MAPK SIGNALING PATHWAYS IN PANCREATIC β CELLS: THE REGULATION OF RAF ACTIVATION BY NUTRIENT STIMULI

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Dedicated to My Family

MAPK SIGNALING PATHWAYS IN PANCREATIC β CELLS: THE REGULATION OF RAF ACTIVATION BY NUTRIENT STIMULI

By

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MAPK signaling pathways in pancreatic β cells:

The regulation of Raf activation by nutrient stimuli

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Abstract: In pancreatic β cells cells, ERK1 and ERK2 participate in nutrient sensing and their activities rise and fall as a function of glucose concentration over the physiologic range. Glucose metabolism triggers calcium influx and release of calcium from intracellular stores which are required for ERK1/2 activity. Calcium influx also activates the calcium-dependent phosphatase calcineurin, which is required for maximal ERK1/2 activation by glucose. Calcineurin controls insulin gene expression by ERK1/2-dependent and -independent mechanisms. This study showed that in β cells, glucose activates the ERK1/2 cascade primarily through B-Raf. Glucose also enhances dimerization of B-Raf with C-Raf. Furthermore, calcineurin up-regulates B-Raf

activity and stabilizes C-Raf/B-Raf in response to glucose. Calcineurin binds to B-Raf in both unstimulated and stimulated cells. B-Raf phospho-T401 is one of the target sites that can be dephosphorylated by calcineurin. This study reveals that cross-talk between Raf and calcineurin is essential for the maximal activation of ERK1/2 in the glucose signaling pathways.

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LIST OF ABBREVIATIONS

Akt/PKB	Protein Kinase B
CaMK	calcium/calmodulin-dependent kinase
CnA	calcineurin
CNK	connector enhancer of KSR
CPH	carboxypeptidase H
CREB	cAMP response element-binding
EGF	epidermal growth factor
ERK1/2	extracellular signal-regulated protein kinasee 1/2
GLUT2	glucose transporter 2
GSIS	glucose-stimulated insulin secretion
GSK3	glycogen synthase kinase 3β
IGF	insulin-like growth factor
KSR	kinase suppressor of Ras
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein-ERK kinase
mTOR	mammalian target of rapamycin
Neuro D1	neurogenic differentiation 1
NFAT	nuclear factor of activated T cells
MafA	V-maf musculoaponeurotic fibrosarcoma oncogene homologue A
Pax	paired box protein
PC1 and PC2	proprotein convertases 1 and 2

PDX-1	pancreatic and duodenal homeobox factor-1
PKA	cAMP-dependent protein kinase, protein kinase A
PKC	protein kinase C
PP2A	protein phosphatase 2A
RKIP	Raf kinase inhibitor protein
S6K	p70 S6 kinase
TCA cycle	tricarboxylic acid cycle

Chapter 1: Overview of pancreatic β-cell function and diabetes

I. Pancreatic β cells and nutrient-sensing mechanisms

A. Pancreatic β-cell functions

The endocrine islets of Langerhans in the mammalian pancreas consist of at least five cell types, α, β, δ, ε, and PP cells, which secrete glucagon, insulin, somatostatin, ghrelin and pancreatic polypeptide (PP), respectively (1,2). Among them, pancreatic β cells and α cells are the two best studied groups. In rodent islets, 65-80% of endocrine cells are β cells. In β cells, insulin is initially synthesized as a single chain 109-amino-acid precursor, preproinsulin in the ribosomes of the rough endoplasmic reticulum (3). Preproinsulin is subsequently cleaved into an 86-amino-acid polypeptide, proinsulin, which is then transported into the Golgi apparatus and further packaged into secretory granules. Finally, proinsulin is cleaved into mature insulin (chains A and B, total 51-amino-acids) and a by-product, C-peptide, by proteases PC1, PC2, and CPH in secretory granules. Insulin can be stored in secretory granules for several days prior to its release (4). Once insulin is secreted from islets into the portal vein, about 50% is immediately removed and degraded by the liver. Thus, the half-life of insulin is very short (min) in the circulating blood. Insulin is the key hormone responsible for maintaining glucose homeostasis and it reduces blood glucose by promoting glucose storage in insulin-sensitive tissues such as liver, muscle and adipose tissues. β cells sense minute-to-minute variations in blood glucose and adjust insulin secretion and synthesis according to the demand (5,6). β cells also secrete multiple small peptides such as C-peptide and amylin (7). Because C peptide is less susceptible than insulin to hepatic degradation, it can be

used as a marker of insulin secretion and allows discrimination of endogenous and exogenous sources of insulin in the evaluation of hypoglycemia. 15-20 % of endocrine cells are α cells in rodent islets. α cells secrete glucagon to release glucose from the liver if blood glucose levels are too low. In addition, secretion from β cells can inhibit glucagon secretion from α cells via releasing suppressors including zinc and glutamate (8,9). Through the inherent glucose-sensing mechanism, β cells and α cells work together to maintain circulating glucose concentration, normally at 5-10 mM (10). Additionally, somatostatin secreted by δ cells and pancreatic polypeptide (PP) secreted by PP cells can regulate the secretion of other hormones and of exocrine enzymes.

B. Insulin secretion

Normal β cells adjust insulin secretion and synthesis by sensing physiologically changes of blood glucose (2–20 mM) (6,11). Glucose is transported into β cells via the predominant glucose transporter 2 (GLUT2), which is a low-affinity transporter with a high capacity Km 15-20 mmol/L, and therefore permits the rapid equilibrium of intra-and extracellular glucose concentration (6, 12). The intracellular glucose in β cells is phosphorylated by glucokinase (GCK) and subsequently metabolized into pyruvate (Pyr) by glycolysis. GCK has a lower affinity and higher Km for glucose than the other hexokinases and is not inhibited by its product glucose-6-phosphate, thus it determines the rate of glycolysis and the generation of pyruvate. Pyruvate preferentially enters mitochondria and is metabolized by the tricarboxylic acid (TCA) cycle. Oxidative metabolism in mitochondria results in a robust rise in the ATP/ADP ratio, which thereby causes closure of the ATP-sensitive K^+ channel and the subsequent depolarization of the

cytosolic membrane. The alteration of electrical signals opens voltage-dependent Ca^{2+} channels and increases intracellular Ca^{2+} concentrations, and finally triggers exocytosis of insulin granules (Figure 1-1). β cells contain at least three types of Ca^{2+}-channels (13, 14). Among them, the nifedipine-sensitive L-type Ca^{2+}-channel is particularly essential for insulin exocytosis because it mediates the Ca^{2+}-influx essential for first-phase secretion of insulin granules. Other types of Ca^{2+}-channels, such as R-type channels, are important for the second phase. Studies showed that insulin is released in a characteristic biphasic fashion consisting of a transient first phase featured with a large peak (lasting 5~10 min), followed by a sustained second phase with a flat or slowly rising rate of secretion.

Mitochondria metabolism generates various nucleotides (ATP, GTP, and NADPH), cyclic nucleotides cAMP as well as metabolites (malonyl-CoA and glutamate) (15, 16). Among them, ATP acts as the dominant messenger in amplifying Ca2+ flux triggered insulin secretion. Other nucleotides and metabolites function as additive messenger factors, further contributing to insulin secretion and synthesis. It is regarded that upon 10-20 mM glucose, glucose-stimulated insulin secretion (GSIS) is regulated mainly via ATP-sensitive K^+ channel-dependent manner because ATP production is linearly proportionate to the rate of glucose oxidation. However, under glucose concentration up to 20 mM, insulin secretion is mainly regulated by NADPH and other metabolites. It is thought that the second phase of glucose-stimulated insulin secretion is due mainly to the K_{ATP} channel-independent pathways.

Glucose is the primary stimulus for insulin secretion. Indeed, other nutrient components such as specific amino acids and lipids may also regulate insulin secretion

via their intermediate metabolites generated in mitochondria. Nutrient-induced insulin secretion is adjusted by multiple hormones including the neurotransmitters acetylcholine and pituitary adenylate cyclase–activating polypeptide (PACAP), gastrointestinal hormones glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP) (17,18).

C. Regulation of insulin synthesis

i) Transcription factors involved in insulin gene expression

Insulin gene transcription is mainly controlled by the 400 bp promoter region proximal to the transcription start site of the insulin gene (19, 20, 21). A group of specific transcription factors interact with discrete cis-acting elements in the insulin gene promoter region, cooperatively contributing to the regulation of insulin gene transcription. Studies showed that human has only one insulin gene and rodents (rat and mouse) have two insulin genes (insulin genes I and II). Although the spacing of cis-acting elements is not same between rodent and human insulin gene promoter regions, their insulin promoters include three conserved regulatory elements A3, E1 and C1, which are bound by the transcription factors PDX-1, BETA2/NeuroD, E47 and Maf A, respectively.

It is sufficient to induce insulin production in non-insulin secreting cells such as hepatocytes transfected with PDX-1, BETA2/NeuroD, and Maf A. In addition, other transcription factors, including NFAT, p300, C/EBPβ, E2A, c-Jun and cAMP-response element binding protein (CREB), positively and negatively affect insulin gene transcription. Although the expression of these factors is not restricted to β cells, their combined expression contributes to insulin synthesis (Figure 1-2).

Pancreatic and duodenal homeobox factor-1 (PDX-1), also known as IDX-1/STF-1/IPF1, is a member of the large family homeodomain-containing proteins. PDX-1 is essential for pancreatic development, β-cell differentiation, and maintenance of mature β-cell function **(22**). PDX-1 is primarily expressed in early endocrine progenitors and directs the growth of the pancreatic buds during pancreatic development. In mature pancreas, PDX-1 expression is restricted to β cells and δ cells. PDX-1 contributes to maintaining mature β-cell functions through up-regulating the transcription of several β cell-specific genes, including insulin, glucose transporter-2 (GLUT2), glucokinase, islet amyloid polypeptide and MafA, as well as auto-regulating its own expression. Insufficient expression of PDX-1 makes β cells susceptible to apoptosis, associated with reduced expression of the anti-apoptotic genes BclXL and Bcl-2 (23). Thus, PDX-1 also acts as an anti-apoptotic factor and a critical regulator of β-cell survival and proliferation. Loss of PDX-1 results in pancreatic agenesis in human and mice, and heterozygous loss-of-function mutation of PDX-1 in humans has been associated with maturity onset diabetes of the young (MODY) 4, supporting the essential role of PDX-1 in pancreatic β-cell development and maintenance of β-cell mass. In addition, PDX-1 is required for maintenance of β-cell features through inhibiting the expression of the α cell-specific gene, glucagon and the pancreatic ductal cell-specific gene, cytokeratin K19 (24).

Glucose modulates PDX-1 function by regulating its localization, DNA-binding ability and interaction with other proteins. In response to high levels of glucose, PDX-1 is activated through PI3K/Akt- or ERK1/2- mediated phosphorylation and is translocated from cytosol to nucleus to bind to A elements of the insulin promoter (26). Activated PDX-1 recruits co-activators such as the histone methyltransferase Set7/9 and the histone

acetyltransferase (HAT) to the insulin promoter and also interacts with other β cell cell-specific transcription factors, such as NeuroD and MafA, thereby promoting insulin gene transcription. PDX-1 may also suppress insulin gene transcription by recruiting histone deacetylase (HDAC)-1 and -2 to the insulin promoter in response to low glucose levels (1–3 mM). Thus, the negative or positive role of PDX-1 in insulin gene transcription is closely controlled by the glucose level.

V-maf musculoaponeurotic fibrosarcoma oncogene homologue A (MafA), a basic-leucine zipper transcription factor, functions as a strong transactivator for insulin gene transcription though binding to the C1 element (27). MafA expression is detected in insulin-producing cells during pancreatic development, but MafA knockout mice have normal pancreatic development before birth. In adult pancreas, MafA expression is restricted to mature β cells and its function is essential for maintaining mature β cell function. MafA knockout mice develop severe diabetes postnatally, with progressively impaired insulin secretion, abnormal islet morphology, and reduced expression of insulin, PDX-1, NeuroD1, and Glut2. In response to high glucose, MafA proteasomal degradation is prevented, thus its protein expression level is quickly increased. Moreover, phosphorylation of MafA promotes MafA binding to the insulin promoter. In addition, PDX-1 and NeuroD can bind to the MAFA gene promoter, thereby enhancing MafA transcription.

Neurogenic differentiation 1 (Neuro D1), also known as BETA2, is a member of the basic helix-loop-helix (bHLH) transcription factor family (28, 29). NeuroD interacts with another bHLH protein E47 and thus associates with the E1 element as a heterodimer. NeuroD1 is initially expressed in endocrine progenitor cells during

pancreatic organogenesis, and subsequently its expression is maintained in all types of mature endocrine cells in adult pancreatic islets. NeuroD1 knockout mice show the decreased endocrine cells in islets and a low level of insulin expression. NeuroD1 as a permissive factor, acts synergistically with PDX-1 to positively stimulate insulin gene transcription. Under normal glucose, NeuroD1 is mainly localized in the cytosol. High glucose stimulates NeuroD1 phosphorylation at multiple sites mainly mediated by ERK1/2, thereby promotes its nuclear translocation and transactivation. In addition, NeuroD1 can recruit co-activators such as p300 or CREB-binding Protein (CBP) and co-repressors such as small heterodimer partner (SHP), thereby controlling insulin gene transcription.

Nuclear factor of activated T cells proteins (NFATs) have five members, NFAT 1-5 (30). The NFAT proteins are a family of calcium/calcineurin-responsive transcription factors. Upon a rise in calcium flux triggered by glucose metabolism, the calcium/calmodulin-dependent phosphatase calcineurin is activated, which dephosphorylates multiple serine residues in NFAT proteins. Dephosphorylation of NFAT causes exposure of the nuclear localization sequences (NLS), thereby promoting NFAT translocated into nuclear. The DNA binding ability of NFAT is relatively weak compared to other transcription factors, thus NFAT requires other partners to regulate gene expression. All five NFAT proteins are expressed in β cells as well as in other hormone-producing islet cells and a subset of acinar cells (website: beta cell atlas). Among them, NFAT1 and 3 interacts with MafA, cooperatively regulating expression of insulin gene expression in β cells. In addition, NFAT activation is required for proliferation and survival in adult β cells. Phosphorylation of NFAT mediated by GSK3

exposes a nuclear export signal (NES), thereby promoting NFAT translocation to the cytosol.

ii**) Regulation of insulin biosynthesis by ERK1/2 and other multiple kinases**

Glucose metabolism in pancreatic β cells increases ATP/ADP ratio and triggers intracellular calcium flux, which leads to activation of multiple kinases (11, 31). Elevated calcium acts as a second messenger, directly activating the phosphatase calcineurin and calcium-dependent kinases including protein kinase C (PKC) and calcium/calmodulin-dependent kinase (CaMK). Calcium influx also activates ERK1/2 probably through the induction of Ca2+-dependent Ras activation. Glucose-induced ERK1/2 activation also requires the activation of the calcium-dependent phosphatase calcineurin. ATP is conversed into cAMP by the enzyme adenylate cyclase (AC). cAMP is responsible for activation of the cAMP-dependent protein kinase, protein kinase A (PKA) (Figure 1-3).

Activated ERK1/2 by glucose promotes insulin gene transcription by phosphorylating crucial insulin gene transcription factors such as PDX-1, MafA and BETA2/NeuroD and enhancing the promoter ability of these transcription factors. ERK1/2 is also involved in the regulation of protein synthesis by activating the p70S6 kinase (p70S6K), therefore contributing to insulin biosynthesis (32).

II. Pancreatic β-cell mass and factors that govern β-cell mass

A. Embryonic pancreatic development

In general, a repertoire of transcription factors controls the differentiation program of endodermal cells into β cells (33, 34)(Figure 1-4). Pancreatic endocrine and

exocrine cells originate from the endoderm. Dorsal and ventral buds of the embryonic mouse pancreas develop from the foregut endoderm at approximately E8.5 and later fuse at E12.5. Early endocrine progenitors within the foregut endoderm are marked by the expression of PDX-1, which is early induced at E8.5. In the same region, the extracellular signaling molecule sonic hedgehog is repressed. Genetic tracing experiments identified that both exocrine and endocrine cells derive from a pool of endodermal cells with expression of PDX-1 and the pancreas transcription factor (Ptf1a). Late endocrine progenitors continue to differentiate and yield endocrine cells marked with PDX-1 and Hnf6 between E13.5 and E14.5. Ngn-3 is transiently expressed in PDX-1 and Hnf6 expressing duct cells. The transient expression of Pax4 specifies the β-cell phenotype. Eventually, differentiated β-cells express PDX-1, Nkx6.1, Nkx2.2, MafA. The fastest expansion of the pancreatic β-cell population occurs in late fetal development. It is thought that the appropriate number of β cells is set in late fetal life.

B. The postnatal growth of β-cell mass

The expansion of the β-cell population in prenatal stages mainly depends on progenitor cell differentiation (35, 36). However, the maintenance and expansion of β-cell population at postnatal stages occurs primarily through proliferation. Similar to most other tissues and organs, the number of β cells after birth is continuously increased until adolescence. In neonatal rodents, a transient burst of β-cell replication and neogenesis due to proliferation of progenitor cells, occurs in the first week of life. β cells in newborn human are also thought to undergo a similar spurt of growth. Later, the rates of β cell replication and neogenesis markedly drop but the β-cell population slowly grows through

childhood. In normal human adults, β-cell mass is maintained at a relatively stable level in response to different stresses. Generally, the life-span of β cells is about 60 days and few adult β-cells (about 5%) undergo apoptosis under normal conditions. The loss of β cells is predominantly supplied through proliferation of pre-existing β cells. In addition, β-cell neogenesis probably from ductal epithelium cells may partially contribute to renewing human β cells. Self-replication is essential for the maintenance of β-cell mass in adult rodents under normal physiological conditions and after partial pancreatectomy. In addition, adult rodent pancreas contains islet cell progenitors and new β cells may be generated from endogenous progenitors with Ngn3 expression in the mouse model of pancreatic duct ligation or autoimmune reaction. The average β-cell size is relatively stable during childhood, but β-cell mass, including β-cell size and β-cell numbers, during adulthood in both human and rodents, continuously rises with age due to increased body weight and insulin resistance.

It appears that the maintenance of β-cell mass and function in postnatal life probably relies on mechanisms distinct from those used to generate β cells during embryogenesis (37,38) (List 1). For an example, expression of the transcription factor Ngn3 is essential for differentiation of embryonic islets, but extremely rare in normal postnatal pancreas. The transcription factors MafA and Foxa expressed in postnatal mature islets are required for maintaining β-cell function, but they are absent or not essential in prenatal β-cell development. Evidence from knock-out mice models suggests that cell cycle progression of prenatal and postnatal β-cell proliferation is controlled by different cell cycle regulators: cyclins and cyclin-dependent kinase (CDKs), and cyclin dependent kinase inhibitors (CKIs). For examples, global deletion of Cyclin D1 or Cyclin

D2 in mice decreases postnatal β cell proliferation, and mature β cell growth in the later age stage, but does not alter the normal complement of β cell population at birth. A similar phenomenon occurs in CDK4 and E2F1/E2F2 knock out mice.

C. Regulation of postnatal β-cell mass by growth factors

Throughout adult life, β-cell mass is dynamically regulated by glucose and multiple mitogens including insulin, IGF-I/II, GLP-1, placental hormones, gastrin, growth hormone (GH), hepatocyte growth factor (HGF) and EGF (39, 40). The net growth of β-cell mass is dependent on the sum of the rate of β-cell replication, size, apoptosis. Once pancreatic β-cells can not produce sufficient insulin levels to compensate changes in the increased body weight, insulin sensitivity, pregnancy, or tissue injury, blood glucose is chronically elevated in the late stage of diabetes.

i) Glucose

Glucose is a major stimulus for maintaining pancreatic β-cell function and mass. Glucose acts as an essential mitogen for β-cell proliferation through activating multiple kinases such as ERK1/2 and PKC, which affect protein synthesis, cell proliferation and anti-apoptotic processes. Studies on primary β-cell cultures showed that the basal glucose concentration (≤ 5mM) reduces the proportion of cycling β cells and 10-20 mM glucose promotes β-cell division. Moreover, glucose infusion in adult rats enhances the β cell mass in 24 hours. Recent studies suggest that glucose also enhances β cell neogenesis from ductal epithelial precursors because most of enhanced numbers of β cell clusters and small islets, scatter among acinar cells in glucose-infused rats by 48 hours. In addition,

glucose augments β-cell responsiveness to other growth factors such as insulin, insulin-like growth factor-1 (IGF-1), growth hormone (GH), GLP-1, and EGF.

ii) Insulin, insulin-like growth factor I, II

Insulin, insulin-like growth factor (IGF)-I and II are vital for maintenance of the adult β cell population. Insulin and IGF-I bind to the insulin receptor (IR) and the IGF-I receptor (IGF-IR), respectively, thereby activating the intrinsic tyrosine kinases of these receptors. In addition, high concentration of insulin also activates the IGF1R or directly binds to IR/IGF1R heterodimers. IGF-II, binds to IGF-II-mannose-6-phosphate receptor or also both the IGF-IR and the A-subtype IR. Activated receptors causes autophosphorylation of inner parts of these receptors and tyrosine phosphorylation of adaptors proteins, including insulin receptor substrate (IRS) proteins, shc, Gab-1, thereby transducting signaling to multiple downstream effectors, such as PI3 kinase, protein kinase B (PKB, also called Akt), p70S6 kinase, Rafs, and PLCγ. Studies on mice with a β cell-specific knockout IR identified that IR signaling is required for maintenance of adult β-cell mass. Similarly, ablation of IRS-2 in β cells leads to a reduction in adult β-cell mass with age, but does not affect embryonic β-cell development. In addition, acute insulin infusion in rats increases β-cell numbers. Thus, these observations support the role of insulin, IGF-I and II in β-cell proliferation and survival.

iii) Glucagon-like peptide-1

Glucagon-like peptide 1 (GLP-1), encoded by the proglucagon gene, shares a 50% amino acid identity to glucagon. GLP-1 binds to the β-cell GLP-1 receptor (GLP-

1R), a Gs-protein-coupled receptor, resulting in increasing intracellular cAMP and Ca^{2+} levels in β cells and activating protein kinase A (PKA), PKB and ERK1/2 kinases. GLP-1 stimulation augments glucose-dependent insulin secretion and synthesis. GLP-1 modulates β-cell mass partially via promotion of β-cell proliferation and reduction of β-cell apoptosis. In addition, GLP-1 may play a role in β-cell neogenesis because it can induce differentiation of duct cell lines into functional β cells in vitro.

iv) Placental hormones

β-cell mass in both rodents and human increases in response to markedly increased insulin demand by pregnancy. Placental hormones, especially placental lactogens, are the chief stimuli for β-cell proliferation during pregnancy. It is reported that placental hormones stimulate β-cell proliferation in isolated islets. β-cell mass recovers to normal levels in 10 days through increased β-cell apoptosis and decreased β-cell proliferation after delivery.

