


1-1-2011

Small gtp-binding proteins in insulin secretion

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SMALL GTP-BINDING PROTEINS IN INSULIN SECRETION

by

BHAVAANI JAYARAM

DISSERTATION

Submitted to the Graduate School of

Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2011

Major: PHARMACEUTICAL SCIENCES

Approved by:

Advisor

Date

DEDICATION

This work is dedicated to my family, especially my grandmother, who supported me with her love and encouragement, my guru, Dr Kowluru, for guiding me and my loving brothers Rahul and Ajan, for being supportive through this journey.

ACKNOWLEDGEMENTS

The climb to PhD was amazing if not for all those helping hands that lifted me up to the top. I would first and foremost like to acknowledge, my teacher, Dr Kowluru for guiding and molding me through my years of study here at Wayne State University. I am grateful to all the Kowluru lab members for the innumerable coffee sessions of science and arguments: Dr Veluthakal, Dr Chandrashekar, Dr Wasanthi, Dr Alka and last but not least Ismail Syed who stood as an inspiring & supportive pillar through the years.

My sincere gratitude goes out to my committee members, Dr Commissaris, Dr Thomas and Dr Jena for their valuable input and advice through the course of my project and career.

I am also thankful to Dr Corcoran and the members of Department of Pharmaceutical Sciences for their continued support and encouragement.

My deepest gratitude goes to my friends who supported me emotionally, put up with all my eccentricities and provided me a second home: Mani, Arvind, Mrudang, Vijay, Vanitha, Mani, Gopi, Chaitu, Anoop, Summi, Jash, Abhi and Saagar.

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LIST OF ABBREVIATIONS

Arf6: ADP-ribosylation factor 6

ARNO: Arf nucleotide binding site opener

GAP: GTPase activating protein

GDI: GDP-dissociation inhibitor

GEF: Guanine nucleotide-exchange factor

GGA3: Golgi-localized c-ear homology domain Arf-binding protein-3

GSIS: Glucose-stimulated insulin secretion

PA: Phosphatidic acid

PIP₂: Phosphatidyl inositol-4,5-bisphosphate

PLD: Phospholipase-D

Smg: Small GTP-binding proteins

DCHFDA: 2', 7'-dichlorodihydrofluorescein diacetate

DPI: diphenylene iodonium

ERK1/2: extracellular mitogen regulated kinase 1/2

Nox: NADPH oxidase

PA: palmitic acid

PIM: Prodo islet media

ROS: Reactive Oxygen Species

Tiam1: T-lymphoma invasion and metastasis 1

ZDF: Zucker Diabetic Fatty rat

ZLC: Zucker Lean Control

T2DM: Type 2 diabetes mellitus

IGT : Impaired glucose tolerance

IAPP : Islet amyloid polypeptide

SG : Secretory granule

GLP-1 : Glucagon-like peptide 1

PLC : Phospholipase C

RRP : Readily releasable pool

Chapter 1

Diabetes

According to World Health Organization, about 220 million people around the world suffer from Diabetes. Surveys conducted by American Diabetes Association, showed about 8.3% of Americans, including children and adults, suffer from diabetes [data from 2011 National Diabetes Fact Sheet]. In US, diabetes ranks 7th as a leading cause of death either as a contributing factor or the underlying cause for morbidity. Diabetes mellitus is a metabolic disorder characterized by abnormally high blood sugar levels. The high sugar in blood leads to classical symptoms of polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger). There are three types of diabetes:

- Type 1 diabetes [T1DM]: Earlier known as juvenile diabetes or insulin-dependent diabetes, is a chronic condition where the body's immune system attacks insulin-producing beta cells. Various factors contribute to T1DM from genetics to viruses. It is typically seen developing at adolescence.
- Type 2 diabetes [T2DM]: A heterogeneous disorder characterized by impaired secretion of insulin and insulin resistance. The disease progresses from a stage of prediabetes to overt T2DM. Essentially there is progressive dysfunction of islet during the course of the disease where insulin secretion is gradually blunted. Along with impairment in insulin secretion, there is evidence for reduction in beta cell mass.

- Gestational diabetes: Develops in pregnant women which might precede the development of T2DM.

Pathogenesis of T2DM

T2DM is marked by a substantial beta-cell failure which starts at an early stage in disease progression and after which deterioration accelerates precipitously. A study conducted in the UK demonstrates a 50% loss in beta-cell secretory capacity by the time fasting hyperglycemia was diagnosed [1]. In support of this, genome-wide studies linked chromosomal loci for T2DM to genes associated with pancreatic beta-cell development and survival and in the control of insulin secretion [2]. Under normal conditions, the beta-cell balances changes in insulin sensitivity with proportionate changes in beta-cell function, demonstrating a hyperbolic relationship between sensitivity to and secretion of insulin. However, in persons with beta-cells predisposed to T2DM, an imperfect compensatory mechanism, dysregulated glycemic levels and abnormal beta-cell function are the triggers. Persons who progressed to T2DM as a result of abnormal beta-cell function were termed as “progressors” and exhibited beta-cell dysfunction even before reaching impaired glucose tolerance [IGT]. With progressing dysfunction, insulin secretion is reduced by ~80% accompanied by a 14% decrease in sensitivity to insulin [3]. Whereas in nonprogressors, an 11% decrease in insulin sensitivity was compensated by 30% increase in insulin secretion. This was indicative of a transitory mechanism from normoglycemia to IGT to diabetes that resulted from ineffective beta-cell compensatory effort for constant insulin resistance in progressors [4].

