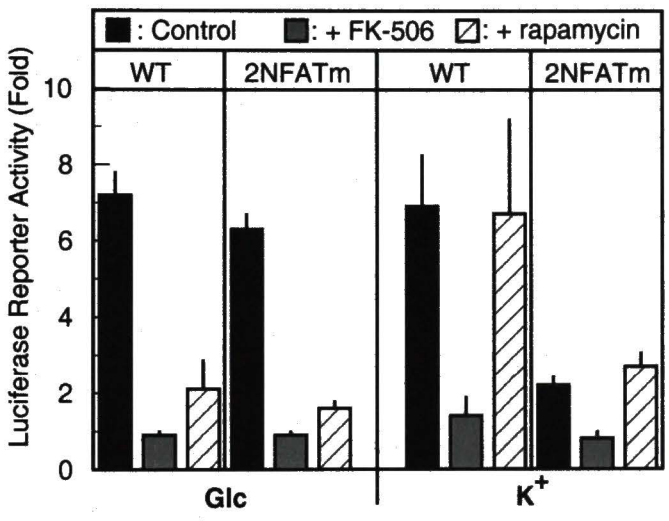
Figure 5: Glucose and K^+ induce NFAT activation in β-cells. INS-1 cells were transfected with a NFAT-Luc reporter construct and incubated with basal (2 mM glucose) or stimulatory (11 mM glucose, Glc, or 30 mM K^+) conditions for 6 h. *Panel A*: Cells were incubated in the presence of 10 μM FK506 (stippled bar) or 10 μM rapamycin (striped bar). Control cells were supplemented with vehicle alone (white and black bars). *Panel B*: Cells were incubated in increasing concentrations of FK506 (filled symbols) or rapamycin (open symbols). Data are expressed as a fold-increase in luciferase activity (normalized to CAT activity) over controls in the presence of 2 mM glucose. Data are means ± S.E. for 3 or more independent determinations.


A
NFAT
Basal
Stimulated
+ FK-506
+ rapamycin
Luciferase Reporter Activity (Fold)
10
8
6
4
2
0
Glc
K+
B
Rapamycin
□: 30 mM KCl
○: 11 mM Glc
FK506
Luciferase Reporter Activity (Fold)
0
2
4
6
8
10
Inhibitor (μM)

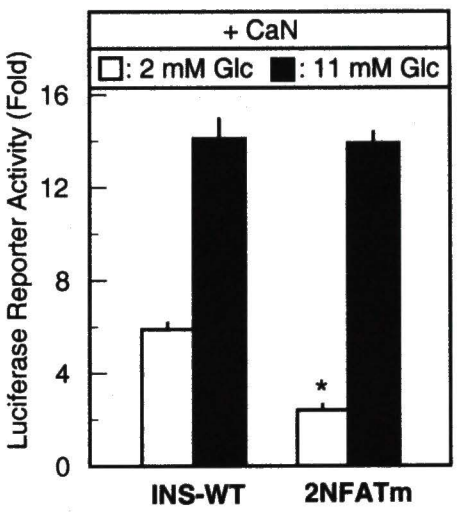
Effect of an NFAT-mutated insulin gene promoter on transcription

To confirm the importance of calcineurin/NFAT in the activation of insulin gene transcription, site-directed mutagenesis was employed to eliminate an NFAT element from the insulin gene promoter. In light of the poorly defined binding of PDX-1 to A-box and the potential overlap with NFAT binding sequences, an NFAT site was chosen (2NFAT) that did not exist within either of the A and E element enhancers (Far-FLAT and Nir-P1) (Fig. 1). The double-point mutated 2NFAT site (2^{nd} NFAT element most proximal to the transcription start site) does not disrupt any known elemental binding sites, such as that for PDX-1 or IEF, which are essential transcription factors for insulin gene promoter activity. There was little difference between luciferase reporter activities in cells transfected with wild-type (pGL2-Rins-1) or mutant (pGL2-2NFATm) promoter constructs in the presence of stimulatory concentrations of glucose (Fig. 6A). In contrast, the mutation of 2NFAT resulted in a marked suppression (~68%) of luciferase reporter expression induced by depolarizing concentrations of extracellular K^+. This mutation also resulted in the dramatic loss (~60%) of the insulin promoter to stimulation by the over-expression of constitutively active calcineurin (Fig. 6B).

Figure 6. Elimination of 2NFAT binding element reduces insulin promoter activity in response to K^+. INS-1 cells were transfected with Rins-1-Luc (WT) or INS-2NFATm (2NFATm). *Panel A:* Cells were incubated with 11 mM glucose or 30 mM K^+ in the absence and presence of FK506 or 5 rapamycin (5 μM each). *Panel B:* Rins-1-Luc or INS-2NFATm were co-transfected with a plasmid expressing constitutively active calcineurin (CaN; pSRαCaNΔCaM-AI). Data are expressed as fold increase in luciferase activity (normalized to CAT activity) over controls in the presence of 2 mM glucose. Data are means ± S.E. for 3 or more independent determinations. * $p<0.05$ *vs.* Rins-1-Luc, 2 mM glc).


■: Control ■: + FK-506 ▨: + rapamycin
WT
2NFATm
WT
2NFATm
Luciferase Reporter Activity (Fold)
0
2
4
6
8
10
Glc
K+

A)


+ CaN
□: 2 mM Glc ■: 11 mM Glc
Luciferase Reporter Activity (Fold)
0
4
8
12
16
*
INS-WT
2NFATm

B)

DISCUSSION

Besides its central role in the coordination of cytokine expression in the activated T-cell, calcineurin is now known to influence transcriptional regulation in a variety of non-immune cells (27). Most dramatic perhaps is its role in the transcriptional regulation of genes associated with hypertrophic growth in cardiac and skeletal muscles (28). Calcineurin has also been implicated in the transcriptional regulation of a non-insulin gene (*i.e.* glucagon) in the β-cell (24). This study has now established that calcineurin has the capacity to directly modulate the insulin gene promoter. This is exhibited directly by the effect of over-expression of constitutively active calcineurin (CaN A) to up-regulate Rins-1 and is further supported by the attenuation of promoter activity by FK506. These data suggest that calcineurin may be required for physiological regulation of insulin gene expression by β-cell stimuli.

Most significantly, the modulation of insulin gene transcription by calcineurin was found to be mediated via NFAT and thus similar to the activated T-cell. The observation that both primary (islet) and clonal β-cells express NFAT was not unexpected based on the widened scope of detection of this transcription factor in non-immune system cells. However, it is not yet determined which of the isoforms of this large multi-gene family of proteins (16) are represented in β-cells. Nevertheless, the application of an NFAT promoter-reporter system demonstrates that the insulin secretagogues, glucose and K^+, both activate NFAT in the β-cell. Despite the fact that NFAT can be

dephosphorylated by a number of phosphatases, the specific involvement of calcineurin is supported by the complete inhibition achieved in the presence of FK506, but not rapamycin. The same discriminatory sensitivity was observed with the Rins-1 insulin gene promoter, at least in response to K^+, arguing that NFAT activation by calcineurin is also required for insulin gene expression under these conditions. This link is strengthened by the observation that the influence of calcineurin and K^+ on insulin gene promoter activity were both significantly dampened by the 2-base pair mutation of a single NFAT site (2NFAT) in this promoter. Collectively, these data establish a functional pathway by which calcineurin can modulate insulin promoter activity through the interaction of NFAT with specific sites and argue that NFAT should be added to the already large repertoire of transcription factors capable of influencing insulin gene transcription.

The lack of effect of rapamycin on K^+-induced insulin promoter activity contrasts a previous study, which emphasized an autocrine effect of insulin secreted, in response to cell depolarization, on insulin gene transcription (29). This autocrine effect may be an important contributor to the regulation of insulin biosynthesis (30), but the identification of a calcineurin/NFAT pathway in the β-cell forwards a direct mechanism by which Ca^{2+}-responsiveness may be conferred on the insulin promoter (31,32). Numerous other studies have established that cell depolarization-induced regulation of gene expression in the β-cell is dependent on Ca^{2+} influx (33,34). In the case of glucagon gene promoter, activation in HIT cells (β-cells) is mediated by calcineurin modulation of CREB interaction with a CRE in this promoter (34,35). Despite the presence of a CRE element

within the -410 rInsI promoter, the context of this site does not appear to permit it to be responsive to FK506 (36). The consideration of these studies suggests that the prevention of NFAT activation is a primary mechanism by which FK506 perturbs insulin gene transcription.

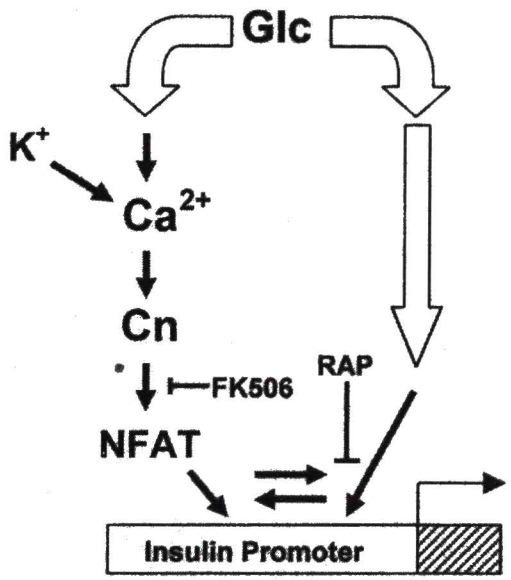
The involvement of calcineurin in glucose regulation of the insulin promoter is less clear and confused by the observation that rapamycin mimics the effects of FK506 on this promoter, effects that have been observed previously (29). This contrasts the indifference of glucose-induced activation of NFAT-reporter construct to rapamycin treatment, suggesting that the early cellular events of calcineurin and NFAT activation are not disrupted. Rather than inhibiting calcineurin, the rapamycin/FKBP12 complex targets mTOR (mammalian target of rapamycin; also named FRAP, RAFT1 and RAPT1)(37) and then PHAS1 (38) and $p70^{S6K}$ (39), which are involved in the regulation of protein translation. The effect of rapamycin is unlikely to be a non-specific, global inhibition of transcription because it has no effect on the insulin promoter activation induced by K^+ or on the constitutive chloramphenicol transferase (CAT) expression from the control vector, pSV-CAT. A potential suggestion, therefore, is that rapamycin affects the biosynthesis of insulin promoter-specific factors required to sustain insulin gene transcription induced by glucose. Alternatively, the inhibition of $p70^{S6K}$, an integral component of the insulin signaling pathway, may interfere with an autocrine effect of secreted insulin to regulate its own transcription in the β-cell (29), but this suggestion is minimized by the lack of effect of rapamycin on K^+-induced activation of –410 Rins-1 (see above). Considering the increased complexity of glucose signaling relative to cell

depolarization (23), it is more likely that rapamycin interferes indirectly with some aspect of transcriptional regulation by glucose. In any case, it is evident that there are at least two distinct pathways arising from glucose metabolism that effect insulin gene transcription: a rapamycin-sensitive pathway and a calcium-dependent (FK506-sensitive) pathway (Fig. 7). The rapamycin-sensitive pathway, which provides factors that are responsible for determining the full effect of glucose-stimulated insulin gene transcription, requires the glucose-induced calcium-dependent pathway.