D. Suppressors of β-cell growth and functions

β-cell growth and function are suppressed by multiple factors including glucotoxicity, lipotoxicity, follistatin, and leptin. Although glucose is required for β-cell survival, high glucose is toxic for β-cell function and increases β cell apoptosis (41,42). Glucotoxicity induced by chronic hyperglycemia contributes to β-cell dysfunction and apoptosis probably through accumulation of reactive oxygen species (ROS), enhancement of endoplasmic reticulum (ER) stress, increased levels of intracellular calcium, and induction of inflammatory factors such as IL-1. Accumulation of oxidative

stress causes DNA damage, activation of p38 MAP Kinase (MAPK) and JNK-stress pathways, finally results in disruption of mitochondrial functions and activation of apoptotic programs. β-cell dysfunction is further promoted by long-term increase inER stress and intracellular calcium. Hyperglycemia also promotes IRS-2 ubiquitination and subsequent proteosomal degradation though chronic mTOR activation, thereby increases β-cell apoptosis. Long-term hyperglycemia reduces the levels of transcription factors such as PDX-1 and Maf A, which are vital for insulin synthesis and β cell proliferation. Certain cytokines including IL-1 β and TNF-α, activate the IκB kinase-β (IKK-β)/nuclear factor-κB (NF-κB) pathways and JNK/p38 pathways, subsequently trigger apoptotic programs and suppress IRS signaling, resulting in impaired β-cell function.

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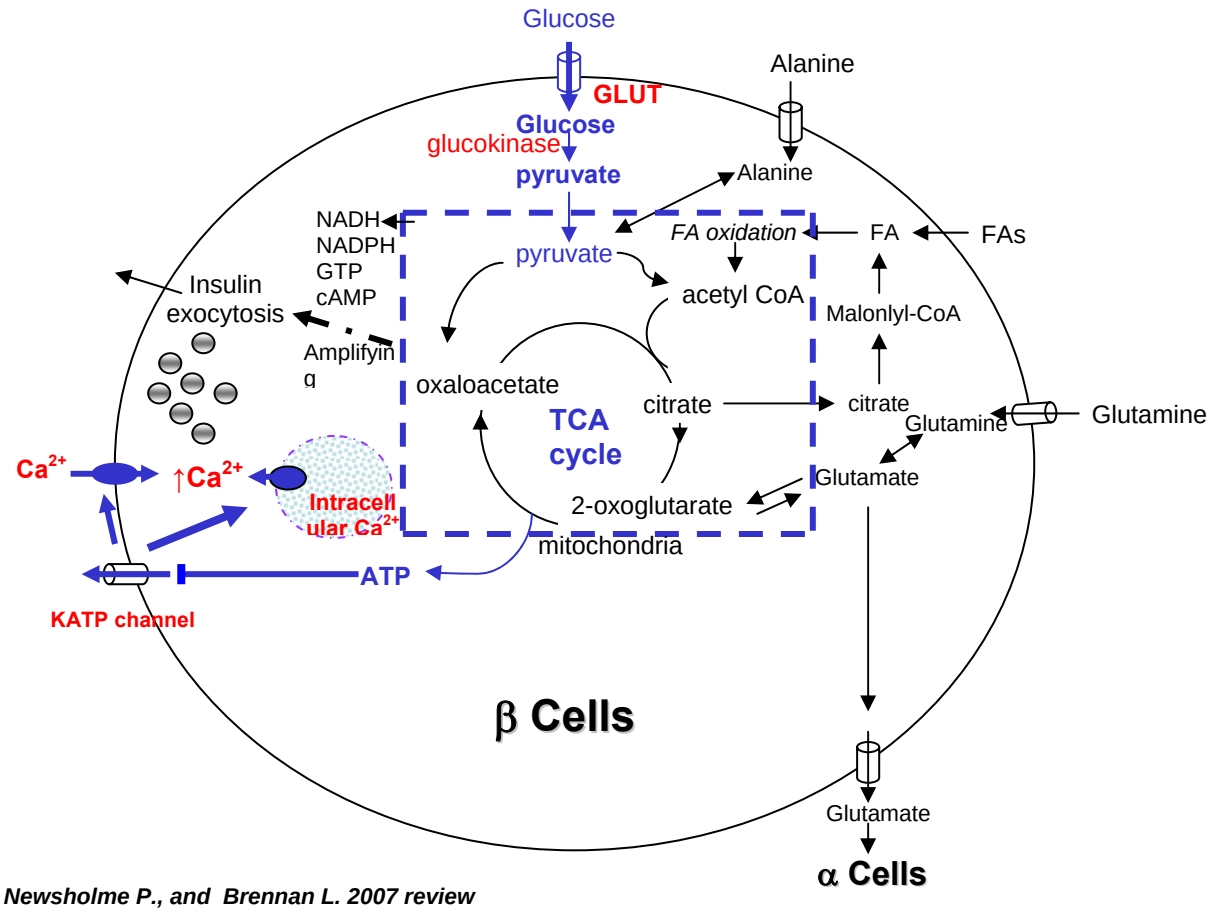

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Figure 1-1. Glucose metabolism and insulin secretion in pancreatic β cells

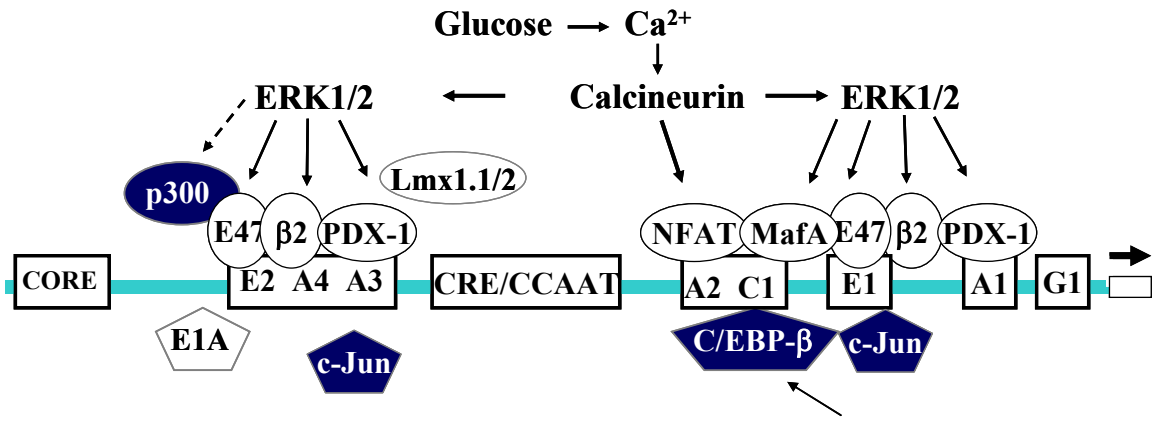


Figure 1-2. Common transcription factors binding to the insulin gene promoter

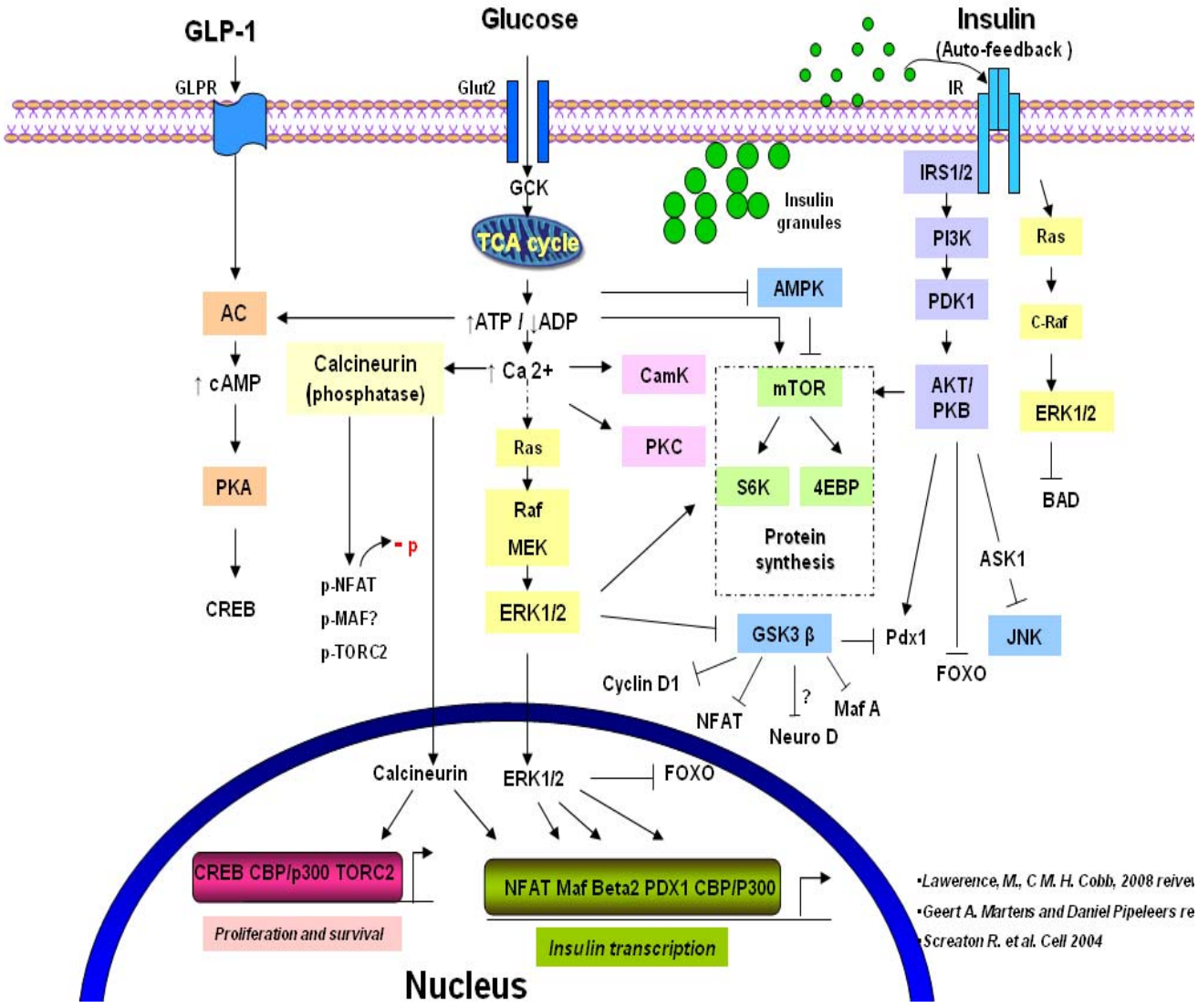


Figure 1-3. Regulation of insulin biosynthesis by ERK1/2 and other kinases in pancreatic β cells

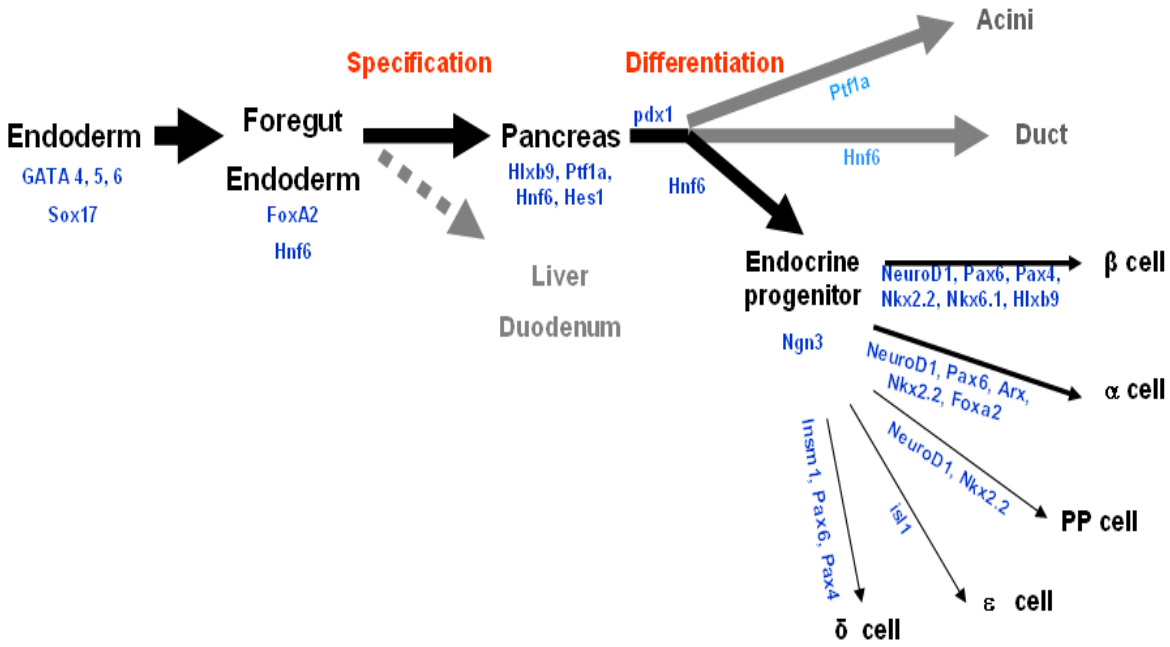


Figure 1-4. Transcription factors involved in regulation of embryonic pancreas development

Transcription Factor	Family	Expression in mature islets	Pancreas-related phenotype in knockout mice
PDX-1	HD	β and δ cells	Absence of pancreas
Hlxb9	HD	β cells	Absence of dorsal pancreas
Isl-1	HD	All islet cells	Absence of islet cells and dorsal pancreatic mesoderm
Pax-4	HD	Endocrine progenitor cells (low expression)	Absence of β and δ cells, increase of α, ε cells
Pax-6	HD	All islet cells	Absence of α cells, decrease of β, δ, and PP cells, increase of ε cells
Nkx2.2	HD	α, β, PP cells	Absence of β cells, decrease of α-, and PP-cells
Nkx6.1	HD	β cells	Decrease of β cells
Ngn3	bHLH	Endocrine progenitor cells	Absence of endocrine cells
NeuroD1	bHLH	All islet cells	Decrease of endocrine cells
Maf A	bLZ	β cells	No effect on islet mass before birth, develop severe diabetes postnatally
NFAT		All islet cells	No effect on islet mass before birth, develop diabetes postnatally

List 1-1. Essential transcription factors in adult mature islets

Chapter 2: Raf kinases, a biochemical review

The mitogen-activated protein kinase (ERK/MAPKs) signaling pathway is a three-tiered kinase cascade consisting of Raf/MEK/ERK1/2, which is involved in regulation of many cellular programs including cell proliferation, survival, differentiation, motility and metabolism (43). Extracellular stimulators including growth factors, cytokines and hormones bind to their surface receptors and activate the small G protein Ras. Commonly, active Ras triggers complex conformational changes of Raf kinases, resulting in plasma membrane translocation and activation of Raf kinases. In addition, intracellular signals including Ca^{2+}, cytoskeletal rearrangement and mitotic progress also activate Raf isoforms (especially B-Raf) in a Ras-dependent or Ras-independent way (43,44). Activated Raf kinases transmit extracellular signals from GTP-Ras to their downstream targets, the dual-specificity protein kinase MEK1 and 2. MEK1/2 phosphorylated and activated by Raf, further phosphorylates and activates the MAPK ERK1 /2. Finally, ERK1/2 phosphorylates multiple downstream factors such as cytosolic proteins and transcription factors to regulate cellular functions.

Raf kinases are the center of the ERK/MAPKs singling cascade. Compared to MEK1/2 and ERK1/2, Raf activation is not only modulated by multiple phosphorylation events, but also regulated by a large number of proteins (45,46). Scaffold proteins such as KSR, CNK and Paxillin interact with Raf and positively regulate Raf activation. Raf kinases are also subjected to regulation by small proteins such as RKIP, SPRED and 14-3-3. The phosphatase PP2A inhibits ERK1/2 activation, while PP2A-mediated

dephosphorylation of the 14-3-3 binding site on the N-terminus of Raf is required for Raf membrane translocation and activation.

I. The structural features of the Raf family

Mammalian cells contain three Raf isoforms: A-Raf (about 68 kDa), B-Raf (range from 75 to 100 kDa), and C-Raf (range from 72 to74 kDa) (45). In 1983, Ulf Rapp and Klaus Bister first cloned retroviral oncogenes v-raf and v-mil, respectively. Later, comparison of v-raf and v-mil DNA sequences found that v-raf and v-mil encoded orthologs of the serine/threonie kinase C-Raf (47). Two new members of the Raf family, A-Raf and B-Raf were also discovered in 1986 and in 1988, respectively (48). The structures of three Raf isoforms C-Raf, B-Raf and A-Raf, can be divided by three highly conserved regions, CR1, CR2 and CR3 (49). The CR1 region contains a Ras binding domain (RBD) and a cysteine-rich domain (CRD), both of which are required for Ras binding and membrane recruitment. The CR2 region is less conserved and contains multiple phosphorylation sites that are responsible for inhibition of Raf activity and recruitment of binding proteins such as the adaptor protein 14-3-3. The CR3 region encodes the catalytic kinase domain, which contains the two conserved activation-segment phosphorylation sites (T491 and S494 in C-Raf, T598 and S601 in B-Raf, T452 and T455 in A-Raf). In addition, the CR3 region contains one 14-3-3 binding site which is essential for maintaining Raf activation. Although MEK1/2 is a common substrate for all Raf isoforms, B-Raf displays the highest basal activity and the strongest catalytic activity toward MEK, and A-raf is the weakest. In contrast to other Raf kinases, B-Raf activation requires fewer activating phosphorylation sites since its N-region, the

negatively charged region is constitutively negatively charged due to residues D 447/D 448, equivalent to Y341 in C-Raf and Y302 in A-Raf. B-Raf is constitutively phosphorylated at S446 (equivalent to S338 in C-Raf and S299 in A-Raf). Secondly, B-Raf has a long amino-terminal region before Ras binding domain, which can facilitate lipid-independent Ras binding and interaction with other proteins (50). These structural features explain why B-Raf possesses high basal activity and is the most efficient MEK1/2 activator compared to A-Raf and C-Raf.

III. Regulation of Raf activation by phosphorylation and dephosphorylation

The activity of Raf isoforms is regulated by numerous phosphorylation sites that are distributed throughout the proteins. Generally, phosphorylation sites for promoting Raf activation (marked in red color) are mainly located in the N-region and the kinase domain, whereas phosphorylation sites for suppressing Raf activity (marked in blue) are scattered throughout the N-terminal regulatory domains and the end of the C-terminus (Figure 2-1). Some phosphorylation sites are conserved in all three isoforms, and are subjected to similar regulatory mechanisms.

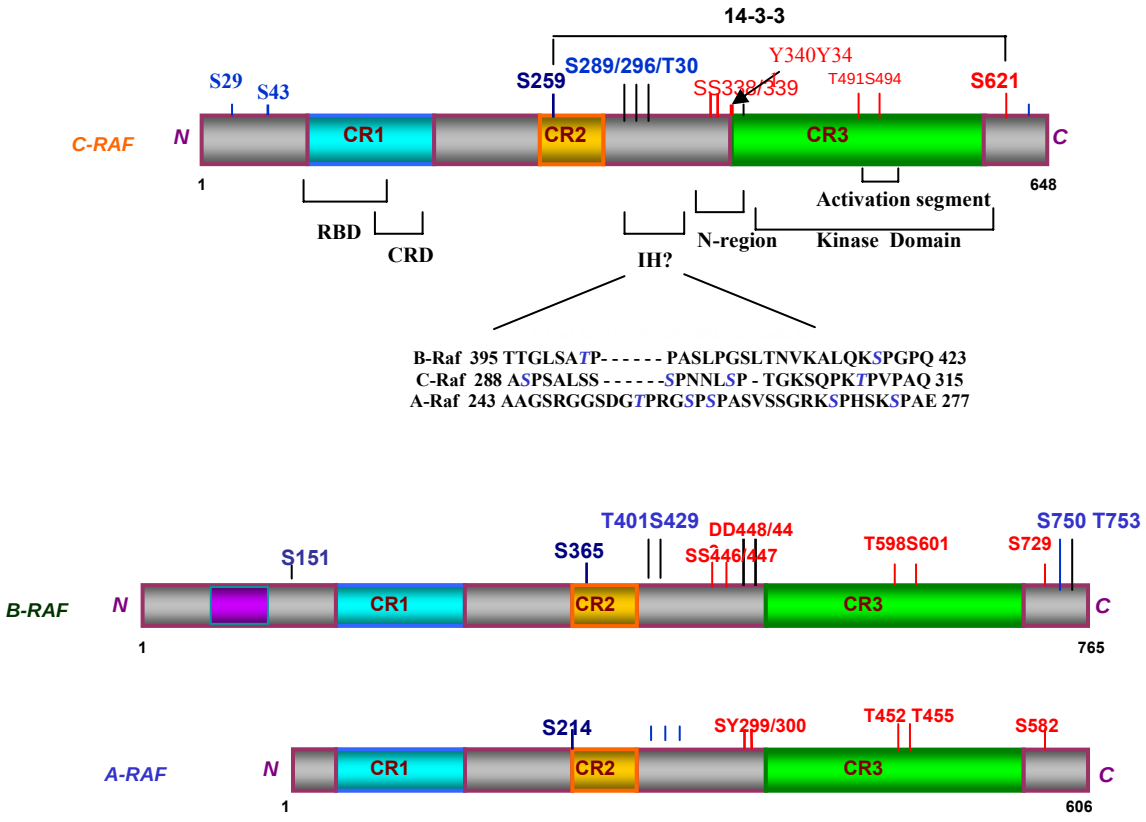


Figure 2-1 Schematic phosphorylation sites in Raf isoforms

C-Raf is the most intensively studied of Raf isoforms (43,51). C-Raf activation requires the autophosphorylation of T491 and S494 in the activation segment. Phosphorylation of S338 and Y341 in the N-region further contributes to C-Raf full activation and membrane recruitment in response to growth factors. S338 phosphorylation is mainly mediated by Pak kinases and Y341 phosphorylation mediated by tyrosine kinases such as src and Jak. C-Raf activation is also negatively regulated by

phosphorylation. Three residues S43, S259 and S621 are phosphorylated by PKA and their phosphorylation correlates with the down-regulation of kinase activity. S259 can also be phosphorylated by Akt/PKB. Phosphorylation of S43 inhibits C-Raf activation via interfering Ras binding, and phosphorylation of S259 and S621 is involved in suppressing C-Raf activation via providing docking sites for 14-3-3 adaptor proteins. Dephosphorylation of S259 or mutated S259A dissociates 14-3-3 binding and therefore facilitates Ras-dependent-C-Raf activation and membrane recruitment (52). Whereas binding of 14-3-3 to phosphorylated S621 appears to be necessary for maintaining kinase activity.

In addition, C-Raf activation is negatively regulated by an ERK1/2-mediated feedback mechanism that inhibits Ras/C-Raf interaction, C-Raf association with the plasma membrane and B-Raf/C-Raf interaction (46). Mass spectrometry identified that multiple residues, S29, S289, S296 and T301, on C-Raf that are phosphorylated by ERK1/2. B-Raf and A-Raf activation is also negatively regulated by ERK1/2-mediated feedback phosphorylation (53,54,55). Active B-Raf at mitosis is mainly located in cytosol and appears not to be regulated by either Ras or membrane association. Negative feedback inactivation of B-Raf by ERK1/2 is probably essential for turning-off B-Raf activity at mitosis to enter other phases of the cell cycle (44,56).

IV. Heterodimerization of B-Raf and C-Raf

In response to active Ras, a Raf isoform forms homodimers with itself or heterodimers with another Raf isoform (57, 58). Raf homo- or hertero-dimerization is necessary for Raf activation in normal cells and cancer cells. The Rushworth laboratory showed that B-Raf/C-Raf heterodimers possess higher kinase activity toward MEK than

Raf homodimers. Active B-Raf also phosphorylates and activates C-Raf through physical interaction. In B-Raf/C-Raf heterodimers, active C-Raf directly phosphorylates MEK or simply acts as a scaffold that facilitates signaling transduction. Recent studies showed that Raf dimerization is especially important for cancer cell growth, survival and drug resistance. An explanation is as follows. Hyperactivated B-Raf mutants such as V600E directly activate cytosolic C-Raf in a Ras-independnet manner, thereby hyperactivating the MEK/ERK1/2 cascade. Some B-Raf mutants with impaired activity interact with C-Raf and, therefore, transducer the activating signal to MEK, although these B-Raf mutants are unable to directly activate MEK1/2. From 2009 to 2010, a series of paper reported that Raf inhibitors effectively inhibit Raf activation and ERK 1/2 phosphorylation in cancer cells with hyperactive B-Raf mutants, but unexpectedly, use of them leads to a paradoxical increase activated Ras (59, 60). Chemical genetic methods identified that Raf inhibitors at low dose drive C-Raf homodimerization, resulting in activating rather than inhibiting the MEK/ERK1/2 cascade.

Raf homo- or hetero-dimers are increased by 14-3-3 proteins, which are known to form dimmers and bind to a 14-3-3 binding site located in the C-terminus of each Raf. Deletion of 14-3-3 or mutation of 14-3-3 binding sites on Raf effectively disrupts Raf dimerization. Recently, research on the crystal structure of the B-Raf kinase domain discovers that two Raf kinase domain make the contact in a side-to-side interface involving αC helix in the N-terminal folding domain (61). The mutation of several key residues located in the side-to-side interface not only disrupts Raf dimer formation, but also reduces Raf kinase activity. In addition, ERK1/2-mediated feedback phosphorylation on B-Raf destabilizes B-Raf and C-Raf interaction by an unidentified mechanism.

IV. Distinct cellular functions of Rafs

A. Development

All three Raf isoforms, A-Raf, B-Raf, and C-Raf are ubiquitously expressed in a wide range of tissues, whereas the three Raf isoforms exhibit different tissue distribution depending on the tissue type (62,63). C-Raf is expressed particularly highly in striated muscle, cerebellum and fetal brain. B-Raf is most highly expressed in neural tissues and A-Raf exhibits relatively high expression in urogenital organs. Genetic studies in mice suggested that all three Raf isoforms are indispensable for embryoric development, but each owns essential functions in different tissues. B-Raf and C-Raf knockout embryos die in midgestation (*in utero* between E10.5 and E12.5 days). Deletion of C-Raf causes developmental defects in placenta, lungs and skin as well as massive apoptosis of embryonic tissues (especially liver apoptosis). Deletion of B-Raf results in growth retardation as well as vascular and neuronal defects in embryos. A-Raf knockout mice survive to birth, but die after 7–21 days due to intestinal and neurological defects. Further studies on conditional knockout mice and chicken supported the idea that each Raf isoform carries out distinct functions and one Raf isoform does not always replace the function of another Raf isoform. For example, both B-Raf and C-Raf are found in neurons, but only B-Raf deletion is lethal for embryonic motorneurons and sensory neurons. More frequently, a cooperation of B-Raf and C-Raf is involved in regulation of cellular functions. For example, B-Raf and C-Raf are required for ERK1/2 activation induced by EGF in fibroblast cells or antigen receptor signaling in B cells (64).

B. Anti-apoptosis

Both C-Raf and B-Raf are necessary for cell survival by antagonizing apoptotic signals because their downstream effectors MEK/ERKs are able to induce expression of caspase inhibitors (IAPs), inhibit cytochrome c release, and block caspases activation(65, 66). Reports also describe the role of A-Raf in apoptosis (67). Genetic studies revealed that during embryogenesis, B-Raf and C-Raf each play essential but unique anti-apoptotic roles. Further studies based on overexpression of active or kinase dead mutants revealed that the anti-apoptotic function of C-Raf is independent of MEK/ERKs activation. C-Raf can directly phosphorylate the pro-apoptotic Bcl-2 family member Bad, resulting in Bad translocation from mitochondria to the cytosol. Furthermore, C-Raf can inhibit activation of the pro-apoptotic kinase ASK1 through interaction with the N-terminal domain of ASK1. In addition, C-Raf can act as scaffold protein, recruiting other kinases such as MEKK-1 to inhibit apoptotic processes (68). Therefore the anti-apoptotic function of C-Raf can be independent of either its kinase activation or MEK/ERKs activation.

C. Tumorigenesis

The Ras-Raf-MEK1/2-ERK1/2 cascade is primarily linked with regulation of cell proliferation, transformation, differentiation and apoptosis. Raf was first reported as proto-oncogene 20 years ago (69). Mutational activation of B-Raf is more frequently detected in human cancers than C-Raf and A-Raf mutation. B-Raf mutations have been discovered in ~ 8% of human cancers. Especially, 66% of malignant melanomas, and 45% of sporadic papillary thyroid cancers (PTCs) have B-Raf mutations. Whereas A-Raf and C-Raf mutations are extremely rare (less than 0.7%) (70,71).

The majority of B-Raf mutations that result in the constitutive activation, involve a single base substitution in the kinase domain, such as ^{V600E}B-Raf (72). Among them, ^{V600E}B-Raf is 500-fold more active than wild-type B-Raf and induces constitutive activation of MAPKs ERK1/2 signaling pathway. Thus, it is perhaps not a surprise that the ^{V600E}B-Raf mutation is found in over 90% of cancers with B-Raf mutation. Some B-Raf mutants such as ^{D594V}B-Raf cause impaired activity, even inactivation, but functional studies show that this type of mutation appears to be sufficient to activate MEK through interacting with and activating C-Raf in a Ras-independent manner. ^{V600E}B-Raf mutation has been found exclusively in human cancers with active Ras mutations, while kinase-dead B-Raf and Ras mutations have a generally high prevalence to occur in cancers.

Chapter 3. Coordination of Raf and calcineurin in the regulation of ERK1/2 activation by glucose in pancreatic β cells

I. Abstract:

In pancreatic β cells, glucose metabolism-triggered calcium influx activates multiple kinases including calcium-sensitive kinases and ERK1/2, which participate in maintaining β cell function and proliferation. In addition, calcium influx activates the Ca^{2+}-dependent phosphatase calcineurin. Calcineurin is essential for regulation of insulin biosynthesis by activating the transcription factor NFAT in β cells. Previously, we found that calcineurin also regulates insulin biosynthesis by regulating ERK/2 activation. Raf kinases are a conserved component of the classical Raf-MEK-ERK1/2 signaling cascade and the two best studied Raf isoforms, B-Raf and C-Raf, are abundantly expressed in β cells. In this study, I show that glucose activates ERK1/2 through induction of the prominent activation of B-Raf, but not C-Raf in β cells. B-Raf, acting as a dominant ERK1/2 activator in β cells, modulates the transient and constitutive ERK1/2 activation in response to glucose stimulation. Despite the low activity of C-Raf in glucose stimulated cells, C-Raf, cooperatively with B-Raf, contributes to elongating glucose-induced ERK1/2 activation. Furthermore, I show that calcineurin up-regulates B-Raf activity and stabilizes C-Raf/B-Raf heterodimer complexes, thereby positively affecting ERK1/2 activity. Finally, I identify B-Raf as a calcineurin substrate; B-Raf phospho-T401 is one of the target sites that can be dephosphorylated by calcineurin. My findings reveal that cross-talk between Raf and calcineurin is essential for the maximal activation of ERK1/2 in the glucose signaling pathways.

II. Introduction

Glucose is a critical stimulus for maintaining pancreatic β-cell function and mass (2, 73). β cells take up glucose via the low affinity glucose transporter type 2 (GLUT2). The subsequent glucose oxidative metabolism in β cells leads to a sustained increase in cytosolic calcium flow that finally triggers insulin secretion. Glucose, together with other nutrients, not only stimulates acute insulin release, but also acts as a proliferation or survival regulator for differentiated β cells in the long term (5,6). Glucose alone is sufficient to activate multiple kinases including ERK1/2 and the calcium-sensitive kinases which are involved in regulating protein synthetic and proliferative activities as well as metabolic needs. Impaired β cell function or decreased β cell mass reduces insulin secretion and synthesis and gradually results in hyperglycemia, particularly in the late stages of type 2 diabetes.

The $Ca2^{+}$ dependent-phosphatase calcineurin is widely expressed in many cell types including neurons, cardiomyocytes, T cells and pancreatic β cells (74,75). Calcineurin inhibitors FK506 and cyclosporin (CsA) bind to the intracellular proteins, FK506 binding protein (FKBP) and cyclophilin, respectively, and subsequently bind to and inhibit the calcineurin complex. Calcineurin interacts with multiple substrates including the transcription factor NFAT, the anti-apoptotic protein Bcl-2, and the scaffold proteins AKAP79 and Cabin1/cain, and are involved in regulation of organ development and cell survival. In β cells, the calcineurin-NFAT interaction is essential for glucose-induced insulin gene transcription. Calcineurin dephosphorylates NFATs and induces their rapid nuclear translocation.

ERK1/2 is a conserved downstream component of the three protein kinase cascade, Raf-MEK-ERK1/2. In β cells, ERK1/2 activation by glucose is glucose-concentration-dependent and also regulated by calcineurin. Active ERK1/2 phosphorylates crucial insulin gene transcription factors, enhances their promoter binding ability and prevents their degradation, thereby increasing insulin gene transcription (76). So activities of ERK1/2 are modulated in a manner that mirrors the demand for insulin synthesis that occurs in response to elevated glucose. In addition, glucose-induced ERK1/2 activity may be required for regulation of acute insulin secretion (77).

The Raf family of serine/threonine protein kinases is a key upstream activator of the MEK/ERK1/2 cascade. Activated Raf transmits extracellular signals from Ras to its downstream targets MEK1/2-ERK1/2. In mammalian cells, there are three isoforms of Raf: A-Raf, B-Raf, and C-Raf, which are indispensable for tissue development. Disruption of the B-Raf and C-Raf genes causes embryonic lethality in mice (43). A-Raf knockout mice are viable but suffer intestinal and neurological defects (78). Among Raf isoforms, B-Raf is thought to be the major MEK1/2 activator, owning to its high basal activity and great catalytic activity toward MEK1/2. C-Raf exhibits lower basal activity, and A-Raf has the lowest activity. In contrast to other Raf isoforms, B-Raf activation requires fewer active phosphorylation events due to negatively charged "phosphomimetic" residues D 447/D 448 in its N-region and constitutive phosphorylation at S446 (equivalent to S338 in C-Raf) . Additionally, B-Raf has a long amino-terminal region before its Ras binding domain, which facilitates lipid-independent Ras binding and interaction with other proteins. It is thought that Ras-GTP alone is sufficient to activate

B-Raf, whereas activation of C-Raf and A-Raf requires coordination of Ras-GTP with other factors (50).

A recent discovery is that Raf proteins can be assembled as homodimers or heterodimers with other Raf proteins. These dimers have been detected in many cells (57, 79). B-Raf /C-Raf heterodimers own higher stimulated kinase activity compared to B-Raf or C-Raf homodimers (57). In B-Raf/C-Raf heterodimer complexes, active C-Raf directly phosphorylates MEK1/2 or simply acts as a scaffold that facilitates signaling transduction. Additionally, wild-type B-Raf activates C-Raf in the cytosol via forming B-Raf/C-Raf heterodimer, and therefore leads to MAPKs ERK1/2 hyperactivation (58).

Studies show that glucose-induced ERK1/2 activation is Raf-dependent. The three Raf isoforms are abundantly expressed in pancreatic β cells. There are conflicting reports about the involvement of Raf proteins in β cells (80,81). It is disputing which Raf isoform, C-Raf or B-Raf, is essential for ERK1/2 activation in the glucose signaling pathway. In this study, I found that B-Raf is the main ERK1/2 activator in response to glucose whereas C-Raf only contributes to the prolonged ERK1/2 activation by glucose in β cells. Moreover, the Ca^{2+}-dependent phosphatase calcineurin positively regulates B-Raf catalytic activity as well as B-Raf/C-Raf heterodimerization upon glucose stimulation. These findings indicate that cross-talk between Raf and calcineurin is critical for the maximal activation of ERK1/2.

III. Materials and methods

Cell culture and reagents. The insulinoma β-cell lines Min6 and INS1 were maintained as described (6). Prior to stimulation, β-cells were starved for 2 h in Krebs-Ringer-

bicarbonate-Hepes (KRBH) buffer. HEK293T cells and C3H10T1/2 were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Purchase information of chemical reagents and antibodies: FK506, FK520, rapamycin and Sorafenib from LC laboratories (Woburn, MA, USA), Cyclosporin (CSA) from Biomol International, U0126 from Promega, Anti-B-Raf(F-7) and Anti-C-Raf (E-10) from Santa Cruz, Anti-pS445 B-Raf (#2696) from Cell Singling Technology, Anti-pS338 C-Raf from Millipore, Anti-Calcineurin (Polyclonal) from Calbiochem, Anti-pT401 B-Raf and Anti-p259 C-Raf from Abgent, Anti-p42/44 ERK1/2 and Anti-Flag (monoclonal) from Sigma-Aldrich. Anti-ERK1/2 is a lab-made polyclonal antibody.

Constructs and transfection. The expression vectors of Flag-CnA and HA-CnA (wild-type calcineurin), HA-truncated active CnA (1-401) and Flag-inactive CnA (1-401 160A) were kindly provided by Dr. Rev Rothermel. The PLNCX-Flag-B-raf expression vector was kindly provided by Dr. Michael White. Human C-Raf and B-Raf cDNAs were amplified by PCR and subcloned into pCMV5 myc-tag expression vector. The Ras-binding domain of human B-Raf (amino acid 1-280) was amplified by PCR and subcloned into BamH1 and XbaI sites of pGEX-KG vectors. A series of B-Raf truncated fragments (1-280, 1-440, 401-776 and 441-776) were amplified by PCR and subcloned into pcDNA-HA expression vectors. Point mutants were introduced by site-directed mutagenesis. Fugene HD (Roche Molecular Biochemicals) was used to transiently transfect Min6 and INS1 cells. Lipofectamine 2000 reagent (Invitrogen Life Technologies) was used to transfect HEK293T cells

Immunoprecipitation and Western blots. Cells (8X106) cultured in 100 mm dishes were lysed at 4°C with 0.65 ml cold lysis buffer (50 mM HEPES, pH 7.5, 150mM NaCl, 0.5 % NP-40, 1mM EGTA, 1.5mM $MgCl_2$, 50mM b-glycerophosphate, 10% glycerol, 0.2 mg/ml PMSF, 0.1 mol/l NaF, 2 mmol/l Na_3VO_4, 10 µg/ml aprotinin, 5 µg/ml pepstatin A, and 5 µg/ml leupeptin). Lysates were homogenized through a 23 gauge syringe and centrifuged for 10 min at 12,000 g in a microcentrifuge at 4°C. Protein concentrations were measured using the BioRad Bradford reagent. Immunoprecipitation was processed at 4°C. 500 µg of lysates were incubated with 3-4 µg of indicated antibodies overnight. Protein immunocomplexes were then incubated with 50 µl protein-A-Sepharose beads for 4 hours. Beads were washed with cell lysis buffer for three times, each time for 5 mins before adding 5 x SDS-loading buffer. Samples were boiled and loaded to 10% SDS-PAGE, and processed for western blots. Immunoblots were developed with ECL reagent.

In vitro B-Raf Kinase assays. Activity of B-Raf and C-Raf kinases was measured in 30 µl of the reaction buffer (10 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM benzamidine, 1 mM DTT, 5 µM ATP, 15 cpm/fmol (γ-32P) ATP, and 2 ug of GST-MEK1 KM. Reactions were incubated at 30 °C for 25-40 min and terminated with 5 x SDS-loading buffer. Samples were boiled and loaded to 10% SDS-PAGE, and processed for Coomassie blue staining and autoradiography.

Ras assays. Ras activity was measured using a glutathione S-transferase (GST) fusion protein containing B-Raf RBD domain. GST-B-Raf 1-280 was expressed in E. coli and purified by glutathione-Sepharose beads. For Ras assays, cells cultured in 100 mm dishes were lysed in 0.6 ml of cold cell lysis buffer (containing 1% NP-40). Cleared extracts

were incubated with 30 μg GST-B-Raf 1-280 pre-coupled to GST-beads at 4°C for 4 h. During this process, active Ras (Ras-GTP) was precipitated by GST-Raf RBD fusion protein. After washing twice with cold lysis buffer, precipitates were separated by SDS-PAGE and analyzed by immunoblots Anti-pan-Ras antibodies.

siRNA knockdown. siRNA oligonucleotides for targeting endogenous mouse B-Raf and C-Raf were purchased from Ambion; Control oligonucleotides were purchased from Dharmacon RNAi Technologies. siRNA oligonucleotides were seen as follows: C-Raf (siRNA ID: s99723), sense: CACGAUUCUUCUAAGACAtt; antisense: UGUCUUAGAAGAAUCCGUGag. B-Raf(siRNA ID: s99634), sense: CCACAGAUGCAUCACGGAAtt; antisense: UUCCGUGAUGCAUCUGUGGga. siRNA oligonucleotides were transfected into cells by utilizing lipofectine maxi (Invitrogen) according to manufacturer's protocol.