A more extensive interaction of *trans*-acting factors on -410 Rins-1, relative to K^+, may account for the lack of effect of 2NFATm mutation on glucose-induced activation of the insulin promoter. Potentially, this site may have greater significance in the context of the action of incretins, which in the case of GLP-1, up-regulate insulin gene transcription to a higher level than induced by glucose alone (40). Divergent heterologous partnering between transcription factors interacting among the distinctly arranged NFAT sites may account for differences in response to a complex combination of integrated signals to which the β-cell is exposed. It is therefore hypothesized that the unaltered NFAT sites may be more important to glucose signaling and may form the primary targets of FK506 under these conditions.

Curiously, of the two NFAT sites that are conserved among mammalian insulin promoters, the site most proximal to the transcriptional start site is in close proximity to the A2 binding for the homeodomain protein PDX1 that is acutely activated by glucose (41,42). This site also overlaps with a CEB binding site for C/EBPβ, a known repressor

Figure 7. Schematic of the activation of the insulin gene promoter by glucose. High glucose (11mM) activates the insulin gene transcription by at least two distinct pathways. The calcium-dependent pathway involves calcineurin and NFAT, whereas the rapamycin-sensitive pathway involves factors derived or activated by glucose metabolism which target the insulin gene promoter.


Glc
K+
Ca2+
Cn
FK506
RAP
NFAT
Insulin Promoter

of insulin gene transcription but only in conditions of persisting hyperglycemia (45). Although deciphering the significance of this NFAT site may prove challenging, *trans*-acting factors to these sites may represent intricate mechanisms by which the β-cell fine-tunes the activity of the insulin gene promoter in response to various signals. Extensive studies in immune-system cells have shown that NFAT commonly, but not always, binds to DNA in concert with a partner, e.g. AP-1, from the bZIP family of transcription factors (12,43). A full understanding of how NFAT regulates the -410 Rins-1 promoter in the β-cell thus hinges on the identification of other factors with which it cooperates and the DNA sequences with which they interact.

In summary, this study has demonstrated that calcineurin regulates insulin gene transcription via a mechanism involving NFAT interaction with specific elements within the insulin promoter. It is suggested that the disruption of this pathway *in vivo* under chronic FK506 treatment contributes to the diabetogenic effect of immunosuppressant therapy involving FK506. It is worth noting that immunosuppressant therapies involving low dose FK506 treatment result in long-term survival of islet transplants (44). The further study of this mechanism is necessary to permit the development of new pharmacological approaches that clinically prevent tissue rejection with reduced risk of post transplant diabetes.

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CHAPTER IV

NFAT REGULATES INSULIN GENE PROMOTER ACTIVITY IN RESPONSE TO SYNERGISTIC PATHWAYS INDUCED BY GLUCOSE AND GLP-1

PREFACE

Based on its unique properties in lowering post-prandial hyperglycemia, glucagon-like peptide-1 (GLP-1) has recently been posed as an excellent candidate for treating diabetes. Produced by L-cells of the intestine, GLP-1 stimulates glucose-dependent insulin secretion and insulin biosynthesis (1). GLP-1 also inhibits glucagon secretion and gastric emptying. In addition, it has been shown that GLP-1 reduces caloric intake by producing a satiety effect in rats and humans (2,3). Because weight management is one of the foremost treatments for type II diabetes, this provides yet an additional means by which GLP-1 reduces adverse effects of diabetes. Unlike most pharmacological insulin-secreting agents, GLP-1 retains its biological actions in subjects many years after the clinical onset of diabetes (4). Despite these promising effects, GLP-1 has a relatively short biological half-life. Regardless of the method of administration, GLP-1 plasma concentrations typically return to base-line levels within 2 hours (5). Nevertheless, type II diabetes patients continuously treated with GLP-1 show normalized blood glucose levels (6,7). Considerably large efforts have been placed on creating GLP-1 analogs, which sustain biological activity (8).

In order to develop effective treatments for diabetes mellitus, it is important to examine β-cell function under physiological conditions. The production of insulin by β-cells is regulated by a complex scheme involving interplay between circulating fuels and hormones. Thus, it is necessary to understand signaling mechanisms by which insulin gene transcription is regulated in response to physiological demand. Glucose is considered the main regulator of insulin production and release, and GLP-1 enhances these effects. Moreover, it has been recently found that elevated glucose metabolism and the hormonal action of glucagon-like peptide-1 (GLP-1) both regulate insulin gene promoter activity in pancreatic β-cells (9). In the following study, evidence is provided to support an important role of NFAT in the regulation of insulin gene transcription via the combined action of glucose and GLP-1. The converging signaling pathways appear to be dependent on calcineurin and protein kinase A (PKA).

SUMMARY

Currently there is intense interest to define the mechanism of action of glucagon-like peptide-1 (GLP-1) to regulate β-cell function including insulin gene transcription. In this study, GLP-1 (100 nmol/l), in the presence of glucose (11mmol/l), induced a ~71-fold increase in insulin gene promoter activity in INS-1 pancreatic β-cells, an effect that was an order of magnitude larger than either stimulant alone. The response of GLP-1 was mimicked by forskolin and largely inhibited by the protein kinase A (PKA) inhibitors, H-89 and myristoylated PKI (14-22) amide, indicating partial mediation via a cAMP/PKA pathway. Significantly, the action of both GLP-1 and forskolin were abolished by the selective Ca^{2+}/calmodulin-dependent phosphatase 2B (calcineurin) inhibitor, FK506, as well as by the chelation of intracellular Ca^{2+} by BAPTA. Glucose and GLP-1 also synergistically activated NFAT-mediated transcription from a minimal promoter construct containing tandem NFAT consensus sequences. Furthermore, two-point base pair mutations in any of the three identified NFAT sites within the rat insulin I promoter resulted in a significant reduction in the combined effect of glucose and GLP-1. These data suggest that the synergistic action of glucose and GLP-1 to promote insulin gene transcription is mediated through NFAT via PKA- and calcineurin-dependent pathways in pancreatic β-cells.

INTRODUCTION

The rate of insulin gene transcription in pancreatic β-cells is regulated by a complex integration of signals derived from nutrients, hormones, and neurotransmitters (10-14). Intracellular calcium ($[Ca^{2+}]_i$) appears to be an important mediator of this process (12,15-19). We have previously identified NFAT as a key regulator of insulin gene transcription in pancreatic β-cells that is activated by the calcium/calmodulin-dependent protein phosphatase 2B (calcineurin) in response to increased $[Ca^{2+}]_i$ (19). NFAT binds to three distinct NFAT elements within the rat I insulin promoter and activates insulin gene transcription. Two signaling pathways arising from glucose metabolism converge to activate NFAT-mediated insulin gene transcription. One pathway results from the direct effect of increased $[Ca^{2+}]_i$, which activates calcineurin, and in turn, it up-regulates insulin gene transcription via NFAT. Glucose metabolism activates this pathway by means of increasing $[Ca^{2+}]_i$ via L-type voltage-dependent calcium channels (VDCCs) by affecting the electrical activity of the cell. The second pathway also involves glucose metabolism, but appears to be driven by glucose-derived factors which target insulin gene transcription independently of $[Ca^{2+}]_i$.

Glucose metabolism is central to the regulation of β-cell function (20), and GLP-1 is a potent hormonal incretin, which has been shown markedly to enhance the stimulatory effects of glucose on β-cells (21,22). For example, GLP-1 potentiates glucose-induced insulin exocytosis. It also increases insulin production by enhancing glucose-induced

insulin gene expression. Both glucose and GLP-1 stimulate insulin promoter activity, stabilize proinsulin mRNA, and increase proinsulin biosynthesis (4). It has also been demonstrated that GLP-1 is capable of restoring glucose-responsiveness to islets and β-cell lines, which have become insensitive to glucose (23,24). Moreover, it has been found that GLP-1 promotes β-cell proliferation and differentiation (25,26). In parallel to these observations, it has been shown in the insulin-producing INS-1 β-cell line that GLP-1 synergizes with glucose to activate expression of immediate-early response genes (IEG) coding for transcription factors implicated in cell proliferation and differentiation, such as c-fos, c-jun, junB, zif-268, and nur-77 (14,27).

GLP-1 displays pleiotropic effects on the β-cell that are correspondingly supported via the activation of multiple intracellular pathways. The principal consequence of GLP-1 action on the β-cell is to elevate cAMP, although GLP-1 also triggers phosphoinositide 3-kinase (PI 3-Kinase) (28). Mechanistically, it is known that GLP-1 increases cAMP in pancreatic β-cells by its action on the Gs-coupled GLP-1-R receptor, which in turn, activates cAMP-dependent protein kinase A (PKA) (29). Cyclic AMP and PKA have been found to augment glucose-induced intracellular Ca^{2+}-signaling in β-cells by modulating VDCCs (30-33). Moreover, it has been shown that cAMP has a direct effect on releasing Ca^{2+} from intracellular stores in β-cells as well (34-36). Thus, cAMP is an important regulator of PKA and calcium metabolism in β-cells, and it appears that PKA and calcium contribute to the incretin effect of GLP-1 on β-cells in the presence of glucose (31).