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.

In Vitro Phosphorylation and and Dephosphorylation Assays. GST-B-Raf 1-440 fusion proteins were expressed in E. coli and purified on glutathione-Sepharose. For in vitro phosphorylation assay, GST fusion proteins were incubated with active ERK1 (lab made) at 37°C for 30 min in kinase buffer (10 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM benzamidine, 1 mM dithiothreitol, 5 μM ATP) supplied with or without 15 cpm/fmol (γ-32P) ATP in a 50 μl of reaction volume. For in vitro dephosphorylation assay, 32P labeled fusion proteins were repurified on GST-beads and incubated with or

without calcineurin (purchased from R&D Systems, Inc) at 37°C for 1 hour in the reaction buffer (1 μM calmodulin, 50 mM HEPEs, pH7.5, 1 mM $CaCl_2$, 5mM $MgCl_2$, 1 mM DTT, 1 mg/ml BSA and 1 mM DTT). After washing with cold PBS twice, proteins were separated by by SDS-PAGE and processed for Coomassie blue staining and autoradiography.

IV. Results

Glucose-induced activation of ERK1/2 occurs through Ras and B-Raf in pancreatic β cells

The small GTP protein Ras is a common upstream component of the Raf/MEK/ERK1/2 cascade. Our previous work showed that expression of G15A Ras (inhibitory) and C-Raf C4B (N-terminal fragment lacking the kinase domain) inhibited ERK1/2 activation by glucose in pancreatic β cells, supporting that Ras is required for glucose-induced-ERK1/2 activation (82). To further verify that ERK1/2 activation by glucose occurs through Ras, we assessed the levels of ERK1/2 phosphorylation and Ras-GTP in the mouse insulinoma cell line Min6 (Fig. 3-1A). Min6 cells were stimulated with 20 mM glucose for 0, 5, 10, 20 minute (s) after starvation with the KRBH buffer (including 2 mM glucose) for 2 hours. Cell lysates were subjected to Ras activity assays and western blots. Activation of ERK1/2 was monitored by specific anti-phospho-ERK1/2 antibodies because the active form of ERK1/2 is doubly phosphorylated at Thr185 and Tyr187. Active Ras (Ras-GTP), but not inactive Ras (Ras-GDP), is precipitated by GST-Raf RBD fusion protein. I therefore measured the level of Ras-GTP utilizing Ras activity assays (Fig.3-1 B). My data show that Ras activity and ERK1/2

phosphorylation were closely related, namely, both of them were peaked between 5 to 10 minutes and partially decreased at 20 minutes upon glucose treatment.

Next, I measured B-Raf kinase activity in Min6 cells and found that B-Raf activity was quickly enhanced by glucose at 5 min and reduced at 20 min (Fig.3-1 C). The pattern of B-Raf activation was similar to changes of ERK1/2 phosphorylation and active Ras, suggesting that glucose-induced ERK1/2 activation occurs through B-Raf in β cells. Sorafenib, a Raf inhibitor, effectively inhibited B-Raf and ERK1/2 activation, further supporting that B-Raf is required for glucose-induced ERK activation (Fig.3-1 D). To clarify which Raf isoform, B-Raf or C-Raf, is essential for glucose-induced ERK1/2 activation in β cells, I isolated endogenous B-Raf or C-Raf in the equal amounts of Min6 cell lysates by using anti-B-Raf or anti-C-Raf antibodies, respectively. As Fig. 3-1E shown, B-Raf owned the high basal and stimulated activity, while C-Raf activity was too low to detectable in the control and glucose-treated cells. Similar results were also detected in rat pancreatic β cell line INS1 (data not shown). C-Raf activation was detected in Hela cells stimulated with phorbol-12-myristate-13-acetate (PMA), (Fig.3-1 F). Together, these data imply that C-Raf may not be strongly activated by glucose in β cells. In addition, A-Raf kinase activity was also undetectable under the same condition (data not shown). Based on these data, I concluded that B-Raf, but not other Raf isoforms, is responsible for the glucose-induced ERK activation in β cells.

The formation of B-Raf/C-Raf heterodimer complexes is induced by glucose

As reported previously, B-Raf/C-Raf heterodimers exist at low levels but are detectable in many cells such as PC12 and Jurkat T cells (19). In B-Raf/C-Raf

complexes, C-Raf directly phosphorylates MEK1/2 or simply acts as a scaffold that facilitates signaling transduction (79). In some condition, B-Raf can activate C-Raf by B-Raf/C-Raf association. To identify if glucose can induce B-Raf and C-Raf association in β cells, endogenous C-Raf or B-Raf was respectively immunoprecipitated by the monoclonal anti-C-Raf or monoclonal anti-B-Raf antibodies and precipitates were detected by western blots. As Fig. 3-2A shown, glucose promoted the formation of endogenous B-Raf/C-Raf heterodimers in β cells. To obtain an additional evidence that glucose initiates B-Raf/C-Raf complex formation, myc-C-Raf constructs were transfected into β cells. I found that glucose quickly enhanced B-Raf/C-Raf association (Fig. 3-2B).

B-Raf is the major Raf isoform activating ERK1/2 in β cells, but C-Raf contributes to the sustained ERK activation in response to glucose.

To gain further evidences that B-Raf, but not C-Raf contributes to glucose-induced ERK1/2 activation, I knocked down B-Raf or C-Raf expression using small interfering RNA oligonucleotides (siRNA), respectively. I observed impacts of B-Raf and C-Raf siRNA knockdown in ERK1/2 activity. As Fig. 3-3A and 3-3B shown, deletion of B-Raf expression by siRNA always impaired ERK1/2 phosphorylation by glucose. Surprisingly, deletion of C-Raf expression by siRNA caused complicate effects on ERK1/2 activation by glucose. In contrast to control cells, cells transfected with C-Raf siRNA presented higher ERK1/2 phosphorylation in 20 mins (Fig. 3-3C), but owed the reduced ERK1/2 phosphorylation in 60 mins (Fig. 3-3B). Next, I detected effects of the continuous glucose treatment on ERK1/2 activation. As Fig. 3-3D shown, ablation of C-Raf expression by siRNA enhanced the basal and transient ERK1/2 activity in early time

points but reduced ERK1/2 activity in late time points, whereas deletion of B-Raf by siRNA decreased ERK1/2 activity at each time point (Fig. 3-3E). Together, these data support that B-Raf is critical for the prominent ERK1/2 activation by glucose, but C-Raf, cooperative with B-Raf, contributes to ERK1/2 activation by glucose in late phase.

To confirm the specificity of B-Raf and C-Raf siRNA oligonucleotides, I used the same B-Raf and C-Raf siRNA to knockdown B-Raf or C-Raf expression in the mouse fibroblast cell line, C3H10T1/2 cells. Both C-Raf and B-Raf are important for ERK1/2 activation by growth factors (62). It is expected that deletion of C-Raf or B-Raf reduced ERK1/2 phosphorylation by EGF in C3H10T1/2 cells (Fig. 3-3F).

B-Raf activity is selectively up-regulated by the Ca^{2+}-dependent phosphatase calcineurin

Upon the elevated calcium influx, the Ca^{2+}-dependent phosphatase calcineurin (CnA) is activated. Based on previous observations that ERK1/2 activity is up-regulated by calcineurin in the Ca^{2+} signal pathway in β cells, I hypothesized that in response to glucose, calcineurin may promote B-Raf activation and therefore promote ERK1/2 activation in β cells. As Fig. 3-4A shown, three calcineurin inhibitors FK506, FK520 and cyclosporin A (CsA) effectively decreased the basal and glucose-induced B-Raf activity. In addition, I found that FK506 negatively affected B-Raf activity induced by sodium chloride (NaCl) triggered-Ca^{2+} influx (Fig. 3-4B). FK506 was unable to inhibit both B-Raf and ERK1/2 activation upon EGF or PMA treatment in the low or no glucose conditions (Fig. 3-4C). Whereas FK506 effectively inhibited EGF-induced B-Raf activity

in presence of 10 mM glucose (data not shown). These data revealed a possibility that calcineurin is selectively regulate B-Raf activation in β cells.

To further verify effects of calcineurin on B-Raf and ERK1/2 activation, I expressed the wild-type calcineurin in Min 6 cells. As Fig. 3-4D and 3-4E shown, cells transfected with the wild-type calcineurin presented the increased ERK1/2 activation as well as B-Raf activity. Together, all of these data support that calcineurin is required for B-Raf activation in the glucose signaling pathway.

Calcineurin contributes to B-Raf/C-Raf heterodimerization

I wanted to identify if calcineurin is involved in regulation of B-Raf/C-Raf heterodimers. 293 cells are easier to be handled to get high transfection efficiency than β cells. So I transfected the wild-type and inactive calcineurin plasmids into 293 cells and detected their effects on B-Raf and C-Raf interaction. As Fig. 3-5A shown, B-Raf/C-Raf complexes were disrupted by expression of the inactive calcineurin, but enhanced by expression of the wild-type calcineurin. Furthermore, I found that FK506 effectively blocked B-Raf/C-Raf heterodimerization in β cells (Fig. 3-5B). Another calcineurin inhibitor CsA also blocked B-Raf/C-Raf associations (Fig 3-5C). C-Raf pS259 and B-Raf pS365 are negative regulatory sites for 14-3-3 docking, but FK506 had no detectable effect on either, implying that calcineurin did not regulate 14-3-3 binding to the N-terminus of Raf kinases (Fig. 3-5D). Together, these observations indicate that calcineurin activity is required for B-Raf/C-Raf heterodimerization.

B-Raf is constitutively associated with calcineurin in vivo

I sought to determine if Raf proteins physically interact with calcineurin in β cells. As Fig. 3-6A shown, B-Raf was interacted with calcineurin even in the absence of glucose, whereas the interaction of C-Raf with calcineurin was induced by EGF, not by glucose. This data indicates that the interaction of B-Raf with calcineurin stably exists. It is conceivable that the interaction of B-Raf with calcineurin provides a convenience for quick respond to the Ca^{2+} signal. To obtain additional evidences of calcineurin interacted with B-Raf or C-Raf, I co-expressed calcineurin with B-Raf or C-Raf into 293 cells. As Fig. 3-6B shown, calcineurin was able to interact with B-Raf or C-Raf. To identify the region of B-Raf required for calcineurin binding, I made a series of B-Raf truncations and examined their ability to interact with calcineurin. B-Raf contains the Ras binding domain (RBD and CRD) and the catalytic kinase domain, which are highly conserved across all Raf isoforms. The middle region (280-457) of B-Raf is serine/ threonine-rich, but less conserved with A- and C-Raf. This region affects Raf activity and localization via various phosphorylatory events and multiple binding proteins. My results showed that calcineurin preferentially interacts with the sequences located in residues from 280 to 440 (Fig. 3-6C).

pT401 on B-Raf can be directly dephosphorylated by Calcineurin in vitro

I wanted to know if B-Raf is a substrate of and dephosphorylated by calcineurin in vitro. Our previous study showed that the PKA inhibitor H89 has no impacts on glucose-induced ERK1/2 activation. B-Raf pS365 and pS445 are target sites of PKA and Akt kinases, but FK506 did not cause the changes of their phosphorylation levels (Fig. 3-5D). Inhibition of ERK1/2 activation by the MEK inhibitor U0126 enhances Raf

activation (83). Recent studies identified multiple residues, S151, T401, S750 and T753, on B-Raf that are phosphorylated by ERK1/2. ERK1/2-mediated feedback phosphorylation on B-Raf effectively disrupts B-Raf/active Ras interaction and B-Raf/C-Raf heterodimerization (46). Based on these published data, I examined if ERK1/2 phosphorylation sites on B-Raf can be dephosphorylated by calcineurin.

As calcineurin mainly interacts with the serine/threonine-rich region (280-440) of B-Raf, I subcloned B-Raf 1-440 into the pGEX-2G vector and purified GST-B-Raf 1-440 protein from bacterial cells. As Fig. 3-7A shown, GST-B-Raf 1-440 was phosphorylated by ERK1/2 in vitro and calcineurin reduced the phosphorylation levels of GST-B-Raf 1-440. Specificity of anti-pT401 B-Raf antibodies was verified in Fig. 3-7B. I found B-Raf pT401 was dephosphorylated by calcineurin in vitro assays, suggesting that B-Raf pT401 is one of calcineurin targets sites (Fig. 3-7C) .

Calcineurin is required for the maximal activation of B-Raf in 293 cells

Next, I assessed impacts of calcineurin on B-Raf activity in 293 cells. Consistent with previous publications, the basal ERK1/2 phosphorylation was elevated in cells transfected with B-Raf but not in cells transfected with C-Raf (Fig.3-8A). Inactive calcineurin effectively reduced ERK1/2 phosphorylation enhanced by B-Raf expression. As Fig. 3-8B shown, B-Raf activity was promoted by the wild-type calcineurin and blocked by the inactive calcineurin. Interestingly, U0126 effectively rescued B-Raf activity impaired by expression of the inactive calcineurin, implying that calcineurin promotes B-Raf activation probably via antagonizing ERK1/2-mediated inhibition.

To verify if B-Raf pT401 is one of target sites by calcineurin in vivo, I made the B-Raf T401A mutant. As Fig. 3-8C shown, B-Raf T401A had similar extents of S365 and S445 phosphorylation. Because ERK1/2-mediated phosphorylation on B-Raf disrupts B-Raf/C-Raf dimerization, I also found that B-Raf T401A presented higher binding ability to C-Raf than the wild-type B-Raf (Fig.3-8D). These findings support the idea that B-Raf pT401 is linked to calcineurin-dependent effects on Raf dimerization.

Data provided above give a evidence that B-Raf pT401 is one of calcineurin target sites, I reasoned that B-Raf T401A would be resistant to inhibitory effects on B-Raf activity by inactive calcineurin and found that it had higher basal activity (Fig.3-8E). The serum-stimulated activity of B-Raf T401A was also unaltered by inactive calcineurin, unlike the wild-type B-Raf. Therefore, calcineurin positively regulates B-Raf activity at least in part by dephosphorylating B-Raf pT401.

Dephosphorylation of B-Raf pT401 by calcineurin promotes B-Baf activation in β cells

I examined the impact of glucose and inhibitors on regulation of pT401 and B-Raf activity in β cells. Both glucose and the calcineurin inhibitor FK506 increased phosphorylation of B-Raf T401 (Fig. 3-9A), consistent with the conclusion that T401 is a site of glucose-stimulated phosphorylation that is dephosphorylated by calcineurin. To identify that phosphorylation of T401 on B-Raf inhibits its activity in β cells, I expressed B-Raf T401A in Min6 cells and found that it had higher basal activity (Fig. 3-9B). Furthermore, glucose-induced B-Raf T401A activity was not impaired by FK506 in comparison to that of wild-type B-Raf.

Finally, I found blocking of ERK1/2 activation with U0126 reduces B-Raf pT401 (Fig.3-9C), supporting the conclusion that ERK1/2 phosphorylates B-Raf on T401 in response to glucose. To confirm if pT401 on B-Raf is a inhibitory site for B-Raf activity in β cells, I assessed the activation of B-Raf T401A in response to glucose and FK506. As expected, B-Raf T401A presented the higher basal activity and glucose-induced B-Raf T401A activity was not impaired by FK506 (Fig.3-9D). Similar data was also detected in 293 cells. These data support the conclusion that calcineurin positively regulates B-Raf activity by reversing ERK1/2 mediated negative feedback phosphorylation of B-Raf T401.

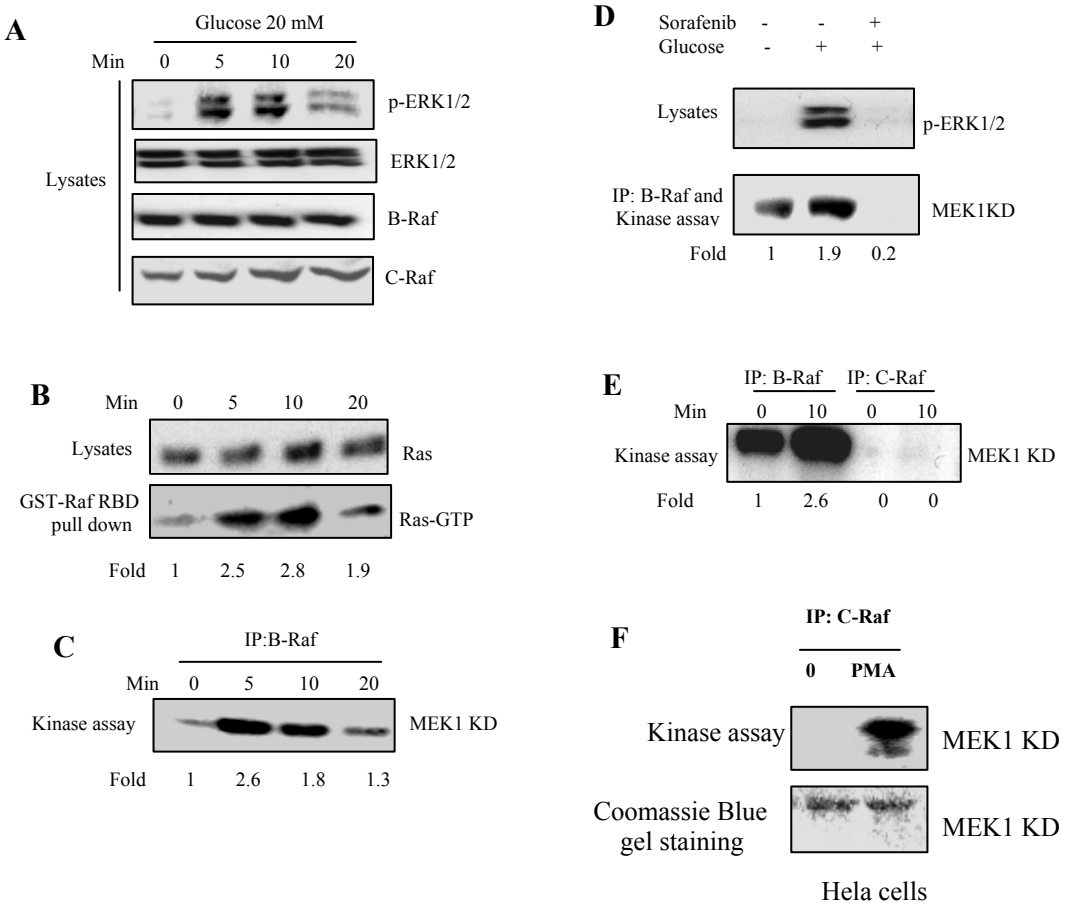


Figure 3-1. Glucose-induced activation of ERK1/2 occurs through Ras and B-Raf in pancreatic β cells. Min6 cells were starved for 2 hours in KRBH buffer containing 2 mM glucose, followed by stimulation with 20 mM glucose for indicated time periods (A, B, C and E). (A) Phosphorylated ERK1/2, ERK1/2, B-Raf, C-Raf in whole cell lysates were determined by western blots. (B) Ras-GTP was pulled-downed by GST-B-Raf–RBD protein and the level of Ras-GTP was detected by western blots. The level of Ras-GTP was quantified with a ImageJ software and is expressed as fold changes. Similar results were seen with three independent experiments. (C) Endogenous B-Raf proteins were immunoprecipitated by anti-B-Raf antibodies. In vitro Raf kinase activity was analyzed by autoradiography. One representative experiment of three is shown. Relative fold changes of Raf activites are showed in the bottom. (D) Min6 cells starved with KRBH buffer were pretreated with 10 μm Sorafenib for 20 min prior to stimulation with 20 mM glucose for 10 min. Cell lysates were used for western blots and B-Raf kinase assays were performed as descriptions. (E) Endogenous B-Raf or C-Raf proteins were immunoprecipitated respectively by anti-B-Raf or anti-C-Raf antibodies from an equivalent amount of cell extracts. Rafs activity assays were performed. Similar results were obtained in two independent experiments. (F) Hela cells were starved in free-serum media for 6 hours and stimulated by 100 nM PMA for 10 min. C-Raf kinase assays were performed.

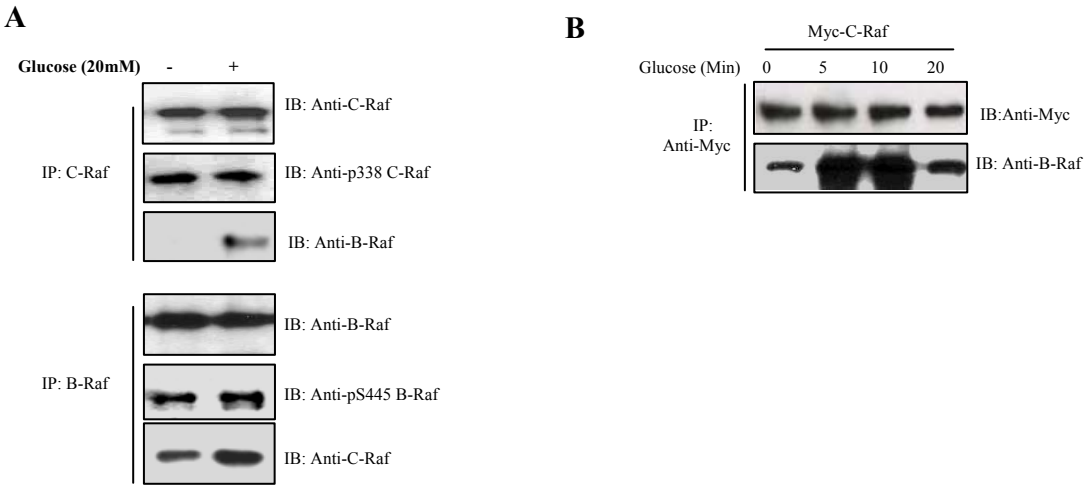


Figure 3-2. B-Raf/C-Raf heterodimerization is stimulated by glucose. (A) Min6 cells were starved in KRBH (2 mM glucose) for 2 hours and then stimulated with 20 mM for 10 min. Endogenous C-Raf or B-Raf proteins were immunoprecipitated and precipitations were detected by western blots to identify bound proteins or phosphorylatory sites on Rafs. All results were repeated for three times. (B) Min6 cells transfected with myc-C-Raf plasmids were starved in KRBH buffer (2 mM glucose) and then stimulated with 20 mM glucose for indicated time points. Myc-C-Raf in lysates was immunoprecipitated by anti-myc antibodies and the presence of B-Raf in precipitation was detected by western blots.

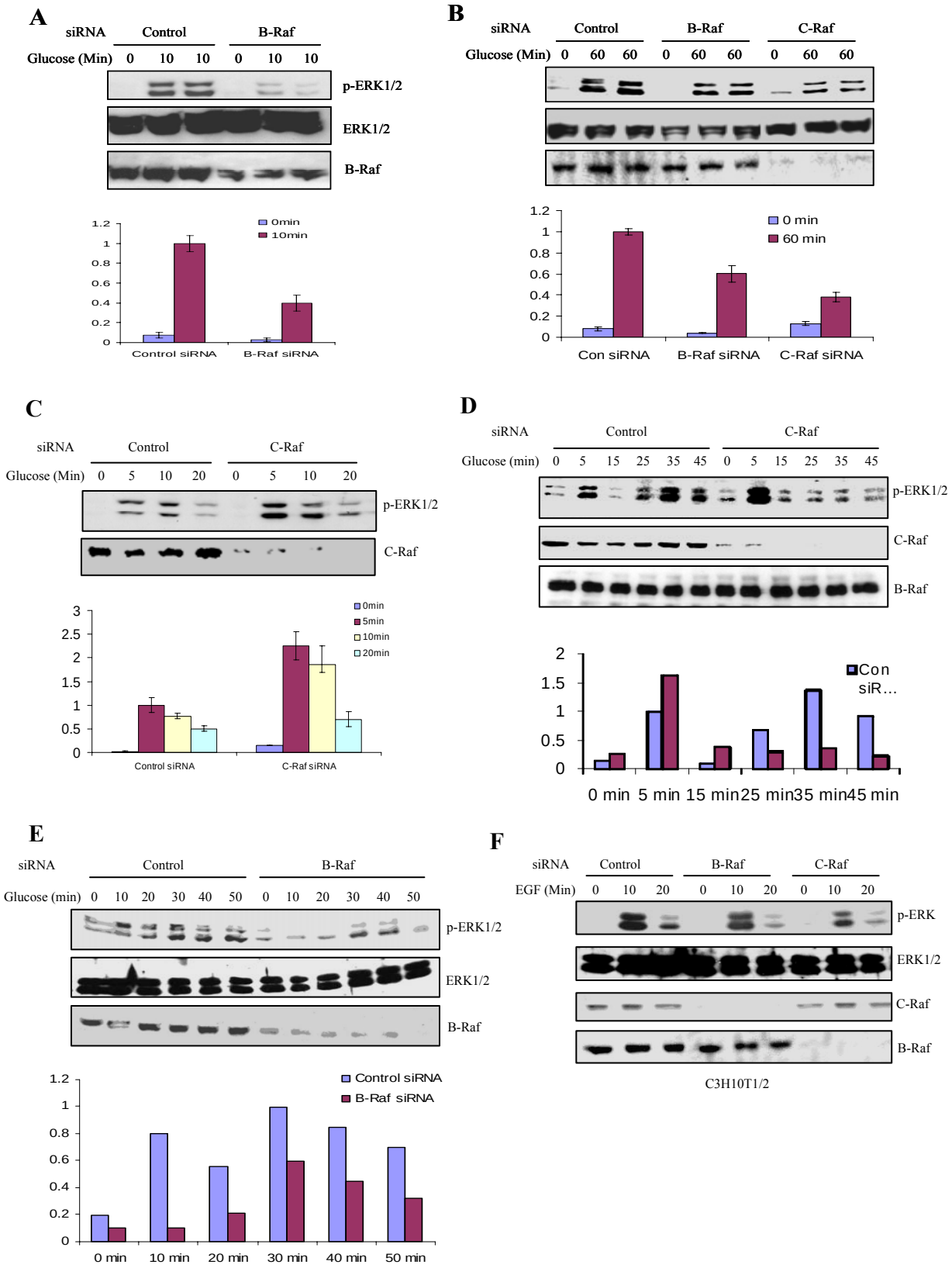


Figure 3-3. Effects of B-Raf and C-Raf siRNA knockdown on ERK1/2 activation in β cells. (A-E) Min6 cells were transiently transfected with indicated siRNA

oligonucleotides. 2 days after transfection, Min6 cells were starved in the KRBH buffer for 2 hours (A, C, D and E) or in free-glucose DMEM media for 4 hours (B) and followed by stimulation with 20 mM glucose for indicated time points. Cell lysates were collected for western blots. Phosphorylatory ERK1/2 was quantified with a ImageJ software and is expressed as fold changes. Results of A, B and C are repeated for three times and expressed as means ± S.E.M. (n = 3). Results of D and E were one representative experiment of two. (F) C3H10T1/2 cells were transfected with siRNA for 2 days, and starved in free-serum DMEM medium for 6 hours before stimulation with 20 ng EGF for indicated time points. Cell lysates were collected for western blots.

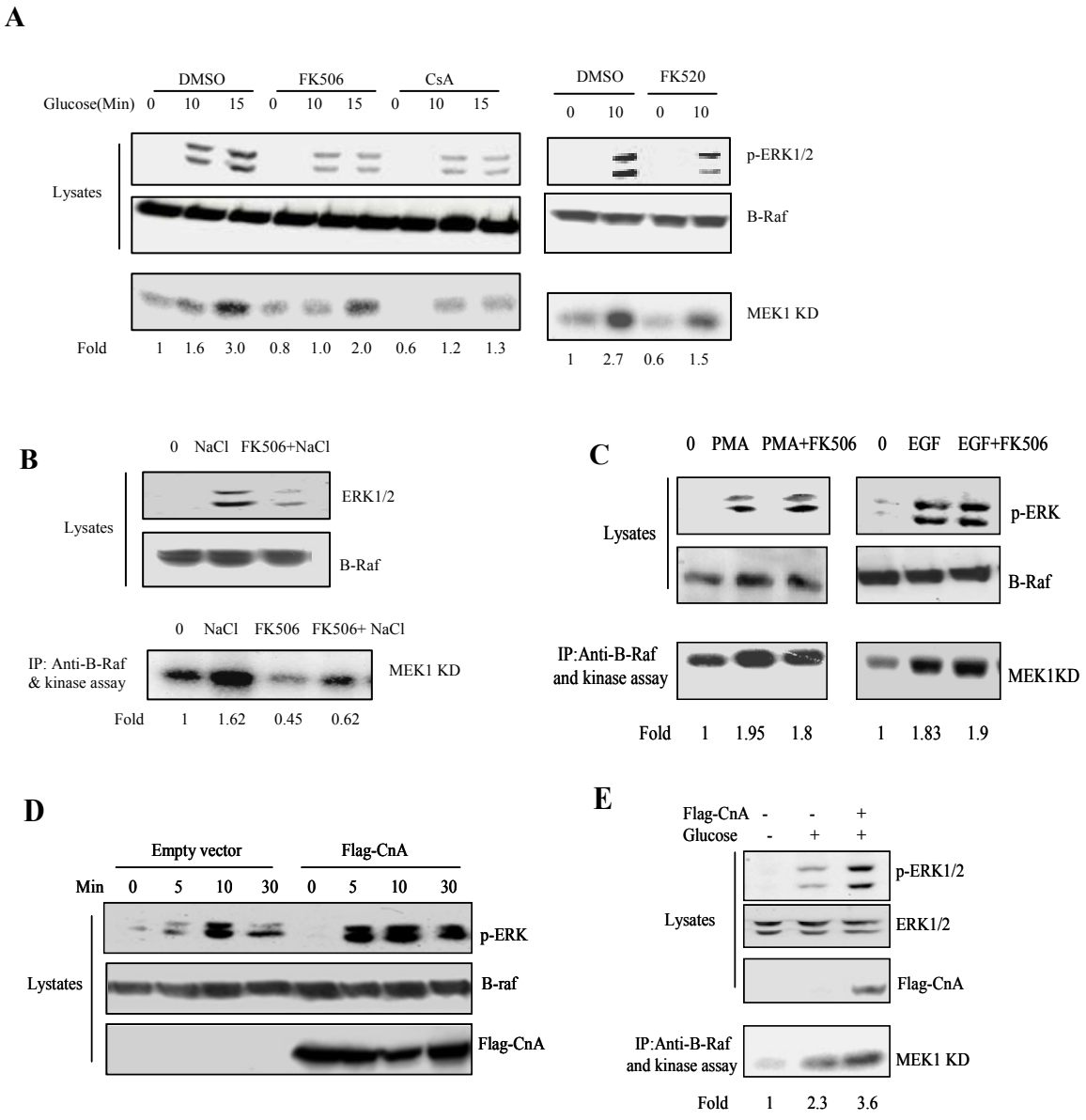


Figure 3-4 Ca^{2+}-dependent phosphatase calcineurin (CnA) selectively regulates activation of the B-Raf/MAPKs cascade in β cells. (A and B) Calcineurin inhibitors reduced ERK1/2 and B-Raf activation upon glucose and NaCl stimuli. Starved Min6 cells were pretreated with DMSO (as a control) or 200 nM FK506, or 100 nM FK520, or 10 uM Crosporin A (CsA) for 20 min before stimulation with 20 mM glucose as indicated time points (A) or with 0.25 M NaCl for 10 min (B). The level of p-ERK1/2 was analyzed by western blots and B-Raf activity assays were performed as descriptions. (C) FK506 did not suppress B-Raf activity upon PMA or EGF stimulation in nutrient-deficient condition. Starved Min6 cells were pretreated with or without FK506 and then stimulated with 100 nM PMA or 20 ng EGF for 10 min. Results were repeated twice. (D and E) Min6 cells were transfected with pCMV5 empty vectors or Flag-CnA plasmids for 2 days. Effects of exogenous calcineurin on glucose-induced ERK1/2 activity were

analyzed by western blots and effects of exogenous CnA on B-Raf activation were analyzed by in vitro Raf kinase assays. All results were repeated twice.

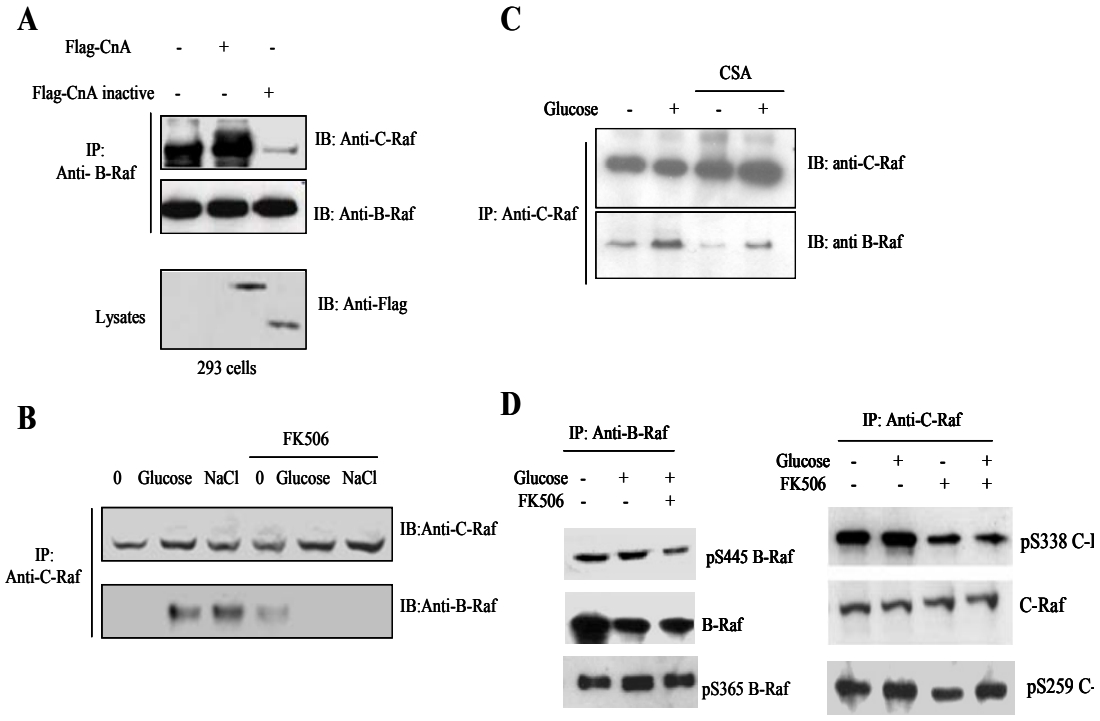


Figure 3-5. Calcineurin (CnA) contributes to B-Raf/C-Raf heterodimerization. (A) Effects of exogenous calcineurin on B-Raf/C-Raf heterodimerization. 293 cells were transiently transfected with wild-type or inactive calcineurin plasmids for 2 days. Cell lysates were used for immunoprecipitation and precipitation was detected by western blots. (B and C) Calcineurin inhibitors FK506 and CsA suppressed B-Raf/C-Raf complexes formation in Min6 cells. Starved cells were stimulated with 20 mM glucose or 0.25M NaCl for 10 min in presence or absence of FK506 or CsA pretreatment. Lysates were used for immunoprecipitation. All results were repeated for three times. (D) FK506 unaltered the levels of pS445 and pS365 on B-Raf or pS259 on C-Raf in Min6 cells. C-Raf or B-Raf was respectively immunoprecipitated by anti-C-Raf or anti-B-Raf antibodies and precipitates were detected by western blots

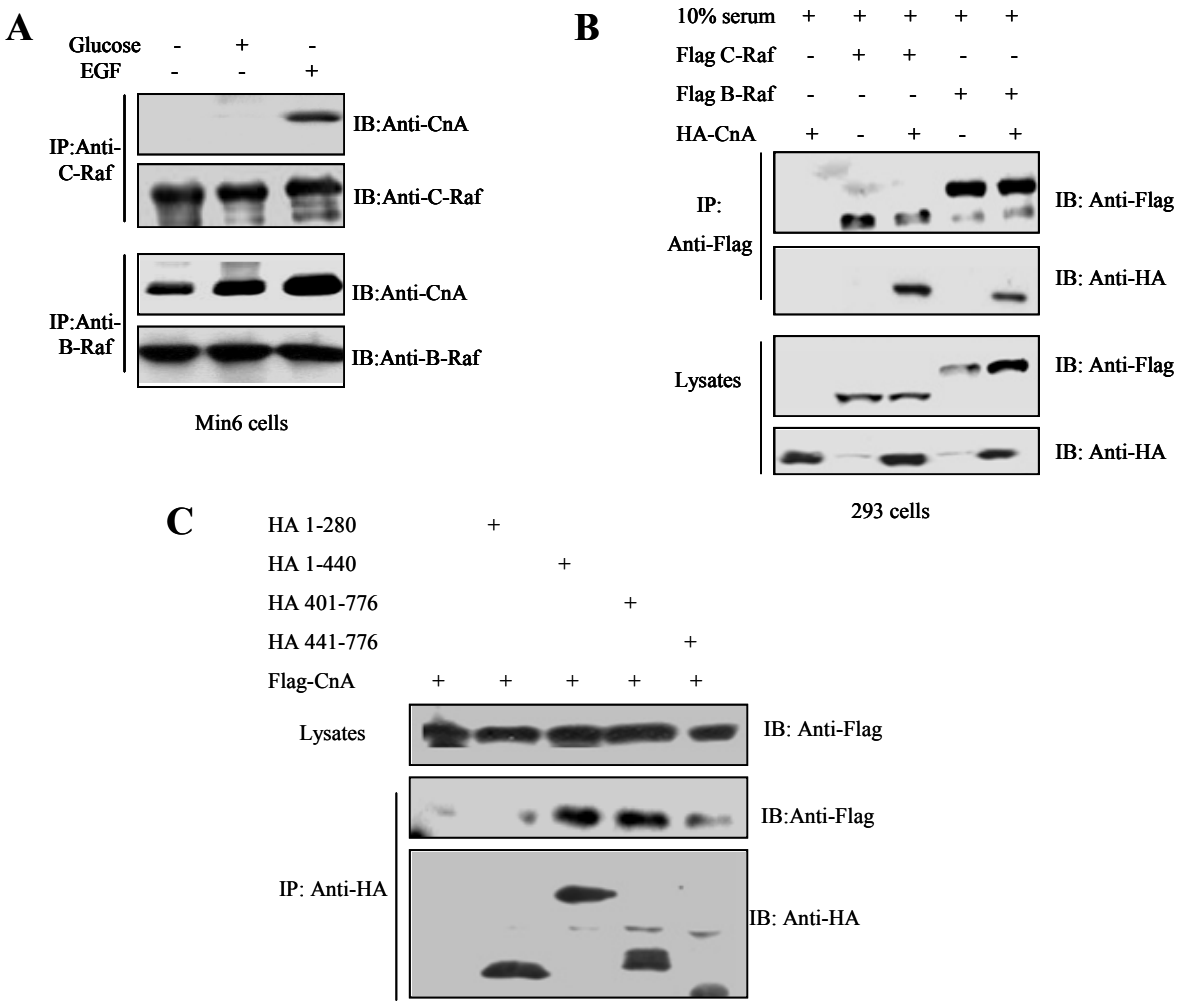


Figure 3-6. Interaction of B-Raf with calcineurin (CnA) in vivo. (A) Interaction of CnA with B-Raf and C-Raf in β cells. Starved Min6 cells were treated with 20 ng EGF for 10min or 10 mM glucose for 2 hour. Cells were lysed and co-immunoprecipitation was performed. (B) Association of CnA with B-Raf or C-Raf in 293 cells. CnA plasmids were co-transfected with B-Raf or C-Raf plasmids into 293 cells. 2 days after transfection, cells were harvested and immunoprecipitation was performed. (D) Showed that the middle region of B-Raf (280-440) is essential for B-Raf/calcineurin interaction. 293 cells were co-transfected with flag-tagged calcineurin and HA-tagged B-Raf truncations. Lysates were used for immunoprecipitation and precipitation was detected by western blots.