It was hypothesized that glucose and GLP-1 provide factors, which synergistically enhance insulin promoter activity via NFAT. This was based on the observation that the calcium-responsive 2NFAT element within the rat I insulin promoter is insensitive to glucose or GLP-1 alone, but in contrast, is responsive to the co-stimulatory effect of these secretagogues in INS-1 β-cells. In the current study, we identify contributing signaling pathways derived by glucose and GLP-1, which play a role in activating NFAT-mediated insulin gene transcription, and determine the relative contribution of each of the three NFAT elements within the rat I insulin promoter in producing this response.

EXPERIMENTAL PROCEDURES

Materials

INS-1 cells were obtained from Dr. Mark Prentki (University of Montreal, the Centre de Recherches du CHUM and Institut du Cancer, Quebec, Canada). FK506 was purchased from Calbiochem, CA. The NFAT promoter-reporter plasmid (NFAT-Luc) was a generous gift of Dr. Gerald Crabtree, CA.

Plasmids and Mutagenesis

Mutagenesis of three NFAT elements identified within the first 410 bp of the rat I insulin gene promoter (1NFAT, 2NFAT, and 3NFAT) (see Fig.1) was performed by site-directed mutagenesis as previously described (19). In brief, the NFAT core sequence of consensus motif was disrupted by altering two base-pairs (5'-GGAAA to 5'-TCAAA) and cloned into the pGL2-Basic mammalian expression vector (Promega, WI) to create pGL2-1NFATm, pGL2-2NFATm, and pGL2-3NFATm.

Cell Transfections and Reporter Assays

INS-1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine, 2-mercaptoethanol, streptomycin (100 μg/ml) and penicillin (100 U/ml) at 37 °C under an atmosphere of 95% air/ 5% CO_2.

Figure 1. NFAT elements within the rat I insulin gene promoter. Relevant position of NFAT elements with respect to other known cis-acting elements (in boxes) within the –410 rat I insulin gene promoter are shown. The three NFAT elements (1-3NFAT) are indicated above the promoter along with the respective NFAT DNA-binding sequences. The underlined base pairs indicate targeted mutations within the NFAT consensus motifs, which were used in the promoter-reporter assays to determine the relative functional contribution of each NFAT element to the rInsI promoter in response to glucose and GLP-1.


3NFAT
TGGAAAATG
2NFAT
AGGAAAAAG
1NFAT
TGGAATTA
Core
E2
A4/3
CRE
A2/C1
E1
A1
G1

The cells were grown to 60-80% confluency in 12-well plates (Falcon) in RPMI 1640 medium, and then brought to 2 mmol/l glucose for 6 h prior to transfection. INS-1 cell transfection was achieved using FuGene-6 (Roche Molecular Biochemicals, IN) according to the manufacturers directions. All cells were co-transfected with a control vector (pSV-CAT) for the normalization of transfection efficiency. Eighteen hours after transfection, the cells were stimulated with 100 nmol/l GLP-1 or 10 mmol/l forskolin in either basal (2 mmol/l) or stimulatory (11 mmol/l) glucose. In experiments involving pharmacological treatments, inhibitors (FK506, 10 μmol/l; H-89 1-50 μmol/l; myristoylated PKI (14-22) amide, 1-50 μmol/l; verapamil, 10-100 μmol/l; BAPTA, 10-100 μmol/l) were added to the media 2 h prior to cell stimulation. The cells were harvested 24 h after transfection (6 h after stimulation) by lysis in Reporter Lysis Buffer (Promega, WI). Following brief centrifugation (~16,000 xg, 5 min) to remove cell debris, the supernatant was assayed for luciferase activity with the Luciferase Assay System (Promega, WI) using a TD-20/20 bioluminometer (Turner Designs) or chloramphenicol acetyltransferase (CAT) expression by the CAT-ELISA method (Roche Molecular Biochemicals, IN). Data expressed on the ordinate of figures represent arbitrary light units normalized against CAT expression determined in the same samples.

Statistical Analyses

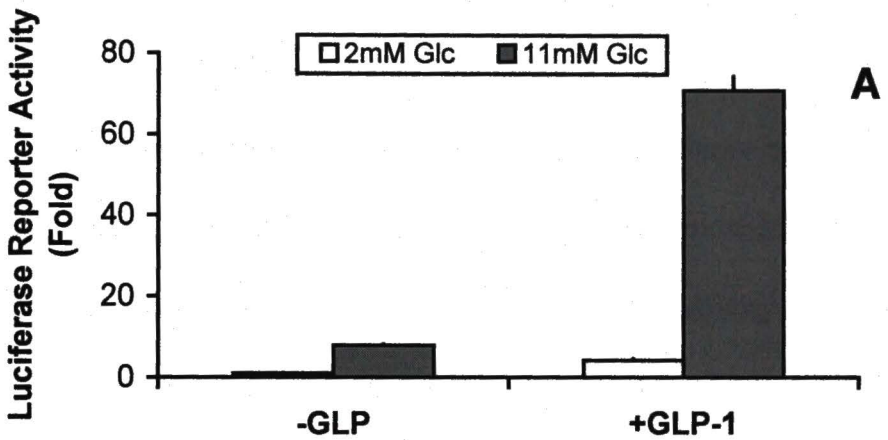
Results are expressed as means ± SEM determined from at least three independent experiments, unless otherwise stated. Statistical significance was calculated by one-tailed unpaired Student's *t*-test.

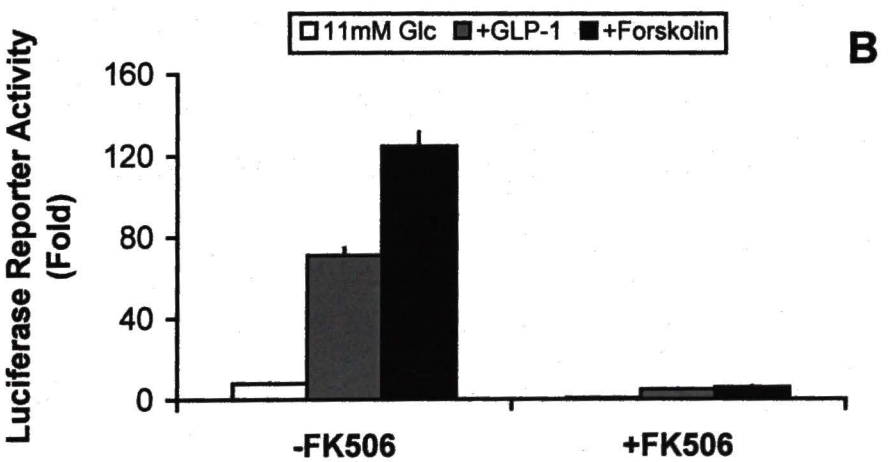
RESULTS

GLP-1 synergistically enhances the effect of glucose on insulin gene transcription

Using the experimental regimen of reducing the glucose concentration of RPMI incubation medium to 2 mmol/l for 18 h prior to INS-1 cell stimulation, the re-establishment of 11 mmol/l glucose (for 6 h) induced a 7.8-fold stimulation of insulin gene promoter-reporter activity (PGL2-rInsI) (Fig. 2A, see also reference 19). Exposure to 100 nmol/l GLP-1 in the presence of 2 mmol/l glucose induced a significant, but smaller (4.1 ± 0.5 fold), increase in insulin promoter activity. When added together, GLP-1 in the presence of stimulatory concentrations of glucose promoted a 71.1 ± 3.5 fold activation of insulin gene promoter activity. This combination thus induced an effect of at least an order of magnitude greater than either agent alone, revealing a marked synergistic interaction of glucose and GLP-1 at the level of insulin gene transcription. This observation emphasizes the previously reported ability of GLP-1 to act as a glucose competence factor (23). The dramatic influence of the combined action of glucose and GLP-1 on insulin gene transcription (appreciably larger than previous reports) is reasoned to be the consequence of the optimization of the cell incubation protocol, which incorporates a specified period (18 h) of cell incubation in a medium containing low (2 mmol/l) glucose. Presumably, this allows cultured pancreatic β-cells to recuperate from long exposure to media supplemented with stimulatory concentrations of glucose.

Figure 2. GLP-1 synergistically enhances the effect of glucose on insulin gene transcription and is dependent on calcineurin activity. A: INS-1 cells were transfected with pGL2-rInsI and then incubated in basal (2 mmol/l glucose) or stimulatory conditions of high glucose (11 mmol/l), GLP-1 (100 nmol/l), or both for 6 h. B: FK506 (10 μmol/l), selective calcineurin inhibitor, blocks the synergistic action of high glucose and GLP-1 (or 10 μmol/l forskolin). Data are expressed as a fold-increase in luciferase activity (normalized to CAT activity) over controls in the presence of 2 mmol/l glucose.