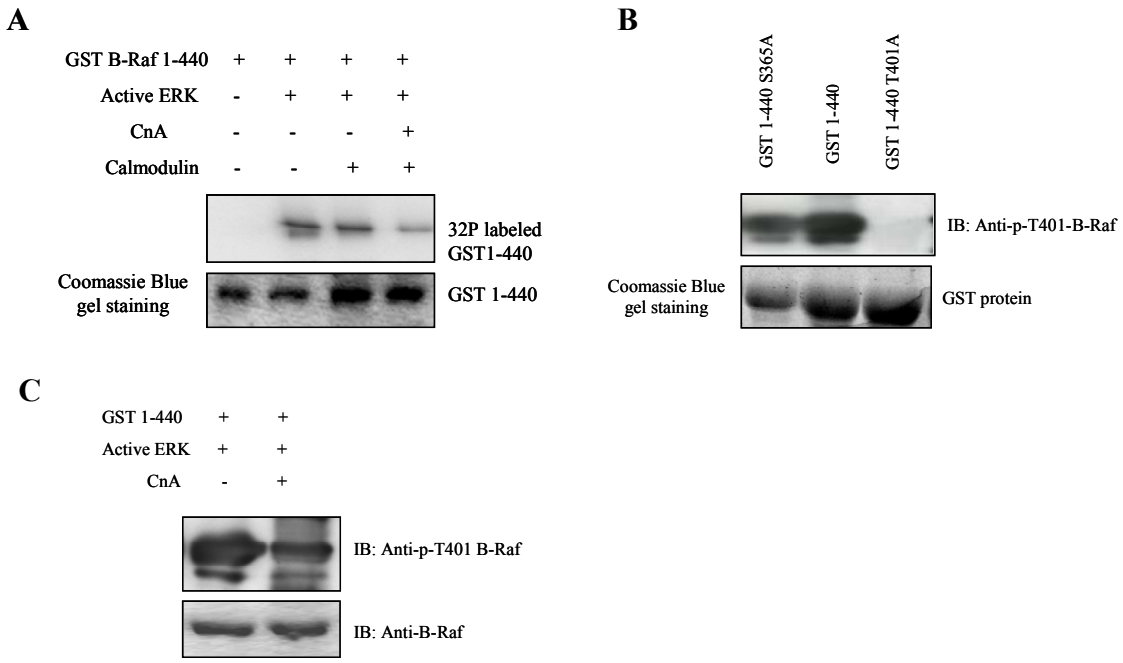


Figure 3-7. pT401 on B-Raf can be dephosphorylated by calcineurin (CnA) in vitro. (A) GST-B-Raf 1-440 fusion proteins were expressed in *E. coli* and purified on glutathione-Sepharose. To label ^{32}P, GST fusion proteins were in vitro phosphorylated by active ERK1. For in vitro dephosphorylation assay, ^{32}P labeled fusion proteins were repurified on GST-beads and incubated with or without calcineurin in the reaction buffer. The proteins were separated by SDS-PAGE and visualized by autoradiography. Loaded GST-B-Raf 1-440 proteins were confirmed by Coomassie blue staining. (B) GST-B-Raf 1-440 fusion proteins were in vitro phosphorylated by active ERK in the kinase reaction buffer (10 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 5 μM ATP, 1 mM dithiothreitol). The level of pT401 on B-Raf was analyzed by western blots with anti-pT401 B-Raf antibodies. (C) Phosphorylated substrates were incubated with calcineurin and the level of pT401 on B-Raf was analyzed by western blots.

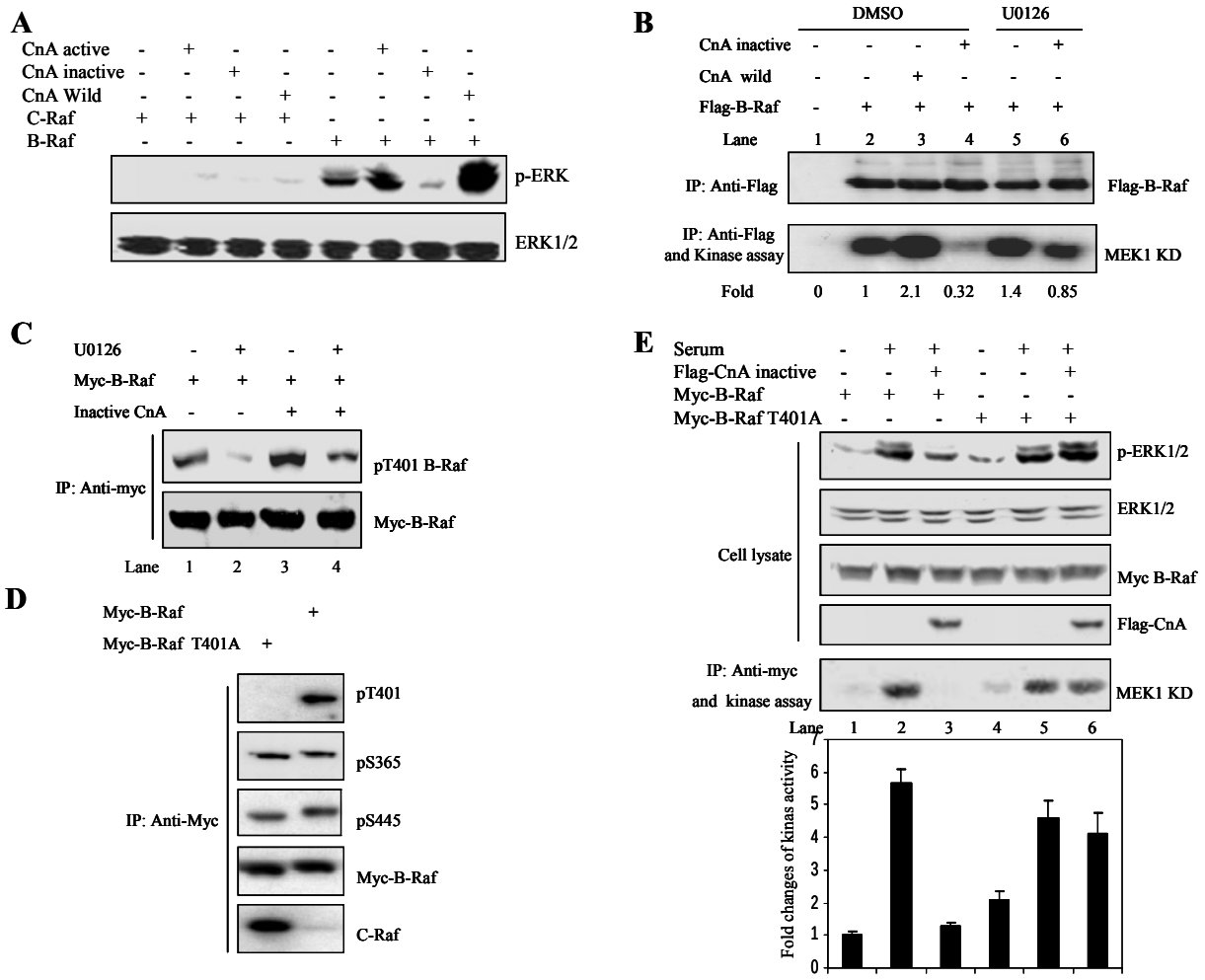


Figure 3-8. Calcineurin is required for the maximal activation of B-Raf in 293 cells. (A) 293 cells were transiently transfected with indicated expression plasmids. 2 days after transfection, cells were harvested and pERK1/2 was examined by western blots. (B) Transfected 293 cells were treated with DMSO (as a control) or 10 μm U0126 for 2 hours. Flag-B-Raf was immunoprecipiated by anti-Flag antibodies and in vitro Raf kinase assays were performed as descriptions. Fold changes of Raf activities are showed in lower panel. Results were repeated three times. (C) 293 cells transiently transfected with indicated plasmids were treated with DMSO or 10 μm U0126 for 2 hours. Myc-B-Raf was immunoprecipitated by anti-myc antibodies and precipitates were examined by western blots with anti-myc antibodies and anti-pT401 B-Raf antibodies. One representative experiment of three is shown. (D) 293 cells were transiently transfected with myc-B-Raf or myc-B-Raf T401A plasmids for 2 days. Cell lysates were immunoprecipiated by anti-myc antibodies and precipitates were examined by western blots. (E) Serum-induced activation of the B-Raf T401A mutant was not inhibited by inactive CnA. 293 cells transfected with indicated expression plamids were starved in serum-free DMEM media for 6 hours and then stimulated 5% serum for 15 min. The level of pERK1/2 in lysates was examined by western blots. Myc-B-Raf and myc-B-Raf

T401A were immunuoprecipitated by anti-myc antibodies for in vitro kinase assays. The results of kinase activities are expressed as means ± S.E.M. (n = 3) and are described as fold increase. A asterisk indicates a p value <0.01.

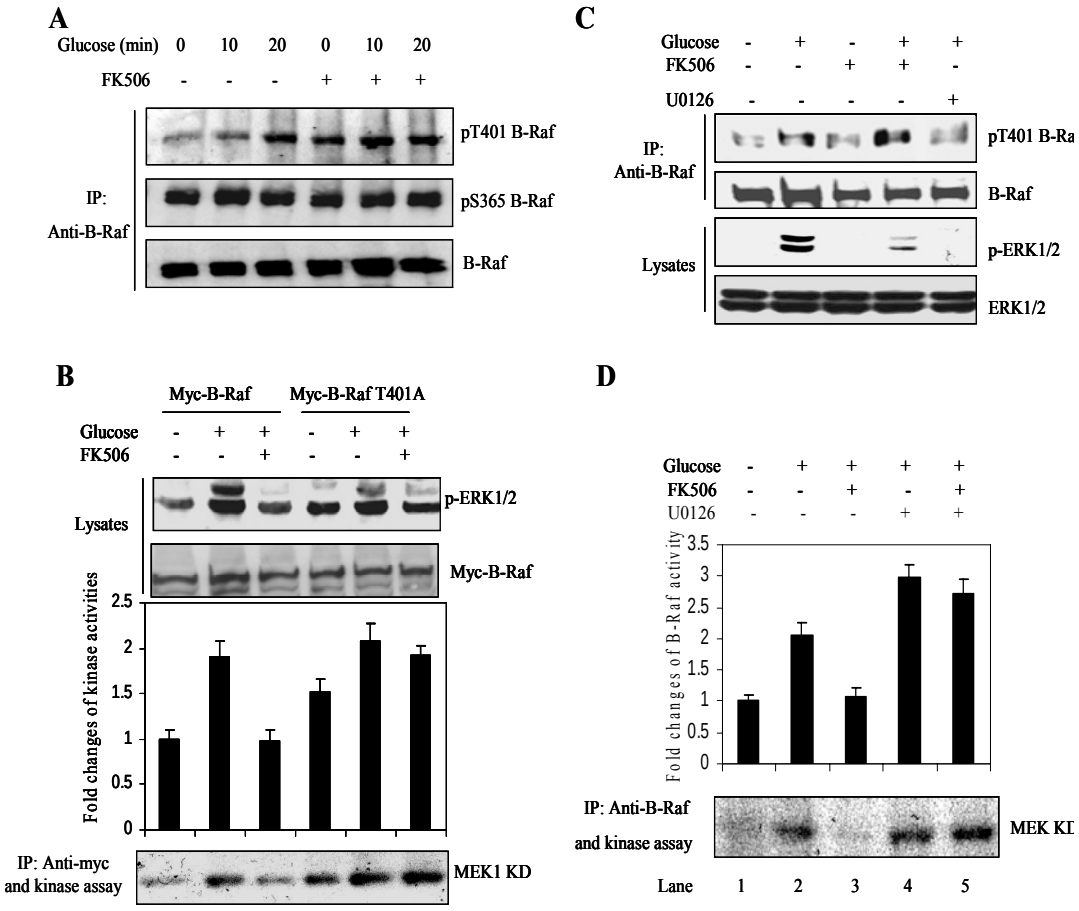


Figure 3-9. Calcineurin up-regulates B-Raf activation via dephosphorylating pT401 on B-Raf in β cells. (A) Impacts of FK506 on B-Raf T401 phosphorylation in β cells. Min6 cells were starved in free-glucose DMEM media for 6 hours and followed by stimulation with glucose for 15 min in presence or absence of FK506. (B) B-Raf T401A was resistant to FK506 negative impacts on B-Raf activity. Min6 cells transfected with myc-B-Raf or myc-B-Raf T401A were starved with KRBH buffer and followed by stimulation with 20 mM glucose for 15 min in the presence or absence of FK506 pretreatment. (C and D) Min6 cells were starved in free-glucose DMEM media for 6 hours and followed by stimulation with glucose for 15 min in presence or absence of FK506 or/and U0126 pretreatment. Cell lysates were immunoprecipitated by anti-B-Raf antibodies for detection of pT401 B-Raf and in vitro B-Raf kinase activity. The results of B-Raf activity expressed as means ± S.E.M. (n = 3) and are described as fold increase.

V. Discussion

Calcium influx triggered by glucose metabolism, activates the Raf/MEK/ERK1/2 cascade and the Ca^{2+}-dependent phosphatase calcineurin in β cells. ERK1/2 and calcineurin are both required for promotion of insulin gene expression via activating essential transcription factors. In addition, ERK1/2 can regulate insulin protein synthesis in β cells through regulating activation of p70 S6 kinase (S6K) and inactivation of glycogen synthase kinase 3β (GSK3 β) (Fig. 3-10) (84, 85). In this study, I found that B-Raf is a main Raf isoform responsible for glucose-induced ERK1/2 activation in β cells. Improtantly, I newly reported that glucose promotes B-Raf activity as well as B-Raf/C-Raf heterodimerzation in a calcineurin-dependent manner.

Two Raf isoforms, B-Raf and C-Raf are abundantly expressed in β cells, but my studies showed that B-Raf, but not C-Raf, is the dominant ERK1/2 activator in β cells. B-Raf deletion by siRNA strongly reduces the immediate and sustained ERK1/2 activation by glucose, while C-Raf deletion enhances the basal and transient ERK1/2 activation but reduces the late phase of ERK1/2 activation. Therefore, B-Raf and C-Raf cooperatively contribute to the late phase of glucose-initiated ERK1/2 activation. A cooperation of B-Raf and C-Raf in ERK1/2 activation is also reported in BCR-signaling pathway in B cells (64). In β cells, the growth factor EGF triggers transient dephosphorylation of pS259 on C-Raf and pS365 on B-Raf that presents Rafs translocation from cytosol to the plasma. But dephosphorylation of pS259 on C-Raf and pS365 on B-Raf is not initiated by glucose in β cells. Indeed, I observed that B-Raf activity strongly exists in cytosol in β cells (data not shown). Raf activation and membrane translocation is mediated by GTP loading Ras, while merely Ras is not sufficient to activate all Raf isoforms. It is reported that B-Raf

can be activated by only Ras, but C-Raf activation needs Ras and other cofactors. Therefore, we propose that glucose-metabolism is not sufficient to activate C-Raf kinase due to lack of other cofactors that are required for to plasma membrane recruitment.

Novel studies discovered that the wild-type B-Raf and the mutant B-Raf can activate C-Raf by forming B-Raf/C-Raf heterodimers, which is well explained by the theory that a side-to-side interface on heterodimers is necessary for triggering and transducting Raf activation (61). It is believed that B-Raf and C-Raf heterodimers possess higher kinase activity toward MEK1/2 than Raf homodimers, and impaired B-Raf mutants can transduct signal to MEK1/2 by utilizing C-Raf (57,58). I observed that glucose metabolism-triggered Ca^{2+} influx promotes the formation of B-Raf/C-Raf heterodimers even in the condition without growth factors. My data also suggest that B-Raf/C-Raf heterodimerization may not always contribute to promotion of ERK/MAPK activity because C-Raf siRNA knockdown in β cells, reversely enhances activation of ERK1/2 in the short time of glucose treatment. Combined with recent reports that C-Raf suppresses B-Raf^{V600E} –induced-ERK1/2 activation via formation of B-Raf^{V600E} /C-Raf heterodimers (24), it is reasonable that C-Raf is able to modulate the wild type B-Raf-mediated signaling transduction in some condition.

I found that the calcium-dependent phosphatase calcineurin contributes to B-Raf activity as well as B-Raf/C-Raf complexes formation in β cells. Several studies demonstrated that Raf activity is dynamically modulated by multiple interactive proteins, lipids, phosphorylation and dephosphorylation events. The cellular phosphatase, PP2A is a key factor for up-regulating Rafs activity and assembling C-Raf/KSR1 complexes in response to growth factors (66, 86). PP2A, associated with Raf, can promote Raf

activation by dephosphorylating inhibitory sites of B-Raf pS365 or C-Raf pS259 (87). I did not observe that the PP2A inhibitor, okadaic acid (OA), has impacts on activation of B-Raf and its downstream ERK1/2 in glucose signaling pathway. In contrast, B-Raf activation triggered by the calcium signal is suppressed by calcineurin inhibitors FK506 and CsA in β cells. So I concluded that calcineurin, but not PP2A is required for B-Raf activation by glucose. In addition, we found FK506 did not alter Ras activity, while CsA minorly reduced Ras activity upon glucose (data not shown). Thus, these founding imply that suppression of B-Raf activity by FK506 may be not via regulation of Ras activation. Different impacts of FK506 and CsA on Ras activity are probably correlated with their different binding partner FK506 binding protein (FKBP) and cyclophilin. I also assessed potential target sites on B-Raf that can be catalyzed by calcineurin. In B-Raf, four sites phosphorylated by ERK1/2 have been identified, including S151, T401, S750 and T753 utilizing massspectrometry assays and mutants assays (46). It was verified that the mutants of ERK1/2 phosphorylation sites on B-Raf increase the basal activity of B-Raf as well as B-Raf/C-Raf heterodimerzation. So I specially focused on ERK1/2 phosphorylation sites in B-Raf and found that pT401 on B-Raf is one of target sites of calcineurin. More experiments still needs to be conducted to verify if other ERK1/2 phosphorylation sites in B-Raf such as pS151, pS750 and pT753 are also targeted by calcineurin.

Taken together, my finding revealed that calcineurin participates in the regulation of the Raf/MAPKs cascade through modulating B-Raf activation. Calcineurin antagonizes ERK1/2-mediated-feedback phosphorylation on B-Raf, thereby promoting B-Raf activity and B-Raf/C-Raf heterodimerization. The cross-talk between the

Raf/MAPKs cascade and calcineurin benefits to maximal activation of insulin gene transcription factors according to strength and duration of calcium influx (Fig. 3-11).

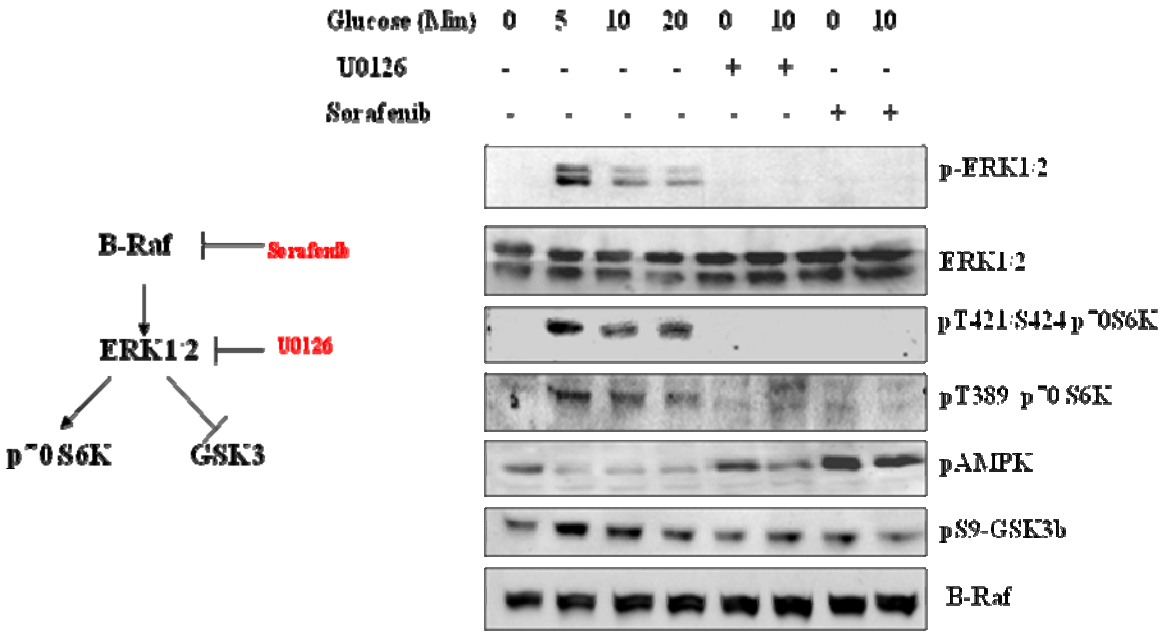


Fig. 3-10. Regulation of p70 S6 Kinase (S6K) activation by ERK1/2 in the glucose signaling pathway. Min6 cells were starved in KRBH buffer for 2 hour. After pretreatment with 10 uM U0126 or 10 uM sorafenib for 20 min, Min6 cells were stimulated with 20 mM glucose for 10 min. Cell extracts were prepared for immunoblots.

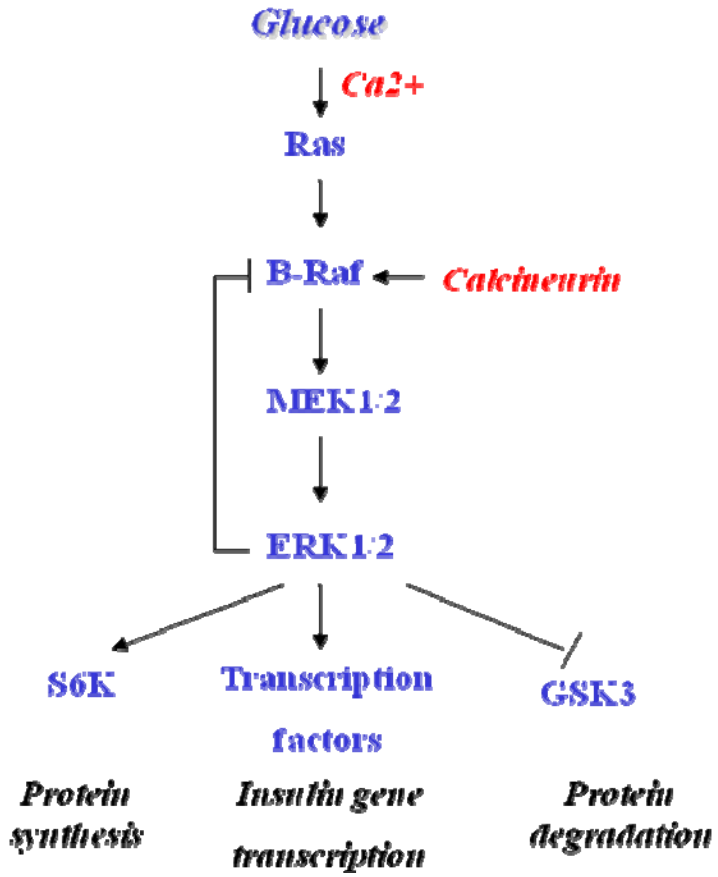


Fig. 3-11. Model for regulation of B-Raf activation by calcineurin in the glucose signaling pathway.