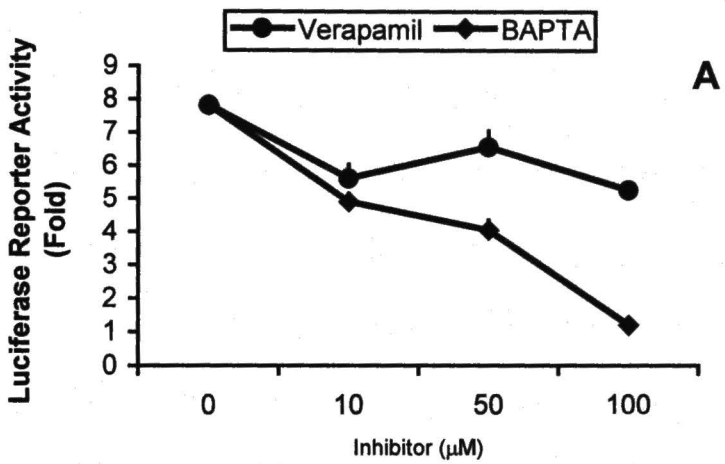

Luciferase Reporter Activity (Fold)
2mM Glc
11mM Glc
A
80
60
40
20
0
-GLP
+GLP-1

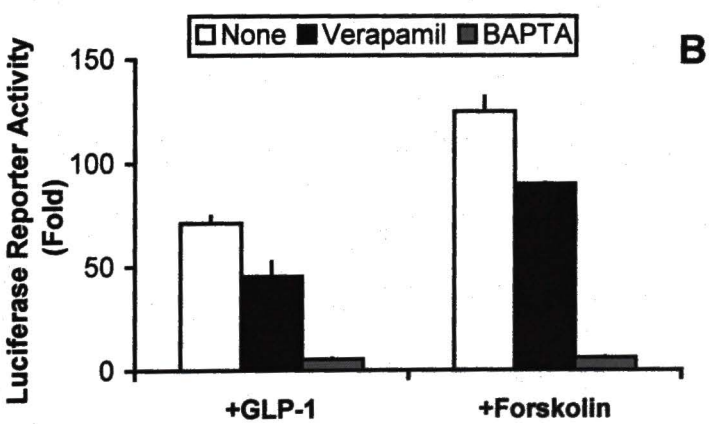

Luciferase Reporter Activity (Fold)
11mM Glc
+GLP-1
+Forskolin
B
160
120
80
40
0
-FK506
+FK506

GLP-1 mediates insulin gene transcription via cAMP, which requires a Ca^{2+}-dependent pathway involving calcineurin

As mentioned, GLP-1-R receptors on the β-cell are coupled to adenylate cyclase via heterotrimeric G proteins, and many of its effects, but not all, are mediated by the generation of cAMP. Not surprisingly, the direct activation of adenylate cyclase, using forskolin, had effects similar to GLP-1 on insulin gene promoter activity in the current experimental model. Specifically, forskolin (10 μmol/l) induced a 6.0 ± 0.2 fold and 124.8 ± 7.0 fold activation of insulin promoter-reporter activity in the presence of 11 mmol/l glucose, respectively (Fig. 2B), suggesting that the effects of GLP-1 were largely attributable to the elevation of cAMP. Novel to this study, each of these responses were profoundly suppressed by the incubation of INS-1 cells with the calcineurin inhibitor, FK-506 (10 μmol/l), indicating that the signaling required for GLP-1 (and forskolin) induction of insulin gene transcription is dependent on the activation of calcineurin.Consistent with this implied involvement of calcineurin, the activation of the insulin gene promoter activity by glucose and GLP-1 was found to be dependent on cytosolic $[Ca^{2+}]_i$. Incubation of INS-1 cells with 100 μmol/l BAPTA, an intracellular Ca^{2+}chelator, prevented insulin promoter activity induced by either glucose (Fig. 3A) or glucose/GLP-1 (Fig. 3B). Verapamil, a selective L-type, voltage-dependent Ca^{2+} channel-blocker, also inhibited glucose/GLP-1-induced activation of insulin promoter activity, although the effects of this compound were only partial (~36%) at a maximal concentration of 100μmol/l. Similar effects of these Ca^{2+} metabolism antagonists were observed when cells were exposed to forskolin in the presence of

Figure 3. Calcium is required for the activation of insulin gene transcription by glucose and GLP-1. INS-1 cells were transfected with pGL2-rInsI and treated with calcium inhibitors verapamil or BAPTA, 2 h prior to stimulation. The cells were stimulated for 6 h with A: high glucose (11 mmol/l) or B: both high glucose and 100 nmol/l GLP-1 (or forskolin). Data are expressed as a fold-increase in luciferase activity (normalized to CAT activity) over controls in the presence of 2 mmol/l glucose.


Verapamil
BAPTA
A
Luciferase Reporter Activity (Fold)
9
8
7
6
5
4
3
2
1
0
0
10
50
100
Inhibitor (μM)


None
Verapamil
BAPTA
B
Luciferase Reporter Activity (Fold)
150
100
50
0
+GLP-1
+Forskolin

glucose. These data indicate that calcium is a requirement for the induction of insulin gene transcription by GLP-1, but further suggest that the elevation of $[Ca^{2+}]_i$ is derived by means other than via voltage-dependent Ca^{2+} influx through L-type Ca^{2+} channels.

Co-stimulatory effect of glucose and GLP-1 on insulin gene transcription is dependent on PKA

The previous experiments provided evidence that GLP-1 enhancement of cAMP-mediated insulin gene transcription was dependent on calcineurin. In order to address signaling via the cAMP-activated protein kinase A (PKA), the effect of two selective PKA inhibitors (H-89 and PKI) on the activation of the insulin promoter by GLP-1 was assessed. Both H-89 and PKI dose-dependently decreased insulin promoter activity (IC_{50} <5 μmol), reaching a maximal inhibition of approximately 80% at 10 μmol/l in both cases (Fig. 4A). A similar dose-responsive, but incomplete, effect of H-89 and PKI was observed for forskolin-induced insulin promoter activity (Fig 4B). These data support a conclusion that PKA plays a crucial role in GLP-1-mediated up-regulation of insulin gene promoter activity, although they also indicate that the activation of this enzyme does not fully account for the synergistic action of GLP-1 or forskolin.

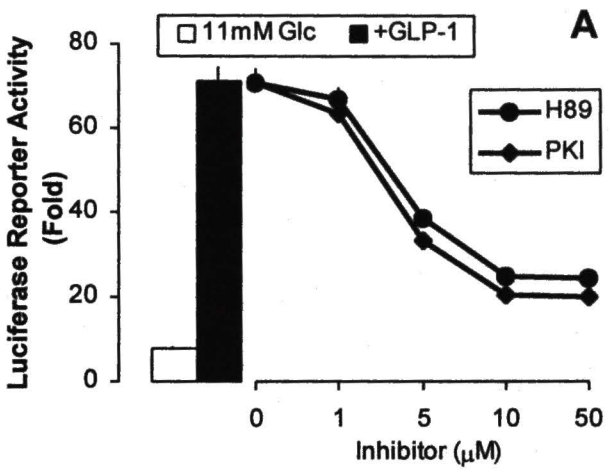
GLP-1 synergistically enhances the effect of glucose on NFAT-mediated gene transcription in β-cells

We have previously shown that calcineurin can activate NFAT in β-cells and that NFAT binds to specific regions on the insulin gene promoter. In order to address a

potential involvement of a similar pathway in the action of GLP-1, INS-1 cells were transfected with a NFAT-reporter construct (NFAT-Luc) in which multiple NFAT-consensus sites were inserted upstream to a minimal promoter (IL-2). Just as in the case of the full length rat I insulin promoter, the co-stimulatory effect of glucose and GLP-1 on NFAT-reporter activity was greater (13.9 ± 0.5 fold) than the additive effect of glucose (2.7 ± 0.2 fold) and GLP-1 (4.1 ± 0.2 fold) alone (Fig 5A). These data demonstrate the ability of GLP-1 to activate NFAT, via calcineurin, but further reveal that synergy between glucose and GLP-1 is achieved in part via the activation of this transcription factor. It was also noted, in contrast to the insulin promoter (Fig 2), that GLP-1 alone had a greater effect on NFAT-reporter activity than high glucose (11 mmol/l), although the significance of this observation is not fully understood.

To determine if the activation of NFAT-mediated transcription in β-cells was mediated by the same signaling mechanisms which activate the full length rat I insulin promoter, we examined the ability of inhibitors of calcineurin, Ca^{2+} metabolism, and PKA to influence NFAT-Luc-transfected INS-1 cells in the presence of 11 mmol/l glucose and 100 nmol/l GLP-1 (or 10 μmol/l forskolin). Each of the inhibitors, H-89, PKI, FK506, or BAPTA, at the maximally effective concentration, completely blocked NFAT-mediated transcription induced by glucose/GLP-1 and glucose/forskolin combinations (Fig 5B). This contrasted with the full-length insulin promoter, which still produced a minimal amount of transcriptional activity in the presence of H-89 and PKI

Figure 4. The synergistic actions of glucose and GLP-1 on insulin gene promoter activity are PKA-dependent. Promoter activity of pGL2-rInsI-transfected INS-1 cells in response to the co-stimulatory effect of high glucose and A: 100nmol/l GLP-1 or B: 10 μmol/l forskolin was ablated by increasing concentrations of selective PKA inhibitors, H-89 (1-50 μmol/l) and myristoylated PKI (1-50 μmol/l). Data are expressed as a fold-increase in luciferase activity (normalized to CAT activity) over controls in the presence of 2 mmol/l glucose.


A
11mM Glc
+GLP-1
H89
PKI
Luciferase Reporter Activity (Fold)
80
60
40
20
0
0
1
5
10
50
Inhibitor (μM)

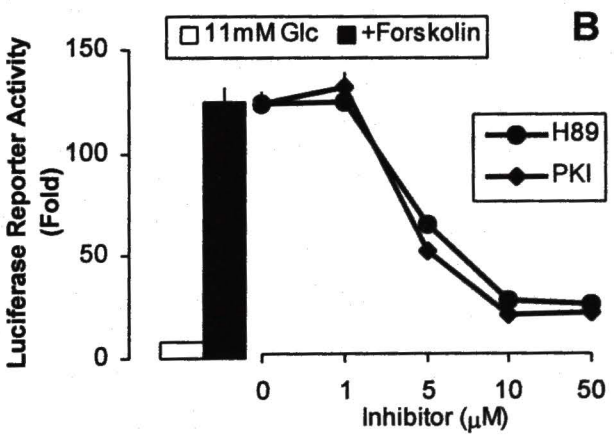
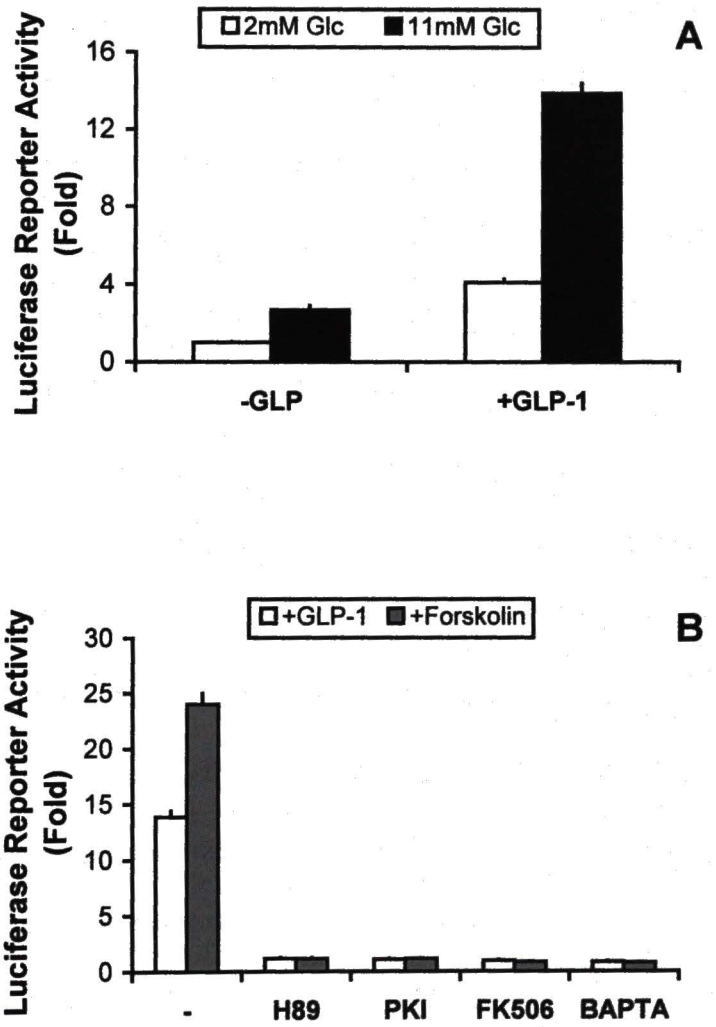

B
11mM Glc
+Forskolin
H89
PKI
Luciferase Reporter Activity (Fold)
150
100
50
0
0
1
5
10
50
Inhibitor (μM)

Figure 5. Glucose and GLP-1 synergistically activate NFAT-mediated gene transcription by mechanisms involving PKA, calcineurin, and calcium. A: INS-1 cells were transfected with NFAT-Luc and then incubated in basal (2 mmol/l glucose) or stimulatory conditions of high glucose (11 mmol/l), GLP-1 (100 nmol/l), or both. B: Inhibitors, H-89, PKI, FK506 (10 μmol/l) or BAPTA (100 μmol/l), were added for 2 h, prior to co-stimulation of INS-1 cells with 11 mmol/l glucose and 100 nmol/l GLP-1 (or 10 μmol/l forskolin). Each of the inhibitors completely blocked NFAT promoter-reporter activity in response to combined glucose and GLP-1 stimulation (or forskolin). Data are expressed as a fold-increase in luciferase activity (normalized to CAT activity) over controls in the presence of 2 mmol/l glucose.


2mM Glc
11mM Glc
A
Luciferase Reporter Activity (Fold)
16
12
8
4
0
-GLP
+GLP-1
+GLP-1
+Forskolin
B
30
25
20
15
10
5
0
-
H89
PKI
FK506
BAPTA

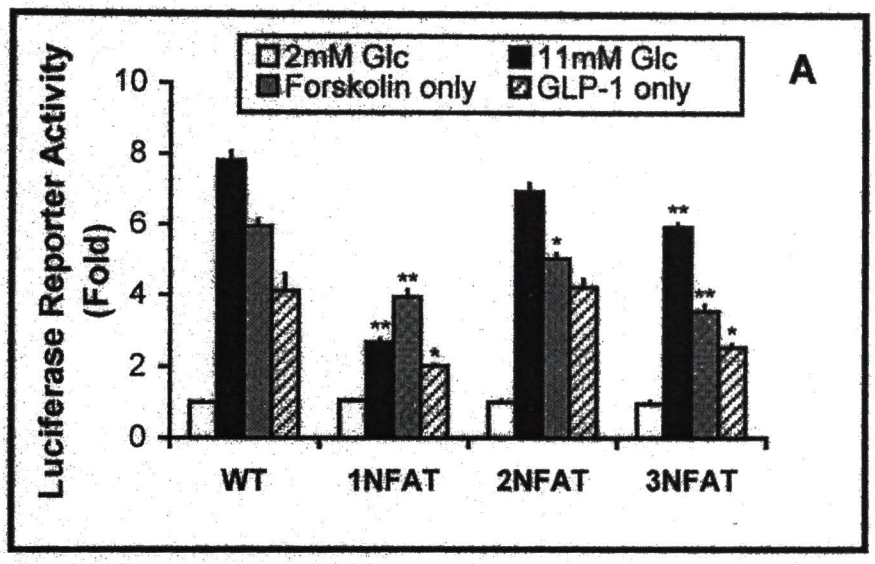
when co-stimulated with glucose/GLP-1 (Fig. 4). These results demonstrate that PKAand calcineurin are required for NFAT-mediated gene transcription in β-cells under these conditions.

Effects of NFAT consensus-site mutations on the insulin gene promoter

It is significant that each of the NFAT binding site consensus sequences are spatially conserved and distinctly arranged among conserved *cis*-acting elements within 410 b.p. of the 5' flanking promoter regions of insulin genes derived from rats, mice, dogs, and humans (with the exception of the 2NFAT element which is conserved only among rats and mice). To determine the relative contribution of each of the NFAT elements in their specific response to glucose or GLP-1, each of the NFAT consensus sites within the rat I insulin promoter were mutated by site-directed mutagenesis. A mutation in the NFAT element most proximal to the start site of transcription (1NFAT) significantly reduced the responsiveness of the insulin gene promoter to glucose (~66%) or GLP-1 (~51%) (Fig. 6A; $p< 0.01$ in either case). In contrast, a mutated 2NFAT element had little effect (~11% reduction) on glucose-induced insulin promoter activity, and had no significant effect ($p> 0.01$) on the induction of insulin promoter activity by GLP-1 (Fig. 6A). A mutated 3NFAT element produced a significant ($p< 0.01$) reduction in both glucose (~24%) and GLP-1 (~38%) induced promoter activity, although not to the extent of the mutated 1NFAT element (Fig. 6A). These data indicate that the NFAT sites within the insulin gene promoter are differentially responsive to glucose and GLP-1. 1NFAT appears to be most important in the activation of insulin promoter activity in

response to either glucose or GLP-1 when added independently. The relative contributions of the NFAT elements became increasingly apparent when the activities of mutant promoters were observed under conditions of co-stimulation by glucose and GLP-1. A mutation in 1NFAT element resulted in a dramatic loss (~71%) in the responsiveness of the insulin gene promoter to glucose/GLP-1 (Fig. 6B). The 2NFAT- and 3NFAT- mutated promoters were also significantly ($p<0.05$) reduced in response to glucose and GLP-1, but to a lesser extent (Fig. 6B). The replacement of GLP-1 with forskolin resulted in a similar trend (Fig. 6B). These data show that the synergistic activation of the insulin gene promoter in response to glucose and GLP-1 is largely dependent upon intact NFAT elements. They also indicate that the 1NFAT element is critically responsive to both glucose and GLP-1 and that 3NFAT is moderately responsive to these stimulants. In contrast, the 2NFAT element was somewhat responsive under conditions of combined effect of glucose and GLP-1, but relatively insensitive to glucose or GLP-1 alone.

Figure 6. NFAT elements within the rat I insulin gene promoter are differentially responsive to glucose and GLP-1 stimulation. INS-1 cells were transfected with pGL2 reporter vectors harboring rat I insulin gene promoters containing NFAT element site-directed mutations and then incubated in basal (2 mmol/l glucose) or stimulatory conditions. A: Transfected cells were stimulated with high glucose (11 mmol/l), 100 nmol/l GLP-1, or 10 μmol/l forskolin. Statistical significance of the data was evaluated by unpaired Student's t-test: *$P < 0.01$, **$P < 0.001$. B: The cells were either treated with high glucose (11 mmol/l) or co-stimulated with high glucose (11 mmol/l) and 100 nmol/l GLP-1 (or 10 mmol/l forskolin). Data are expressed as a fold-increase in luciferase activity (normalized to CAT activity) over the wild type pGL2-rInsI (WT) in the presence of 2 mmol/l glucose. Statistical significance of the data was evaluated by unpaired Student's t-test: *$P < 0.05$, **$P < 0.001$. The statistical significance was based on comparisons of the mutant rInsI promoters (1-3NFAT) to the wild type rInsI promoter (WT) with respective condtions.