Chapter 4: The role of Raf kinases and the scaffold protein KSR2 in the regulation of β-cell function and proliferation

I. Abstract

Kinase suppressors of Ras 1 and 2 (KSR1 and KSR2) are known for their function as molecular scaffolds of Raf/MEK/ERK1/2 cascades, facilitating Ras signaling transduction and positively regulating ERK1/2 activation. KSR proteins, as Raf scaffolds, are involved in regulation of cell differentiation, proliferation and survival. Further study revealed that KSR proteins interact with multiple other proteins, not only core components of the Raf signaling cascade. For examples, KSR2 interacts with and modulates AMPK, thereby regulating glucose and lipid metabolism. This study shows that KSR2, but not KSR1, is abundantly expressed in pancreatic β cells. KSR2 contributes to regulation of glucose-induced ERK1/2 activation. Despite positive effects of KSR2 on glucose-induced ERK1/2 activation, my data suggest that KSR2 may be not an essential regulator of insulin synthesis. Finally, I show that KSR2, together with B-Raf and C-Raf kinases, is involved in regulation of β-cell proliferation.

II. Introduction

The Raf/MEK/ERK signaling cascade plays an essential role in the regulation of cell differentiation, proliferation and metabolism (43). Raf kinases are the center of the Raf/MEK/ERK signaling cascade, and regulated by the Ras small GTPase as well as other proteins, kinases, phosphatases and scaffolding proteins. Several Raf interacting scaffold proteins have been reported, including kinase suppressor of Ras (KSR), connector enhancer of KSR (CNK), Raf kinase inhibitor protein (RKIP) (RKIP) (45, 88). Among them, the KSR family has been extensively studied and shown to positively regulate ERK1/2 activation. KSR1 was originally discovered in 1995 through genetic screens in Drosophila and C. elegans (89). Subsequently, analysis of the C. elegans genome further identified the second KSR isoform, KSR2. In C. elegans, functions of KSR1 and KSR2 are redundant and only double knockdown of KSR1 and KSR2 results in vulva defects. (valval induction explaining system a little). Both KSR isoforms, KSR1 and KSR2 have been identified in mice and humans. Mammalian KSR proteins, known as scaffold proteins of the Raf/MEK/ERK cascade, contribute to regulation of Ras signal transduction including cell proliferation, adipogenesis and tumorigensis. It is not a surprise that ablation of KSR1 with antisense oligonucleotides effectively inhibits the growth of pancreatic β tumor xenografts. KSR proteins not only function as Raf cascade scaffolds, but also regulate activation of other kinases including AMPK and GSK3. KSR2 specifically interacts with AMPK to regulate cellular thermogenesis, fat oxidation and glucose metabolism (90,91).

The structure of KSR proteins is similar to Raf family members, but KSR proteins lack kinase activity because an essential lysine residue in kinase domain is substituted by

an arginine residue (92). KSR proteins do not contain a Ras-binding domain (RBD) although active Ras appears to induce KSR protein activation and membrane translocation. Mass spectrometry identified that KSR proteins interact with many molecules and form a multi-protein complex (90,93). KSR proteins are thought to increase the efficiency and specificity of MAPKs ERK1/2 activation by multiple mechanisms. KSR proteins as scaffold proteins, directly interact with the core components of the Raf signaling cascade including Raf, MEK and ERK1/2, as well as many regulators of the Raf signaling cascade including phosphatases and other kinases, thereby facilitating Raf/MEK/ERK1/2 signaling. Furthermore, KSR proteins directly promote Raf kinase activity by forming a 14-3-3-dependent heterodimer. In addition, KSR proteins can regulate spatial activation of ERK1/2 by directing the localization of the cascade components. KSR1 and KSR2 share common binding partners including Raf, MEK and ERK1/2, but interact selectively with certain binding proteins. Mass spectrometry determined that calcineurin, AMPK and GSK3 are specially present in KSR2 complexes, and Raptor and BIRC6 are specially in KSR1 complexes. The selective binding of KSR1 and KSR2 to distinct proteins suggests that KSR1 and KSR2 may contribute to different cellular processes.

KSR proteins are widely present in most mouse and human tissues, but their expression levels are too low to be detected by immunoblotting in most of tissues (94). KSR proteins are highly expressed in neuronal cells and play roles in inducing neurite formation. In this study, I found that KSR2 is also abundantly expressed in β cells. To identify the role of KSR2 in regulation of β-cell function, I observed effects of KSR2 on insulin synthesis. The data show that different from B-Raf and C-Raf, KSR2 has little

effect on insulin synthesis. Finally, I showed that B-Raf, C-Raf and KSR2 are involved in regulation of β-cell proliferation.

III. Materials and methods

Cell culture and reagents. The insulinoma β-cell lines Min6, INS1, and the α-cell line αTC1 cells maintained as described (6).

Constructs and transfection. Constructs of pGL3-rIns1-Luc and pRL-SV40 have been described previously (6). Human C-Raf and B-Raf cDNAs were amplified by PCR and subcloned into pCMV5 myc-tag expression vector. For transfection of Min6 and INS1 cells, I used Fugene HD (Roche Molecular Biochemicals) according to manufacturer's protocol.

RNA isolation and detection by reverse transcription PCR.

Total RNA was isolated from the pancreatic β-cell line Min6 and the α-cell line αTC1 using TRI Reagent (Ambion, Austin, TX). The mRNA was reverse transcribed using High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). cDNA samples were frozen at –80°C until used. PCR amplification was carried out using 1 cycle of 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 55 °C for 30s, 72 °C for 1min, 1 cycle of 72 °C for 5 min. Primers used for PCR are shown as follows:

KSR2 Forward: 5'- AAC CTC TGA GTT TGC AAA AAG C-3'

Reverse: 5'- CTC CAT ACT TCT CCA CGG TCT C-3'

KSR1 forward: 5'-AGG ATC ACC TTC CTC CCA CT-3'

reverse: 5'-GTG GCA CTG GAG GGA TTA GA-3'

Insulin(ins1) forward: 5'- TGT TGG TGC ACT TCC TAC CC-3'

Reverse: 5'-CAC TTG TGG GTC CTC CAC TT-3'

Glucagon forward: 5'-CAG AG GAGA ACC CCA GAT CA-3'

Reverse; 5'-TGA CGT TTG GCA ATG TTG TT-3'

Real-Time Quantitative PCR.

Insulin and β actin mRNA levels were assessed by quantitative real-time PCR. β actin was used as an internal expression control. Real-Time quantitative PCR was performed on the ABI 7500 DNA Sequence Detection System, using iTaq SYBR Green Supermix with ROX kit (Bio-Rad). PCR was carried out in a final volume of 50 µl containing 1 µl of diluted cDNA sample, 10 µl of 5x reaction buffer, 1 µl 10mM dNTP mix, 2 ul of primers and 0.5 ul of iProof DNA polymerase. Thermal cycling conditions were specified by the manufacturer: 50°C for 2 min, 95°C for 10 min for one cycle, an additional 40 cycles at 95°C for 15 sec, and ramp to 60°C for 1 min.

Luciferase reporter assays.

Cells were plated in 12-well plates and co-transfected with either pGL3-rInsI and pRL-SV40 by using the FuGENE HD reagent (Roche Molecular Biochemicals). 48 h later, cells were harvested with passive lysis buffer (Promega). The lysates were vortexed for 30 s and the supernatants were collected following centrifugation for 30 min at 14,000 rpm at 4 °C in a microcentrifuge. The supernatants were utilized for promoter activity assays by the Dual Luciferase Assay System (Promega, WI) and a TD-20/20 bioluminometer (Turner Designs).

siRNA knockdown.

All siRNA oligonucleotides for targeting endogenous mouse B-Raf, C-Raf and KSR2 were purchased from Ambion; Random oligonucleotides were purchased from Dharmacon RNAi Technologies. siRNA oligonucleotides were as follows: C-Raf (siRNA ID: s99723), sense: CACGAUUCUUCUAAGACAtt antisense: UGUCUUAGAAGAAUCCGUGag B-Raf(siRNA ID: s99634), sense: CCACAGAUGCAUCACGGAAtt KSR2 (siRNA ID: s116943) sense: CCUCAGGAAUGUGCACAAAtt. siRNA oligonucleotides were transfected into cells utilizing Lipofectine maxi (Invitrogen) according to manufacturer's protocol.

Insulin ELISA.

Mercodia Insulin ELISA (human) kit was used to measure insulin contents. Min6 cells transfected with siRNA oligonucleotides for 3 d were washed with cold PBS and then harvested in 1% NP-40 lysis buffer. 20 μg of cell lysates were loaded per well.

Cell proliferation.

To detect the effect of siRNA knockdown on cell growth, Min6 cells (3.6×10^5/well) transfected with siRNA oligonucleotides were plated in 12 well plates. Cells were collected by trypsinization at 3, 5, 6 days. Cell numbers were measured by counting the number of trypan blue-excluding cells.

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.

IV. Results

Blocking B-Raf activity reduces insulin gene promoter activity in β cells

ERK1/2 activation is required for insulin synthesis, especially in high glucose (16 mM) (xx). Based on my observation that B-Raf is the major Raf kinase responsible for glucose-induced ERK1/2 activation in β cells, I asked if B-Raf, as an upstream activator of ERK1/2, is involved in regulation of insulin synthesis. The B-Raf mutant T598S601AA (B-Raf AA) is kinase dead form and is no longer activated by mitogenic signals. T598 and S601 residues are located in the kinase activation loop and their phosphorylation is essential for B-Raf activity (49). The data showed that overexpression of the B-Raf mutant T598S601AA effectively blocked ERK1/2 activation induced by glucose in both Min6 and INS1 cells (Fig. 4-1A). Next, I cotransfected wild-type B-Raf or the mutant B-Raf T598S601AA with insulin promoter constructs into β cells. Relative insulin promoter activities were effectively enhanced by expression of wild-type B-Raf, but reduced by expression of B-Raf AA in both Min6 and INS1 cell lines (Fig. 4-1B). This suggested that B-Raf participates in regulation of insulin biosynthesis through mediating ERK1/2 activation by glucose.

Effects of B-Raf and C-Raf siRNA knockdown on insulin synthesis

Previous data shoId that knockdown of B-Raf by siRNA reduces the transient and sustained ERK1/2 activation by glucose. Despite of the low C-Raf kinase activity in response to glucose treatment, knockdown of C-Raf by siRNA decreases the sustained ERK1/2 activation by glucose. This suggests that not only B-Raf, but C-Raf affects glucose-induced ERK1/2 activation. To know if both B-Raf and C-Raf contribute to insulin synthesis, I analyzed insulin mRNA and protein expression levels in Min6 cells transfected with B-Raf or C-Raf siRNA, respectively. I found that deletion of B-Raf or C-

Raf by siRNA effectively decreased the level of insulin mRNA and insulin protein in Min6 cells (Fig. 4-2A and 4-2B).

Identification of the MAPK scaffold protein KSR2 expressed in pancreatic β cells and effect of KSR2 in glucose-induced ERK1/2 activation

The scaffold KSR, positively regulates MAPKs ERK1/2 activation via assembling Raf/ MEK/ERK1/2 complexes. To identify the expression pattern of KSR1 and KSR2 in β cells, I examined KSR1 and KSR2 mRNA expression in a mouse pancreatic β cell line, Min6 and an α cell line, αTC1 by using semi-quantitative reverse transcriptase-PCR. My data shoId that the KSR2, not KSR1 gene was expressed in Min6 and αTC1 cells (Fig. 4-3A upper). To determine cDNA quantity, I detected insulin and glucagon mRNA expression because insulin and glucagon genes are regarded as markers for β and α cells, respectively. In my RT-PCR product, insulin mRNA was detected in Min6 cells, but not in αTC1. But I found that the glucagon gene was expressed not only in αTC1 cells, but also in Min6 cells. Another group also reported that the glucagon gene is expressed in insulinoma cells (95). I further examined KSR1 and KSR2 protein expression by using specific anti-KSR1 and KSR2 antibodies and immunoblotting (Fig. 4-3A bottom). Immunoblotting results supported that KSR1 and KSR2 are selectively expressed in different cell lines(Fig.4-3A bottom). I found that KSR2, but not KSR1 was expressed in the β cell lines Min6, INS1, and an α cell line aTC1. KSR1 expression level is very low in both β cell lines Min6 and INS1, but high in Hela cells.

Both KSR1 and KSR2 interact with three components of Raf/MEK/ERK1/2 cascade and positively regulate ERK1/2 activation. To investigate the effect of KSR2 on

glucose-induced ERK1/2 activation in β cells, I transfected wild-type KSR2 constructs into Min6 cells. Expression of exogenous KSR2 enhanced ERK1/2 activation and prolonged the duration of ERK1/2 activation in response to glucose treatment (Fig.4-3B). This supports that KSR2 contributes to ERK1/2 activation by glucose. The inhibitor U0126 effectively inhibits MEK activation, whereas calcineurin inhibitors FK506 and CsA can directly inhibit Raf activation. My data showed that the positive impact of KSR2 on ERK1/2 activation was blocked by the MEK inhibitor UO126 and attenuated by the calcineurin inhibitor FK506 (Fig.4-3C).

Effects of KSR2 siRNA knockdown on ERK1/2 activation induced by glucose and insulin biosynthesis

Next, I knocked down KSR2 expression by using siRNA oligonucleotides to further examine effects of KSR2 on glucose-induced ERK1/2 activation. Ablation of KSR2 expression by siRNA did not alter glucose-induced ERK1/2 activation at 5 min, but significantly decreased it at 15 min (Fig.4-4A). The data suggests that the positive role of KSR2 in regulation of ERK1/2 activation by glucose. I asked if KSR2 plays a role in the regulation of insulin synthesis. Surprisingly, overexpression of KSR2 did not obviously enhance the insulin promoter activity (Fig.4-4B). In addition, deletion of KSR2 by siRNA had no obvious effects on the expression levels of insulin mRNA and protein (data not shown). These data suggest that KSR2 may be not essential for regulation of insulin synthesis. Considering that glucose, not only stimulates acute insulin release, but also acts as a long term regulator of proliferation and survival for differentiated β cells,

KSR2 may promote β-cell proliferation by adjusting ERK1/2 activation induced by glucose.

Effects of B-Raf, C-Raf and KSR2 on β-cell proliferation

Growth factors acting through receptor tyrosine kinases (RTKs) including EGF, insulin, IGF, effectively up-regulate β-cell proliferation (96). Upon stimulation with these growth factors, the Raf/MEK/ERK1/2 signaling cascade is activated. As the scaffold protein KSR2 contributes to ERK1/2 activation and Raf kinases are upstream activators for ERK1/2 activation, I examined their effects on EGF-induced ERK1/2 activation by using siRNA oligonucleotides. In contrast to cells transfected with random siRNA oligonucleotides, cells transfected with B-Raf and KSR2 siRNA oligonucleotides shoId reduced ERK1/2 activation in response to EGF treatment (Fig.4-5A). In contrast, knockdown of C-Raf expression by siRNA resulted in constitutive ERK1/2 activation in β cells cultured under glucose-deficient conditions. As B-Raf and C-Raf have reverse effects on ERK1/2 activation by EGF, I considered the possibility that they have different roles in promoting β-cell proliferation. I found that knockdown of B-Raf and KSR2 by siRNA decreased β-cell proliferation (Fig.4-5B). In comparison to B-Raf and KSR2, C-Raf has less impact on β-cell proliferation. These studies suggest that B-Raf and KSR2 play more important roles than C-Raf in regulation of β-cell growth. As expression of C-Raf is required for cell survival, reduction of C-Raf expression by siRNA decreases β-cell numbers probably though activating apoptotic signaling pathways.

PDX-1 is one of transcription factors that is essential for maintaining β-cell function and proliferation (24). I considered the possibility if Raf signaling cascade

impacts PDX-1 expression. I showed that overexpression of KSR2 and C-Raf enhanced PDX-1 protein levels and ablation of B-Raf, C-Raf and KSR2 by siRNA reduced PDX-1 expression (Fig.4-5C and Fig.4-5D).

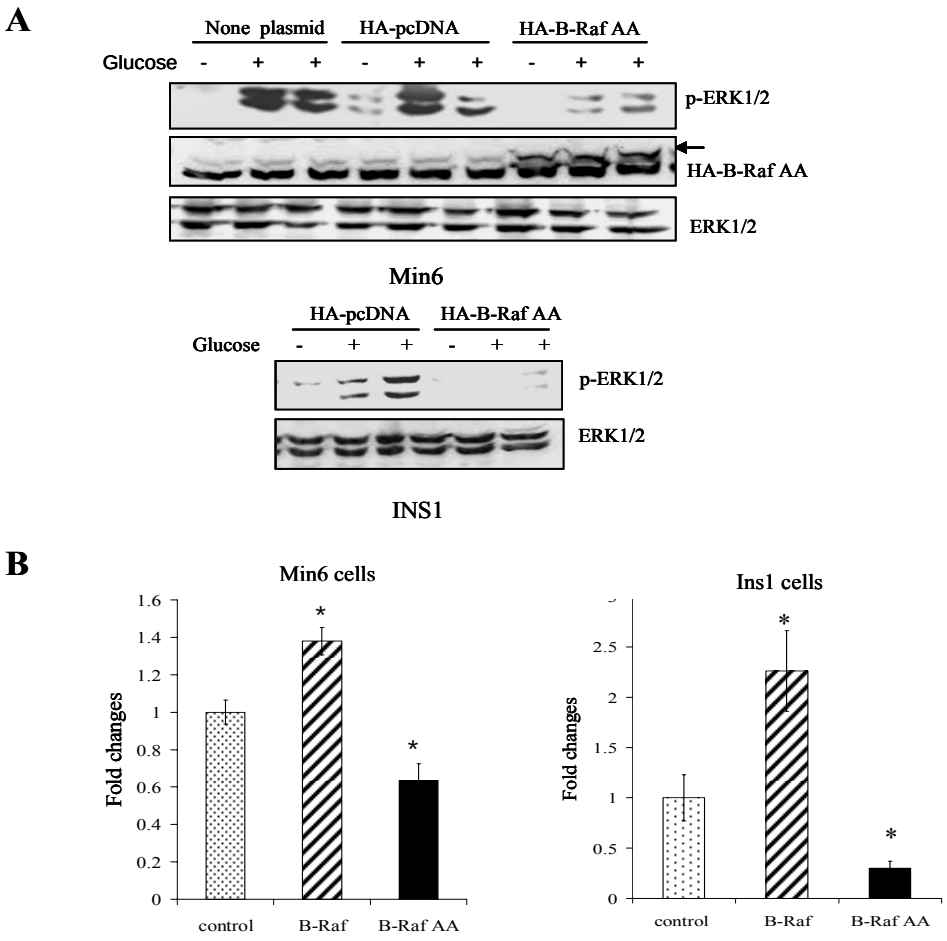


Figure 4-1. Effects of B-Raf AA on ERK1/2 activation and insulin gene promoter activity in β cells. (A) Expression of inactive B-Raf AA inhibited ERK1/2 activation upon glucose stimulation. Mouse β cell line Min6 and rat β cell line INS1 were transfected with HA-B-Raf AA (kinase dead) constructs or empty vectors for 3 days. Cells were starved with KRBH buffer (2mM glucose) for 2 hours, then stimulated with 20 mM glucose for 10 min. The level of ERK1/2 phosphorylation was analyzed by western blots. (B) Effects of wild-type B-Raf and mutant B-Raf AA on rat I insulin promoter activity. Cells were cotransfected with rIns1-Luc, pRL-SV40 and the indicated expression plasmids for 2 days. The activation of rIns1 promoter was reflected by luciferase activity normalized to SV40 promoter activity. Results are expressed as means ± S.E.M. (n = 3) and are described as fold increase. Statistical significance was analyzed using a two-tailed t-test with p-values as indicated. An asterisk indicate a p value <0.01.