2mM Glc
11mM Glc
Forskolin only
GLP-1 only
A
Luciferase Reporter Activity (Fold)
10
8
6
4
2
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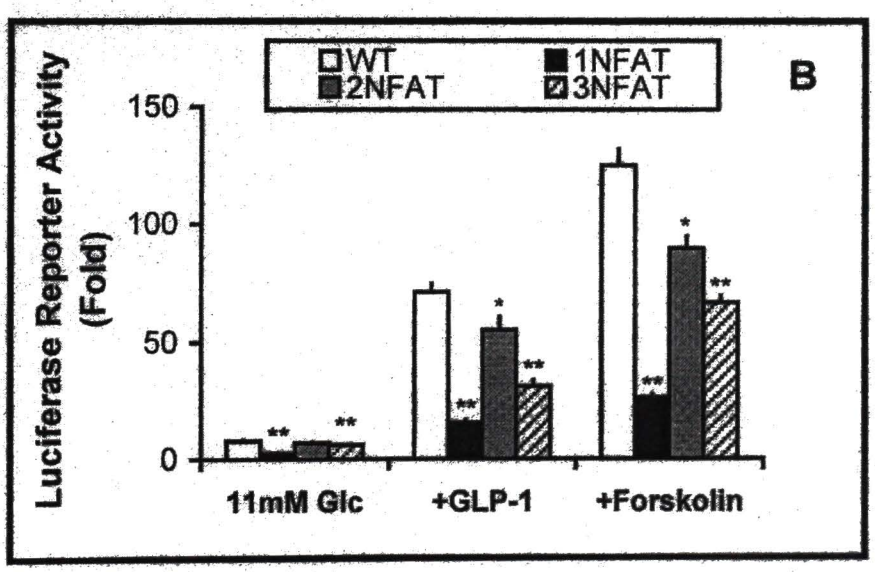
WT
1NFAT
2NFAT
3NFAT


WT
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2NFAT
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B
Luciferase Reporter Activity (Fold)
150
100
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0
11mM Glc
+GLP-1
+Forskolin

DISCUSSION

Glucose is an essential regulator of insulin gene expression from β-cells, and it is likely that glucose is required during any physiological scenario when increased insulin production is needed (11,20,37-39). In the present study, we identify signaling mechanisms by which GLP-1 synergistically enhances insulin gene transcription in the presence of glucose. Glucose and GLP-1 have similar effects on β-cells arising from distinct and overlapping signaling pathways. For example, glucose elevates intracellular calcium, but also provides factors to enhance insulin gene transcription by pathways distinct of calcium (19,40,41). GLP-1 increases cAMP and activates PKA, both factors which are involved in calcium-dependent and calcium-independent pathways in pancreatic β-cells (42-44). Thus, both glucose-factors and GLP-1-factors exert multiple effects on the β-cell, and the points of convergence, which synergistically up-regulate insulin gene transcription, are not well understood.

The obvious common effect produced by glucose and GLP-1 is the elevation of $[Ca^{2+}]_i$. Calcineurin, the calcium/calmodulin dependent protein phosphatase 2B, has been determined to be a major target of calcium in regulating insulin gene transcription in the β-cell (19,45,46). We have recently identified NFAT as a sequential downstream target of calcineurin in the activation of insulin gene transcription by both glucose and depolarizing concentrations of K^+ (19). The current study further suggests that this pathway is also a conduit for the action of GLP-1 to enhance the up-regulation of insulin

gene expression by glucose. This suggestion is supported by the primary demonstrations that GLP-1-induced activation of insulin gene expression, alone or in combination with glucose, was attenuated by the selective calcineurin inhibitor, FK-506, or by the site-directed mutagenesis of identified NFAT binding sites in the insulin gene promoter. Furthermore, GLP-1 was capable of activating NFAT in a calcineurin-dependent manner based on increased activity of a transfected NFAT-reporter construct. Collectively, these data provide further support for an important role of this transcription factor in the physiological regulation of insulin biosynthesis in the β-cell.

GLP-1 heightens $[Ca^{2+}]_i$ in addition to the effect of glucose on β-cells by the potentiation of glucose-induced closure of K_{ATP} channels and subsequent cell depolarization and also by direct influence via PKA-mediated phosphorylation (32,47). Glucose/GLP-1 induced insulin gene transcription is clearly dependent on $[Ca^{2+}]_i$ based on its elimination in the presence of the intracellular Ca^{2+}chelator, BAPTA. However, the action of glucose and GLP-1 was only partially blocked by verapamil. This indicates that while L-type calcium channels are involved to some degree, they are not the only source of Ca^{2+}. In addition to its action on verapamil-sensitive (L-type) VDCCs, GLP-1 can also stimulate the opening of Ca^{2+}-activated non-selective cation channels (NSCCs) that are permeant to Ca^{2+} as well as Na^+ (48). Moreover, ω-agatoxin-sensitive (P-type) VDCCs have been identified in β-cells, which contribute to glucose-induced insulin exocytosis (49). Furthermore, GLP-1 has been reported to elicit fast transient elevations in $[Ca^{2+}]_i$ via Ca^{2+}-induced Ca^{2+} release through type 2 ryanodine receptors on the endoplasmic reticulum (36). These observations may provide insight to other potential sources of

calcium, which may contribute to glucose/GLP-1-induced insulin gene transcription, although no attempt was made in the current study to directly address the involvement of these specific calcium channels.

The actions of GLP-1 on insulin promoter activity in this study were mimicked closely by forskolin, indicating that they are primarily mediated via the activation of adenylyl cyclase and the generation of cAMP. However, the effect of forskolin on insulin and NFAT promoter activation was inhibited by FK-506 in a manner equal to GLP-1 implies that the effects are also dependent on calcineurin. The effect of GLP-1, at least at the level of the insulin gene promoter, cannot be completely explained by the activation of PKA consistent with a previous report. This latter study concluded that there is a direct effect of cAMP on the insulin gene promoter although the identity of this cAMP receptor was not identified (50). This is contrasted by the complete effectiveness of inhibitors of PKA to prevent GLP-1 and forskolin activation of NFAT. These data suggest that the synergistic action of GLP-1 (or forskolin) and glucose on NFAT is mediated by signaling mechanisms involving both calcineurin and PKA. This could be achieved, for example, at the level of Ca^{2+} signaling via the known effects of PKA to influence Ca^{2+} influx and mobilization via the phosphorylation of L-type Ca^{2+} channels and ryanodine receptor complexes, respectively (47,51). It has been proposed that the synergistic enhancement of increased intracellular calcium by GLP-1 in β-cells is accomplished by PKA-mediated sensitization of type 2 ryanodine receptors to Ca^{2+} invoked by L-type VDCCs, i.e. by Ca^{2+}-induced Ca^{2+} release (CICR) (35,36). Alternatively, another point of convergence could be mediated via the co-localization of

calcineurin and PKA to the plasma membrane via interaction with A-kinase anchoring proteins (AKAPs). Indeed, evidence has recently been provided to suggest that the activation of PKA promotes increased activation of calcineurin by promoting its release into the cytoplasmic compartment of the cell where it would presumably be free to dephosphorylate and activate NFAT. AKAP79 was shown to target PKA to VDCC's in β-cells, and disrupting the targeting function of AKAP prevents both cAMP-mediated elevation of $[Ca^{2+}]_i$ and insulin secretion elicited by GLP-1 (47). Thus, this potentially provides a mechanism whereby GLP-1 facilitates the availability and activation of calcineurin via a mechanism involving the activation of PKA by cAMP.

It is also likely that the interaction of glucose and GLP-1 signaling pathways are mediated by heterologous partnering of critical transcription factors at the level of the insulin gene promoter. Three functional NFAT elements have been identified within the first 410bp of the rat I insulin promoter. Two of these elements (1NFAT and 3NFAT) are conserved among other known insulin gene promoters of other mammals including dogs, mice, and humans. Each NFAT site displays its own distinct responsiveness within the context of the rat I insulin promoter. It is clear that the most proximal NFAT element (1NFAT) to the start site of transcription is most responsive to glucose. The potency of 1NFAT is most likely a result of its embedding within glucose-responsive *cis*-acting elements such as the overlapping A2/C1 binding sites (RIPE3b), which have been reported to bind to the glucose-responsive trans-acting factors, PDX-1, RIPE3b1, and A2-specific factors (52). PDX-1 (formerly STF-1, IDX-1, IPF-1, GSF, IUF-1) is a homeodomain family transcription factor, which has been indicated to bind to the A

elements of the insulin promoter and interact with transcription factors that bind to neighboring E elements (53). Both glucose and GLP-1 are known to increase PDX-1 activity and expression in β-cells (54). More recently, glucose and GLP-1 have been shown to stimulate PDX-1 nuclear translocation in β-cells by a cAMP/PKA-dependent mechanism (55). The glucose-sensitive RIPE3b1-binding complex, expressed strictly in pancreatic β-cells, is known to bind to specific sequences of both the C1 and A2 elements within the RIPE3b region of the rat II insulin gene promoter and interact with the recently identified A2-specific factors (A2.1, A2.2, A2.3) (52). Given the proximity and similarities in glucose-responsiveness, 1NFAT potentially interacts with these elements. In support of this, we have found that NFAT DNA-binding complexes formed with a probe harboring the 1NFAT element display altered banding patterns in electrophoretic mobility shift assays based on the specific conditions and handling of stimulated islets and INS-1 β-cells (data not shown).

The mechanism of NFAT activation is best understood in immune-system cells where NFAT is demonstrated to bind to DNA in complex with members of the basic-region leucine-zipper (bZIP) transcription factors, namely c-fos/c-jun (AP-1) (56,57). Based on these observations, it is of interest to identify bZIP transcription factors that potentially interact with NFAT within the insulin gene promoter. An attractive candidate in this respect is the activating transcription factor-2 (ATF-2 or CRE-BP1) of the bZIP family, which in β-cells can be activated by the Ca^{2+}/calmodulin-dependent kinase IV in response to glucose. The calcium-responsive ATF-2 transcription factor binds to four identified CRE elements of the insulin gene promoter (58). A common theme to the

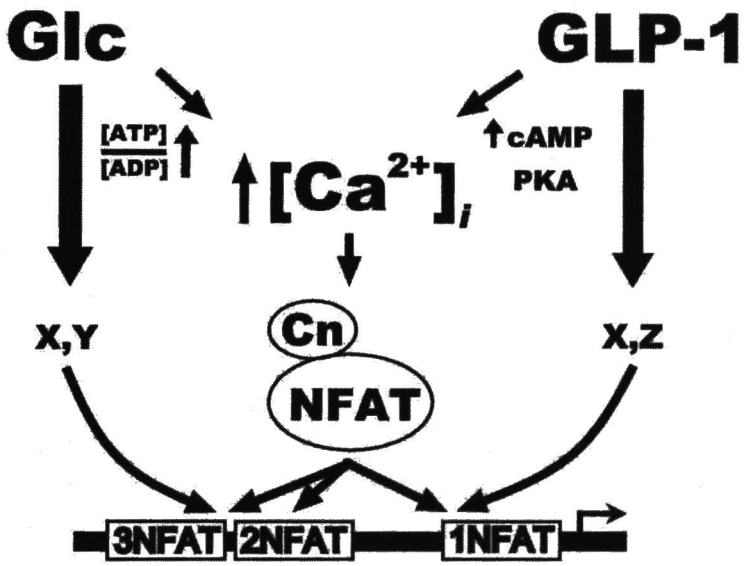
stimulation of cytokine gene transcription by NFAT involves the activation of AP-1 by a lymphokine receptor simultaneously with elevated calcium (56). In a scenario where CaMKIV phosphorylates ATF-2 and calcineurin dephosphorylates NFAT, calcium would suffice to activate both interacting partners in β-cells. Alternatively, both calcineurin and PKA are known to directly activate CREB in pancreatic β-cells (12). Thus, a role of calcineurin and PKA in insulin gene transcription may involve the activation of a bZIP member or other required co-activator to induce the insulin promoter. Furthermore, the 1NFAT element itself contains an inverted and overlapping functional bZIP element (CEB/P) which can act as a repressor during prolonged exposure of β-cells to high glucose conditions (53). A model for heterologous partnering between NFAT elements within the insulin gene promoter and other transcription factors of the β-cell is yet to be determined.

In any case, it is likely that interactions between NFAT and other *cis*-acting elements within the insulin promoter are required to produce a maximal stimulatory response. This co-dependency of given factors likely contributes to the synergistic action of glucose and GLP-1 at the insulin gene promoter, such that the individual effect of each stimulator on insulin promoter activity appears to be insignificant in comparison to the extent of activation observed during co-stimulation. We have recently shown that the 2NFAT element is calcium-responsive and that blocking the calcineurin pathway prevents its activation. However, a mutation of the 2NFAT element does not appreciably affect insulin gene transcription induced by either glucose or GLP-1. This is a unique characteristic of the 2NFAT element with respect to 1NFAT and 3NFAT, which are

responsive to either glucose or GLP-1 alone. In contrast, the co-stimulatory effect of glucose and GLP-1 was significantly ($p<0.05$) blunted by a 2NFAT mutation. These observations indicate synergism at the promoter level. Hence, glucose and GLP-1 contribute factors, which enhance the calcium-responsive NFAT-pathway in addition to the effect that they have on elevating $[Ca^{2+}]_i$ in β-cells. The current study provides further evidence to support this in that the effect of glucose/GLP-1 signaling on isolated NFAT elements in β-cells is completely dependent on PKA, whereas glucose/GLP-1 exerts effects on the full-length promoter (rInsI), which are partially PKA-independent. Furthermore, the fact that the NFAT elements within the insulin gene promoter respond differentially to stimulatory conditions of glucose, GLP-1, or glucose/GLP-1, in itself, indicates that other transcription factors are modifying the effect of NFAT on insulin gene transcription (or vice-versa).

In summary, glucose and GLP-1 synergistically up-regulate the insulin gene promoter by pathways, which converge to enhance NFAT-mediated insulin gene transcription (Fig. 7). This activation requires elevated intracellular calcium, and ultimately the activation of calcineurin, in addition to distinct factors provided by glucose and GLP-1. These factors converge at the level of signal transduction as well as the level of transcription to produce the synergistic effect. The extent to which glucose and GLP-1 synergize to enhance insulin gene transcription supports the notion that combinatory signaling in pancreatic β-cells bears a larger physiological relevance to β-cell function than what is observed by glucose signaling alone.

Figure 7. A simplified schematic of signaling pathways, which synergistically activate the rat I insulin gene promoter via NFAT-mediated transcription. Three major pathways that regulate NFAT-mediated insulin gene transcription are derived by 1) glucose factors 2) GLP-1 factors and 3) elevation of $[Ca^{+2}]i$. The first pathway begins with glucose metabolism. The second pathway is largely dependent on PKA. The third pathway is influenced by both the glucose and GLP-1 pathways and is absolutely required. The combination of all three pathways provides a synergistic effect on insulin gene transcription. The synergistic effect, in part, is regulated at the promoter level. Abbreviations: Glc, glucose; GLP-1, glucagon-like peptide-1; PKA, protein kinase A; Cn, calcineurin. Key: X= common factors which are activated by both glucose and GLP-1; Y= factors that are activated by glucose; Z= factors that are activated by GLP-1.


Glc
GLP-1
[ATP]
[ADP]
[Ca2+]i
cAMP
PKA
X,Y
Cn
NFAT
X,Z
3NFAT
2NFAT
1NFAT

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CHAPTER V

CONCLUSION

SUMMARY

The works described in this dissertation have revealed a novel pathway within pancreatic β-cells, which regulates insulin gene promoter activity in response to glucose and GLP-1. This pathway involves elevated intracellular calcium and the activation of the calcium/calmodulin protein 2B phosphatase (calcineurin) to elicit NFAT-mediated insulin gene transcription. Both glucose and GLP-1 result in increased calcium in β-cells, and also provide distinct factors (signaling molecules, transcription factors, pathways), which converge and synergize with the calcineurin/NFAT pathway (see Fig.7, Chapter 4). One such pathway induced by glucose has been identified in these studies as a rapamycin-sensitive pathway. Another pathway evoked by GLP-1 is largely dependent on protein kinase A (PKA). Each of the distinct pathways provided by glucose and GLP-1 converge at the level of signal transduction as well as the level of gene transcription to produce the synergistic effect.

SIGNIFICANCE

Direct relevance to post-transplant diabetes

The immunosuppressant drug, FK506, which is commonly used to increase the success rate of tissue transplantations, imposes a risk (10-30%) of post-transplant diabetes mellitus among other life-threatening diseases (1,2). The studies presented in this dissertation were the first to define a mechanism whereby the immunosuppressant drug FK506 inhibits the production of insulin (via calcineurin/NFAT) at the level of gene transcription. Determining differences among factors in β-cells versus immune-system cells represents a viable means to developing alternative therapeutic agents for preventing tissue rejection, which are less invasive to β-cells. For example, FK506 has been found to bind differentially to the FK506-binding proteins (FKBP's) and FKBP's have been found to be differentially expressed among tissues (3). Exploiting differences among these factors by various derivatives of FK506 may potentially provide an analogue, which could (within an effective concentration range) effectively target the immune system without the currently observed side-effects. This may also provide an effective treatment to prevent the full progression of type I diabetes in patients who have been diagnosed with pre-symptoms of the disease. Moreover, it remains to be determined, which isoform(s) of NFAT is predominately expressed in pancreatic β-cells to regulate insulin gene transcription. This offers yet another set of factors, in which differences occur among β-cells and immune-system cells which may be used in the development of

enhanced pharmacological agents for improving the success of tissue transplantation and/or the prevention of autoimmune diseases.

Direct relevance to GLP-1/glucose signaling

The work described in the second published article of this dissertation emphasizes the importance of the combined effects of physiological regulators (namely glucose and GLP-1) on insulin gene expression. Glucose has been commonly viewed as the major regulator of insulin biosynthesis and secretion. However, the response of insulin gene transcription to glucose in INS-1 cells appears to be insignificant when compared to the combined effect of glucose and GLP-1. The extent to which glucose and GLP-1 synergize to enhance insulin gene transcription indicates that combinatory signaling in pancreatic β-cells bears a larger physiological relevance to β-cell function than what is observed by glucose signaling alone. While not directly tested herein, such as synergistic relationship is also likely to be important for the primary islet β-cell. Furthermore, these studies provide new insights to the mechanisms of GLP-1 to enhance insulin gene transcription. Understanding the molecular basis of GLP-1 action on β-cells forges the potential development of novel therapeutic approaches to mimicking or improving the promising effects of GLP-1 as an anti-diabetogenic agent.

Relevance to therapeutic approaches to type I and type II diabetes

Remedial approaches to diabetes mellitus based on the molecular mechanisms underlying the physiological behavior of pancreatic β–cells have been viewed in terms of two major aims: to produce an elaborate model which can be utilized to 1) develop a

means to repair/restore β–cell function or 2) engineer β–cells for replacement. The first aim has been somewhat successful, most noticeably in the fact that (due to the discovery of insulin) patients diagnosed with type I diabetes survive much longer than before. However, the life expectancy of those clinically diagnosed with type I diabetes remains significantly shortened. Recent reports from trials indicate that aggressive insulin therapy can significantly suppress the progression of the disease, but the regimen is inconvenient in practice and requires intense discipline (4). GLP-1 treatment of type II patients is also promising. However, the treatment requires continuous infusion of GLP-1 to maintain acceptable blood glucose levels (5). The second aim (engineering β–cells for replacement) is an extension to the currently established methods of allo- and xeno- β-cell transplantation. Material for allo-transplantation is scarce, which amplifies other related imposing problems such as high cost, technical difficulty, and immunity rejection (6). Xeno-transplantations have similar drawbacks as well, and have increased risk for infection by endogenous animal viruses (7).

The results obtained from the current studies contribute information at the level of gene expression to the models required for the previously mentioned major aims to treating diabetes mellitus. Addressing the first aim (to develop a means to repair/restore β–cell function), it has been found that GLP-1 restores glucose-responsiveness to β-cells that have become glucose-insensitive due to prolonged exposure to supraphysiological glucose conditions (8-10). Based on the current study, the activation of NFAT is a key component of GLP-1- and glucose- induced insulin gene expression. Thus, NFAT potentially represents a mechanism whereby GLP-1 elicits its effects by modulating the

expression of insulin and/or genes related to β-cell restoration. In light of the advent of gene delivery methodology for the treatment of diabetes (11-13), NFAT elements combined with modular β-cell-specific elements could provide a useful means to construct vectors, which target and modulate the expression of replacement genes or pharmacological peptides in β-cells. In the current study, it was demonstrated that isolated NFAT elements transfected into β-cells could be activated by glucose, GLP-1, or cell depolarization. Rapamycin could only interfere with this activation when NFAT elements were presented in the context of its natural neighboring cis-acting elements of the insulin gene promoter. Mutation of an NFAT site within the insulin promoter reduced its response to cell depolarization. Therefore, it is conceivable that increasing copies of NFAT elements placed within a strategic combination of modular components would presumably increase the potency of the promoter activity, while the β-cell specific transcription factor components would establish targeted specificity. Indeed, a transgene composed of strategically aligned glucose-responsive elements of the rat L-pyruvate kinase gene inserted into the insulin-sensitive, liver-specific components of the insulin-like growth factor binding protein promoter has been constructed and successfully shown to produce and release insulin from liver cells in response to glucose (13). Transgenes have also recently been used to treat hyperglycemia and diabetes in animal models (11,12). Lastly, there also lies the potential of targeting calcineurin/NFAT genes into β-cells to enhance the effect of NFAT-mediated transcription. The present study has shown that transfecting a constitutively active form of calcineurin into β-cells shifts the glucose-

concentration/response curve of insulin gene transcription to the left. Prospects of gene therapy for diabetes mellitus are reviewed by Efrat (14).