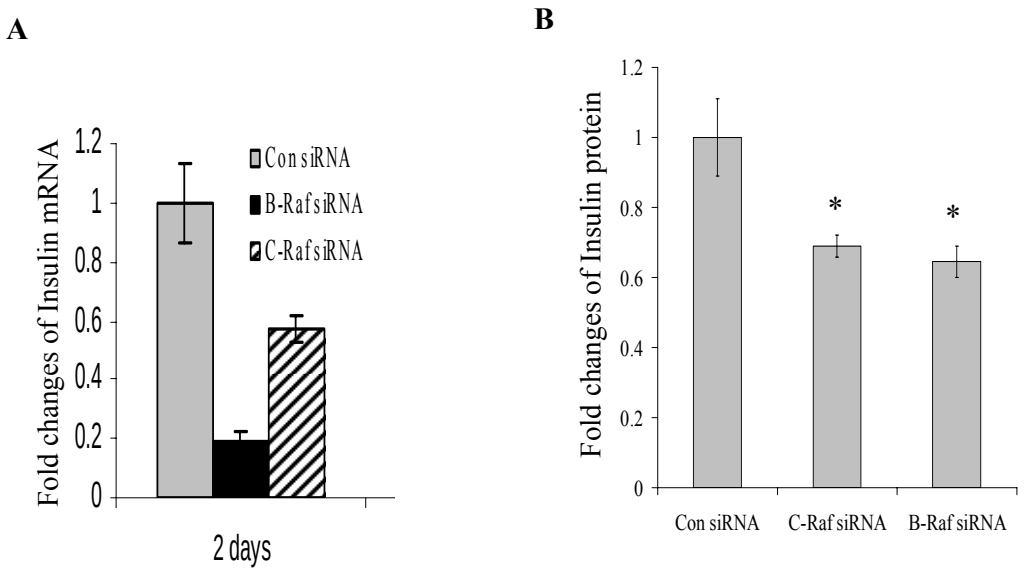


Figure 4-2. Effects of B-Raf and C-Raf siRNA knockdown on insulin biosynthesis. (A) Min6 cells were transiently transfected with control, B-Raf, or C-Raf siRNA for 2 days. The levels of insulin mRNA were analyzed by real-time RT-PCR (*n* = 3). (B) Min6 cells were transfected with indicated siRNA for 3 days. The level of insulin protein in lysates was measured by ELISA kit (n=3). These results are expressed as means ± S.E.M. (n = 3) and are described as fold increase. An asterisk indicates indicate a p value <0.01.

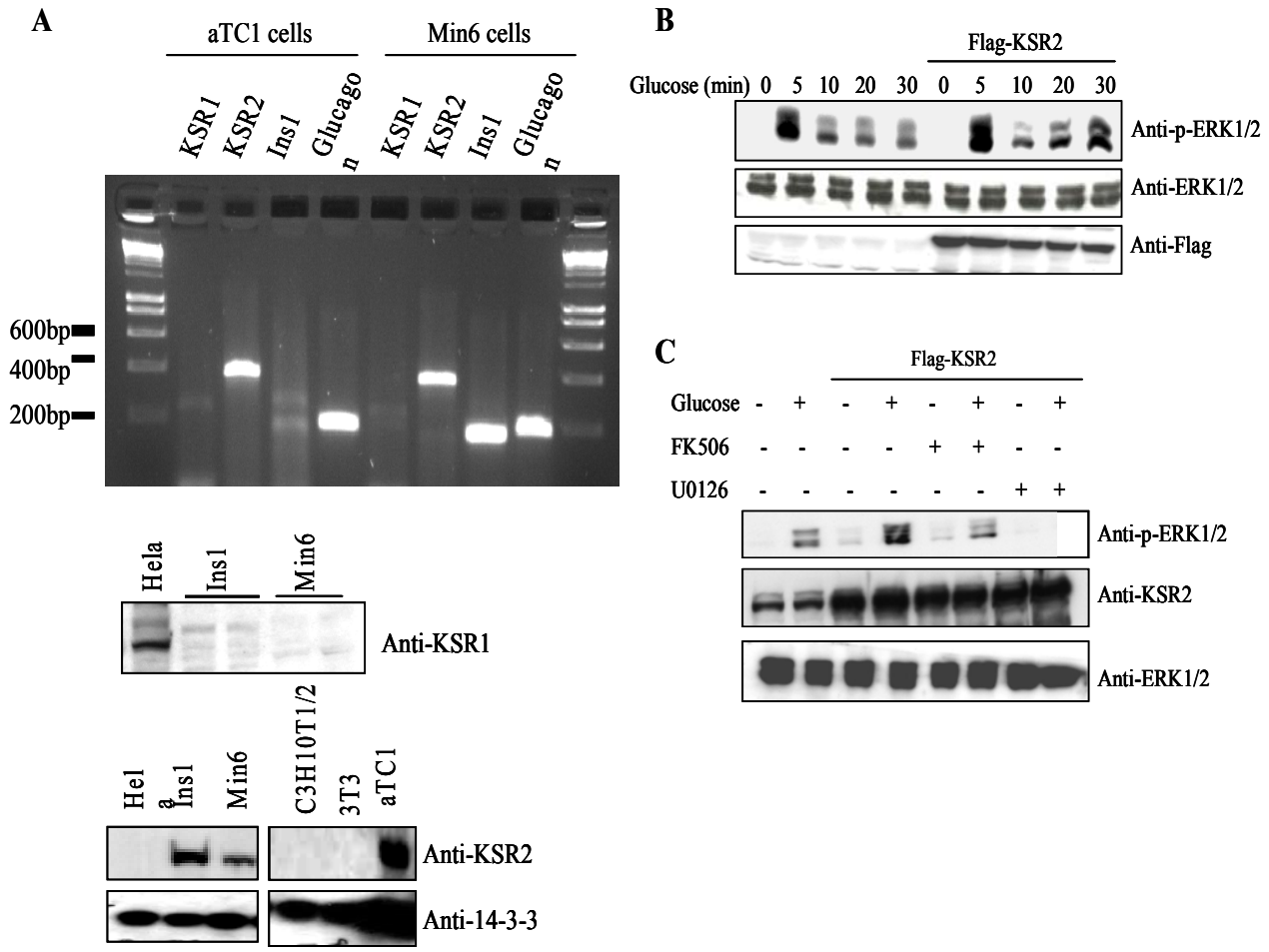
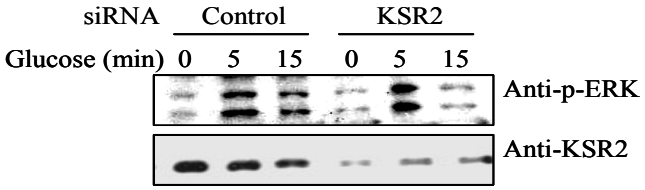


Figure 4-3. Effects of KSR2 on glucose-induced ERK1/2 activation. (A) Idenfication of the expression pattern of KSR1 and KSR2 in various cell lines using PCR and immunoblotting. (B) Effects of KSR2 on glucose-induced ERK1/2 activation. Min6 cells were transfected with pCMV5 empty vectors and Flag-KSR2 plasmids, respectively. 2 days later, Min6 cells were starved in 2mM glucose KRBH buffer for 2 hours and then stimulated with 20 mM glucose for the indicated timepoints. (C) Min6 transfected with Flag-KSR2 were starved and then were treated with 20 mM glucose in the presence or absence of 200 nM FK506 and 10 M U0126.

A



B

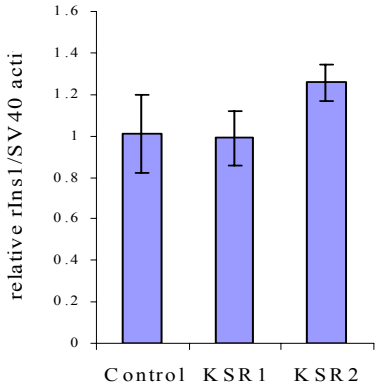


Figure 4-4. Effects of KSR2 siRNA knockdown on ERK1/2 activation induced by glucose. (A) Min6 cells were transfected with KSR2 siRNA oligonucleotides. (B) Effects of KSR1 and KSR2 on rat I insulin promoter activity.

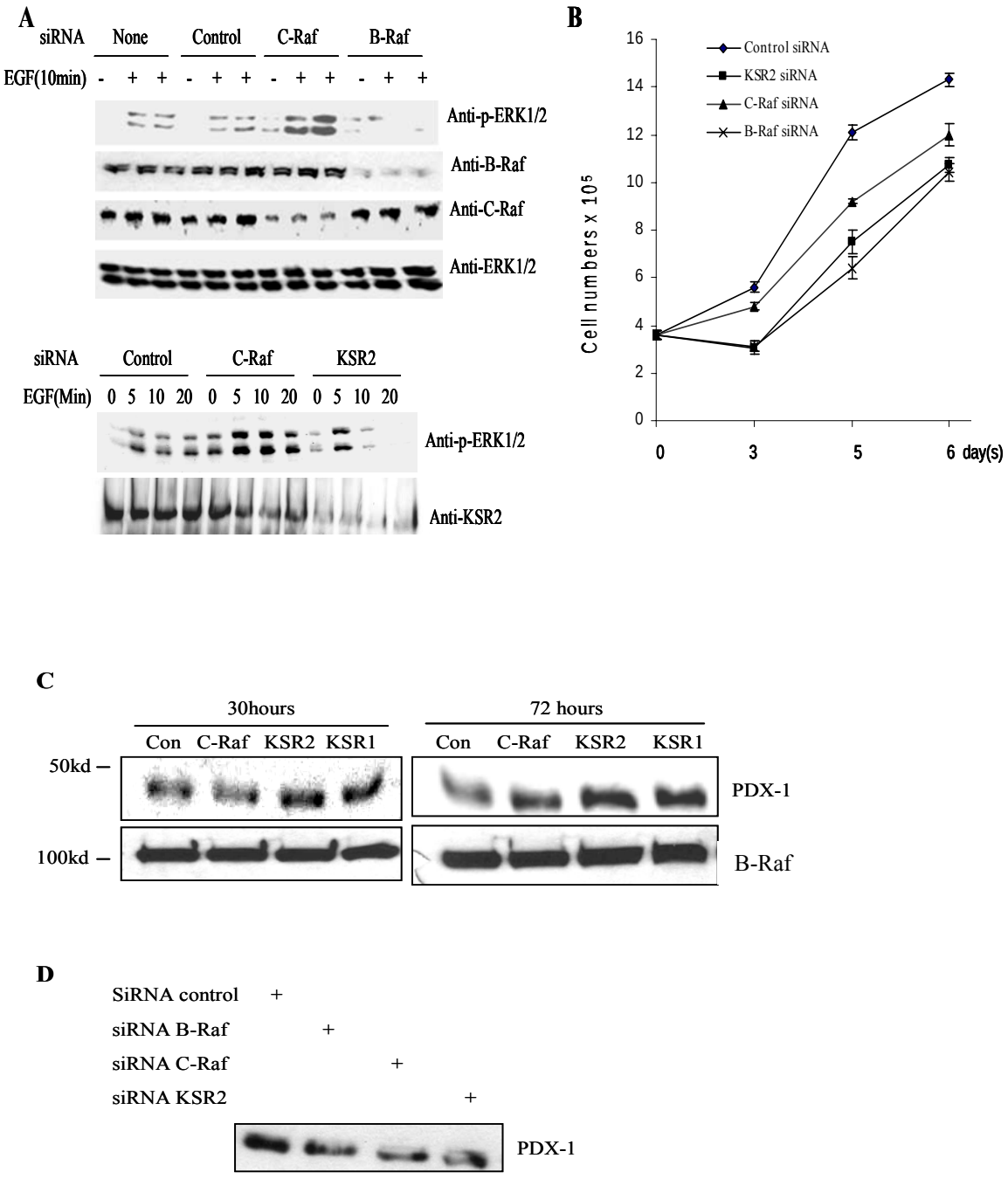


Figure 4-5. Effects of B-Raf, C-Raf and KSR2 on β-cell proliferation. (A) Effects of B-Raf, C-Raf and KSR2 siRNA knockdown on ERK1/2 activation induced by EGF. (B) Effects of B-Raf, C-Raf and KSR2 siRNA knockdown on cell proliferation. (C) Min6 cells were transfected with KSR1, KSR2 and C-Raf. The expression level of PDX-1 was detected by western blots.(D) Min6 cells were transfected with the indicated siRNA oligonucleotides. The expression level of PDX-1 was detected by western blots.

V. Discussion

In this study, I identified that KSR2, together with Raf kinases, increases β-cell growth through positively regulating ERK1/2 activation in response to glucose and EGF. Two Raf isoforms, B-Raf and C-Raf, regulate insulin synthesis by mediating ERK1/2 activation by glucose, while KSR2 seems to exhibit no effects on insulin synthesis. PDX-1, an essential transcription factor for maintaining β-cell proliferation and function, is activated by ERK1/2-mediated phosphorylation. In addition, activated ERK1/2 suppresses GSK3 activation, thereby preventing PDX-1 degradation mediated by GSK3. Insufficient PDX-1 expression results in reduced insulin synthesis and enhanced β-cell apoptosis. As Raf kinases and KSR2 are required for maintaining normal PDX-1 expression levels, Raf kinases and KSR2 contribute to maintaining β-cell proliferation as well as differentiation, probably by regulation of the PDX-1 expression profile. Recent studies showed that KSR2 interacts with multiple proteins, forming complexes to control Raf signaling and kinases related to energy metabolism. It is an intriguing question if KSR2 and Raf kinases control β-cell proliferation through suppressing these energy sensor proteins including AMPK and GSK3.

Adult β-cell mass dynamically changes according to altered metabolic demands. β-cell proliferation is markedly enhanced in the presence of a range of physiological stimuli including obesity, insulin resistance and pregnancy. Once the amount of β-cell death outweighs the amount of β-cell proliferation in diabetes patients, pancreatic islets no longer produce sufficient insulin, resulting in high glucose levels in the blood. New adult β cells are believed to regenerate from replication of differentiated β cells and neogenesis, but the primary mechanism of adult β-cell proliferation is likely replication

rather than neogenesis. Currently, there are many studies focusing on manipulating β-cell mass by up-regulating β-cell proliferation in vitro for diabetic therapy. Both KSR1 and KSR2 exhibit an ability to promote cell proliferation. KSR1 is richly expressed in pancreatic tumors, whereas KSR2, but not KSR1 is richly expressed in normal β cells. Significantly, it has been shown that KSR2 affects insulin secretion of β cells. Together, these suggest that functions of KSR1 and KSR2 are slightly different. Further investigation on mechanisms that KSR2 contributes to maintaining β-cell proliferation and differentiation may help to better understand adult β-cell replication and proliferation.

Chapter Five: Future Directions

Calcium influx activates the Ras signaling pathway as well as the calcium/calmodulin-dependent phosphatase calcineurin in various cells types including neurons, lymphocytes, cardiomyocyte cells, and pancreatic β cells. It has been known that calcineurin and ERK1/2 activation are required for maintaining normal functions of pancreatic β cells. Calcineurin contributes to ERK1/2 activation by glucose in β cells and suppression of calcineurin decreases insulin synthesis via attenuation of ERK1/2 activation and inhibition of NFAT activation. In this study, we found that calcineurin constitutively binds to B-Raf and modulates B-Raf activation and B-Raf/C-Raf heterodimerizatin via dephosphorylating inhibitory phosphorylation sites in B-Raf in β cells. Recent studies identify that B-Raf/C-Raf heterodimerization is essential for effective activation of the MAPK cascade and causes drug resistance to Raf inhibitors in Ras mutant tumors.

Calcineurin inhibitors were firstly introduced as immunosuppressive drugs and later also used in treatment of diseases including skin disorders (97,98). It is disputed whether calcineurin inhibitors increase the risk of cancers, especially skin cancer. However, other studies show that calcineurin inhibitors exhibit cancer-protecting capabilities and suppress growth of various tumors such as breast cancer, glioblastoma and chronic lymphatic leukemia (98, 99). Considering the impact of calcineurin on B-Raf activation and B-Raf/C-Raf heterodimerization, analysis of types of Ras and Raf mutants during application of calcineurin inhibitors in cancer treatment may help to understand mechanisms of cancer-protecting and cancer-promoting effects of calcineurin inhibitors.

The scaffold proteins KSR1 and KSR2, known as Raf-related pseudo-kinases, promote ERK1/2 activation through coordinating the assembly of multi-protein complexes including core components of the Raf/MEK/ERK1/2 cascade. Recently, KSR2 was reported to interact with calcineurin and impact Ca^{2+}-regulated ERK/2 activation in neurons. In this study, we also showed that KSR2 is involved in regulation of ERK1/2 activation upon glucose-metabolism triggered calcium influx in β cells. It is an intriguing question whether calcineurin impacts Raf kinases and KSR2 membrane recruitment and therefore promotes the Ras-Raf interaction. Because active Ras recruits Raf kinases and KSR scaffold proteins from the cytosol to membranes, I examined the subcellular distribution of B-Raf, KSR2, calcineurin and Ras. I found that in contrast to B-Raf and C-Raf, KSR2 protein is evenly distributed in cytosolic and microsomal fractions and calcineurin is prone to localize in the microsomal fractions (Fig. 5-1). It is possible that KSR2 and calcineurin direct or support membrane localization of core components of the Raf signaling cascade and enhance Raf and Ras interaction.

Recently, many studies focus on identifying mechanisms and sources for adult β-cell regeneration. It is thought that altered metabolic demands and pancreas injury caused by partial pancreatectomy may stimulate the proliferation of adult β cells from existing β cells or neogenesis from progenitor cells. Most opinions think that proteins required in the proliferation of adult β cells are distinct from those in embryonic pancreatic development. Studies on calcineurin b1-knockout mice suggest that calcineurin is essential for adult β-cell growth but not for β-cell neogenesis during embryonic development (75). Calcineurin b1-deficient mice retain normal embryonic pancreatic morphology and display normal glucose tolerance initially, but develop age-dependent

diabetes characterized by decreased β-cell proliferation, reduced pancreatic insulin content and hypoinsulinemia. Recently, it was reported that KSR2 knockout mice also develop age-dependent obesity and insulin resistance which are possibly caused by the reduced energy expenditure (91). My study showed that KSR2 contributes to glucose and EGF-stimulated ERK1/2 activation and also plays a positive role in β-cell proliferation. Reduction of KSR2 expression may cause impaired β-cell mass expansion, which might contribute to age-dependent diabetes. Further investigation needs to be performed to identify whether KSR2 is one of the factors required for maintaining adult β-cell proliferation and functions in human.

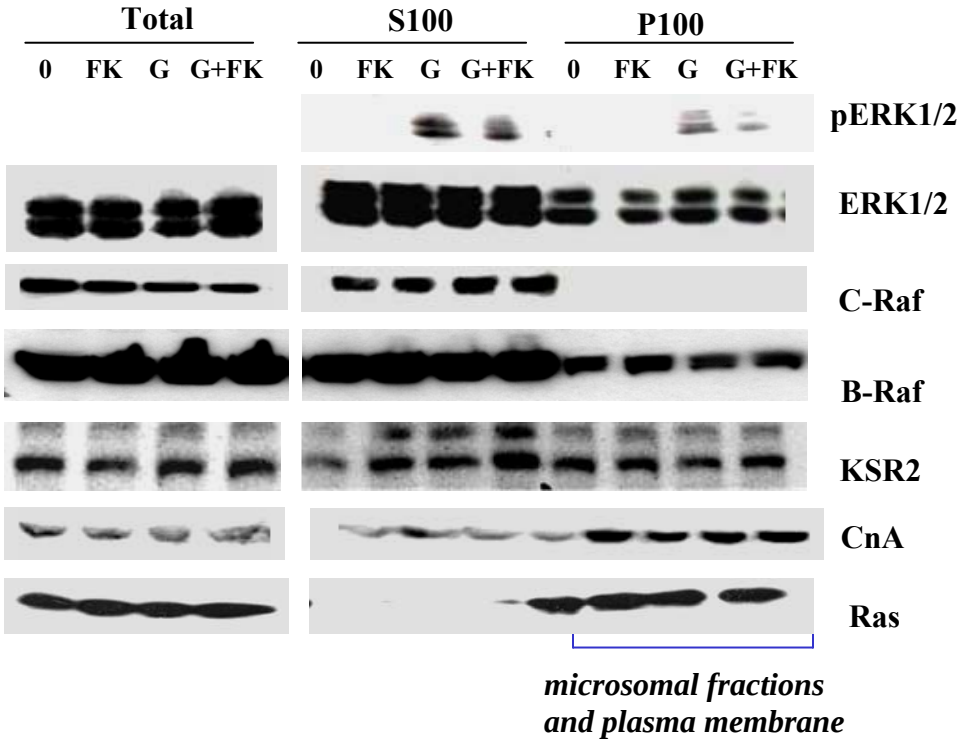


Figure 5-1. Cellular distribution of B-Raf, C-Raf, KSR2, calcineurin and Ras. Min6 cells were washed with cold PBS and scraped on ice in 0.5 ml of hypotonic buffer (10 mM Tris/HCl pH 7.2, 5 mM MgCl2, 1mM DTT, 1 mM EGTA, 100 µM NaVO4, 1 mM PMSF). After incubation for 20 min on ice, cells were homogenized with 25 strokes in a tight-fitting Dounce homogenizer, and the nuclei were removed by the low-speed centrifugation. 50 µl of the postnuclear supernatants cell extracts (Total) were kept for Blots. The left postnuclear extracts were spun at 100,000 × *g* and the supernatant (S100) was collected. The pellet (P100) was rinsed and resuspended in 200 µl of ice-cold lysis buffer (including 1% NP40). The P100 membrane fraction contains plasma membranes and various intracellular membranes, including the endoplasmic reticulum, Golgi apparatus, and endosomes.

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