In reference to the second major aim (engineering β–cells for replacement), NFAT should be considered to be a major candidate involved in β-cell/islet development. Although no data have been collected to directly support this view, the observations that GLP-1 can induce β-cell differentiation (15-17) and that GLP-1 is a potent activator of NFAT-mediated gene transcription in β-cells, suggest that NFAT may play a role in islet development. There have been several recent reports showing that pancreatic non-β-cell lines or pluripotent stem cells can be induced to differentiate into pancreatic β-cells (18-23). The problematic issues surrounding this technology center around the ability to produce β-cells that 1) sustain their differentiation (and thus produce insulin), 2) cease their proliferation once transplanted, and 3) are resistant to immune rejection/assault. Transgenes may offer a means to modulate the differentiation of pancreatic cell lines or stem cells designed for transplantation. Utilizing a model of GLP-1 activation of NFAT may be particularly useful in this case to artificially mimic or amplify the effect. For example, a transgene harboring the GLP-1 receptor could increase the sensitivity of engineered β-cells to GLP-1 (24). Ideally, the transgene would be placed under the regulation of an artificially constructed promoter (such as one that contains glucose/NFAT-responsive modules), which in conditions of hyperglycemia would presumably provide feedback to up-regulate the expression of GLP-1, thus maintaining β-cell differentiation. Lastly, transgenes which contribute to increased β-cell immunity can be targeted to pancreatic β-cells developed for replacement therapy, as it has recently been shown that the expression of immunoregulatory genes (early region 3 genes (E3)) cloned from the adenovirus prolongs islet survival and reduces the incidence of type I

diabetes in mice (25-28). Thus, in a scenario as mentioned above, an artificial promoter containing glucose/NFAT-responsive modules to regulate the immunoregulatory E3 genes provides an opportunity to create enhanced artificial β-cells which can survive the autoimmune response. This creates the potential of combined transgene and replacement therapies of autoimmune-related diseases beyond the scope of type I diabetes mellitus. However, it should be mentioned that this would also impose a long-term risk regarding the natural selection or spontaneous transformation of artificial cells that may proliferate and be resistant to immune destruction. A more elaborate description on the genetic engineering of β-cells is reviewed by Soria, et al (6) and Efrat (29).

FUTURE DIRECTION

Determining NFAT as a critical component of the induction of the insulin gene promoter in response to glucose and GLP-1 is a major step toward understanding how signals arising from physiologic regulators converge to amplify the production of insulin in pancreatic β-cells. Although it is clear that the synergy among signals arising from glucose and GLP-1 occurs at both the level of signal transduction and transcription, there are multiple points of convergence that are yet to be determined. For one, it is not clear how glucose metabolism affects insulin promoter activity independently of the calcium-responsive pathway (30-34). Similarly, it is largely unknown how cAMP/PKA activates insulin gene transcription, although it appears that there are multiple sites of action (35-42). Therefore, it is important to identify these pathways in order to determine how they feed into the calcineurin/NFAT pathway.

Regarding cooperative interactions, which occur at the promoter level, it is most important to identify the NFAT isoform(s) responsible for insulin gene transcription. Recently, NFAT isoforms have been detected in β-cells by immunoblotting and RT-PCR (unpublished observations, Easom et al.), but the predominant form responsible for insulin gene transcription is still unclear. It should also be mentioned that other NFAT-dependent promoters are likely present in islets, such as 5'-flanking regions of the amylin and glucagon genes. The amylin gene promoter, which is closely regulated by the same

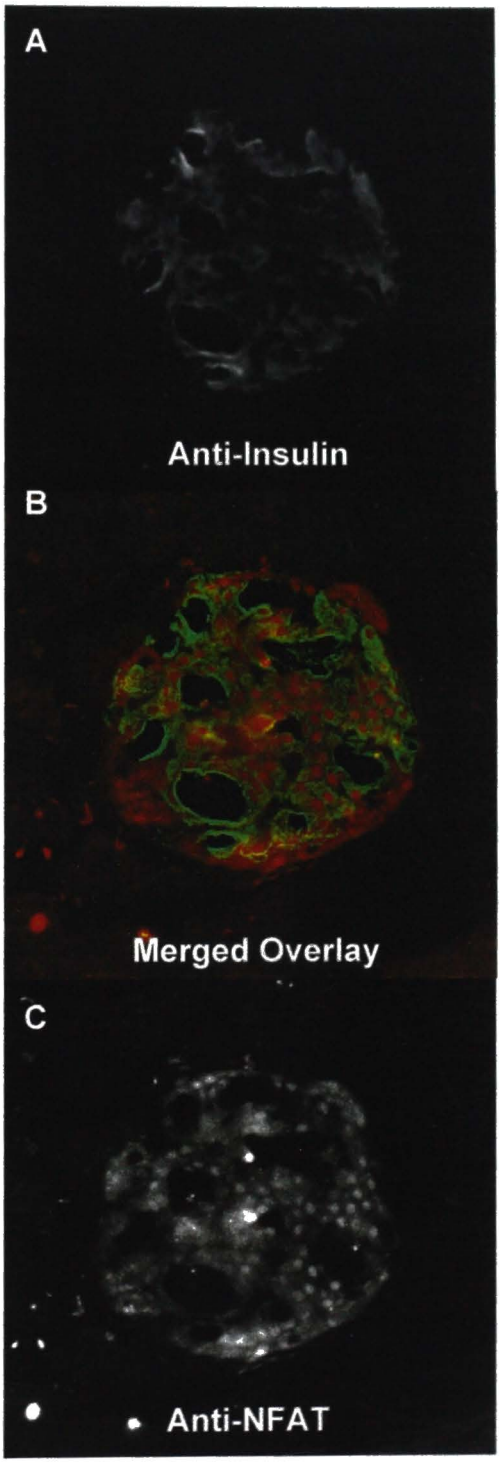
signaling as the insulin gene promoter in β-cells, also contains NFAT-consensus sites, although the functions of these sites have yet to be examined. NFAT motifs exist within the glucagon gene promoter as well. To substantiate one of these putative NFATsites, it was shown that the NFAT element within the G2 region lends calcium-responsiveness to the glucagon promoter. Hence, NFAT is expressed and has multiple functions in islets. Spliced variants of the NFAT family likely represent a mechanism of specificity among cell types and functions. It also cannot be excluded that distinct NFAT isoforms may differentially cooperate with *trans*-acting factors interacting with the insulin gene promoter based upon the particular conditions to which the β-cell is exposed. In the current study it was shown that each NFAT element contributed differentially to the transcriptional response of the insulin gene promoter to both glucose and GLP-1. This suggests that neighboring elements and the spatial orientation of the NFAT elements play a role in the regulatory response as well. Identifying and cloning NFAT isoforms present within β-cells will allow further studies to determine interactions, which occur between NFAT and transcription factors (or co-activators) important not only in insulin gene transcription, but also in activating other genes relevant to NFAT signaling in islets.

Pancreatic sections from male Wistar rats have shown NFAT-immunoreactivity localized specifically within the islets. Interestingly, it was observed that in one pancreatic preparation (in which the rat was allowed to feed), NFAT was clearly localized in the nucleus of the insulin-producing cells (β-cells) (Fig 1). NFAT-immunoreactivity was also detected in the outer region of the islets corresponding to non-insulin producing cells (likely α-cells), although nuclear localization was less apparent.

This method of detection will allow the opportunity to monitor the activation of NFAT in whole animals in response to physiological conditions, and it is also a potential marker to indicate the activation of insulin gene transcription. Further information regarding the phosphorylation status, subcellular localization, and transactivation functions of islet-specific NFAT isoforms in response to physiological stimuli is required.

Future studies to determine the specific isoforms of NFAT within islets and identify specific factors that cooperate with NFAT in the activation of islet-specific genes will expand our knowledge of how a broad range of external and internal signaling networks converge to produce specific transcriptional responses within the endocrine pancreas. This will also involve the characterization of the individual functions of NFAT elements and isoforms alike. Moreover, it will be useful to further explore pathways by which calcineurin and other signaling molecules overlap to produce a unified response in gene transcription. Determining major factors, which direct these signaling pathways in response to physiological stimuli will provide insight to the molecular processes underlying endocrine function. Understanding the functional behavior of the endocrine pancreas at the molecular level within the context of physiological processes will allow further opportunities in developing methods to repair or restore endocrine cells and/or engineer artificial replacement cells to restore endocrine function in patients diagnosed with diabetes mellitus.

Figure 1. Expression and localization of NFAT within the nuclei of islet cells of male Wistar rat pancreas. Immunofluorescent staining of pancreatic sections with either A) anti-insulin or C) anti-NFATp primary antibodies. Secondary antibodies conjugated with Cy-2 (green) and Texas-red fluorescein markers were used for the detection of insulin and NFAT, respectively, by fluorescence microscopy. B) Merged representation of panels A and B showing insulin in the cytosol and NFAT in the nucleus of the β-cells.


A
Anti-Insulin
B
Merged Overlay
C
Anti-NFAT

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