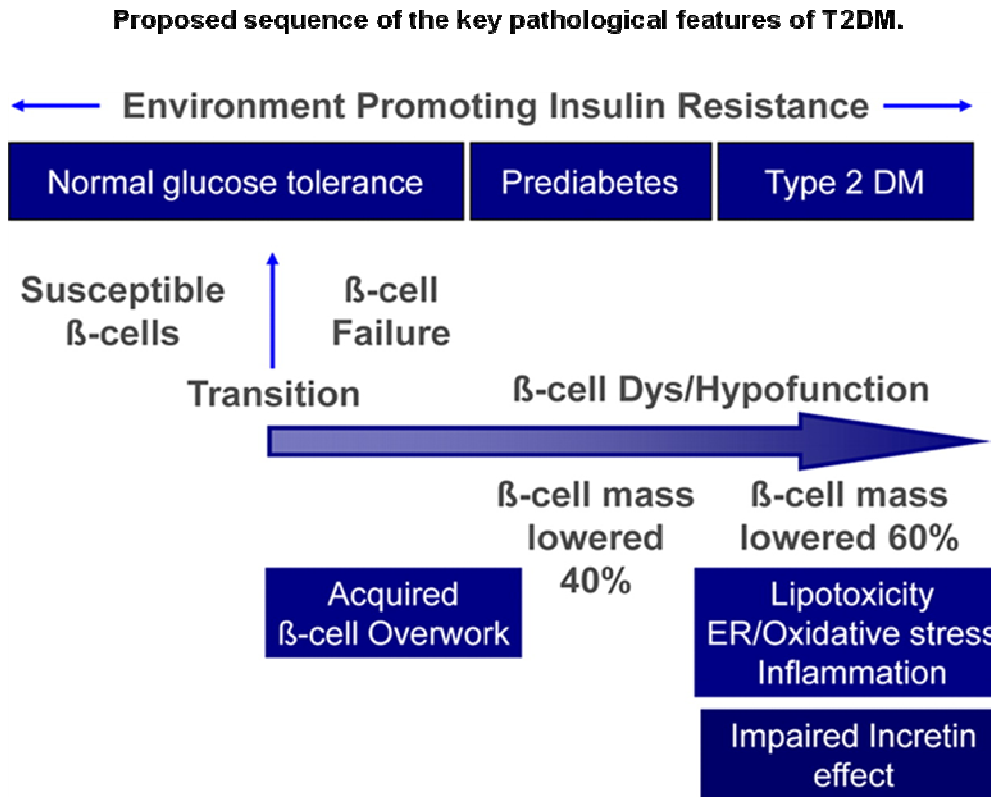


Figure 1-1. Proposed scheme for progression of T2DM [5].

Both genetic and acquired factors contribute to progressive beta-cell failure. While genes encoding proteins responsible for glucose metabolism and insulin signaling maybe compromised, acquired factors like glucotoxicity, lipotoxicity, inflammatory cytokines and islet amyloid polypeptide [IAPP] deposition also contribute to beta-cell failure [6, 7]. Numerous studies have demonstrated the functional, qualitative and anatomic manifestations of beta-cell failure associated with T2DM. A qualitative defect in release of insulin is marked by disrupted pulsatile secretion and processing of the precursor proinsulin to fully active insulin [8, 9]. This is consistent with the loss of first-phase insulin secretion in both insulin dependent and non-insulin dependent diabetes. The above facts indicate the importance of studying the mechanisms that regulate and

control beta-cell physiology. Understanding beta-cell function may pave the way for a better understanding of the pathophysiology of beta-cell dysfunction and aid in discovering new therapeutic agents to protect beta-cells from defects.

Islet of Langerhans

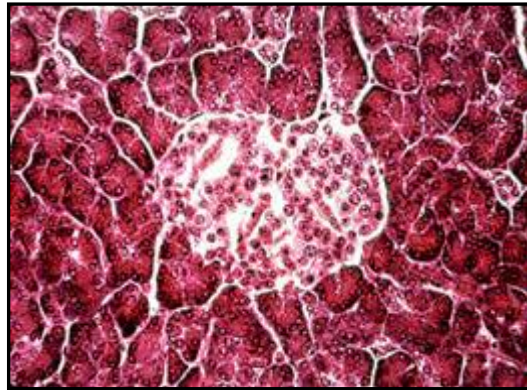
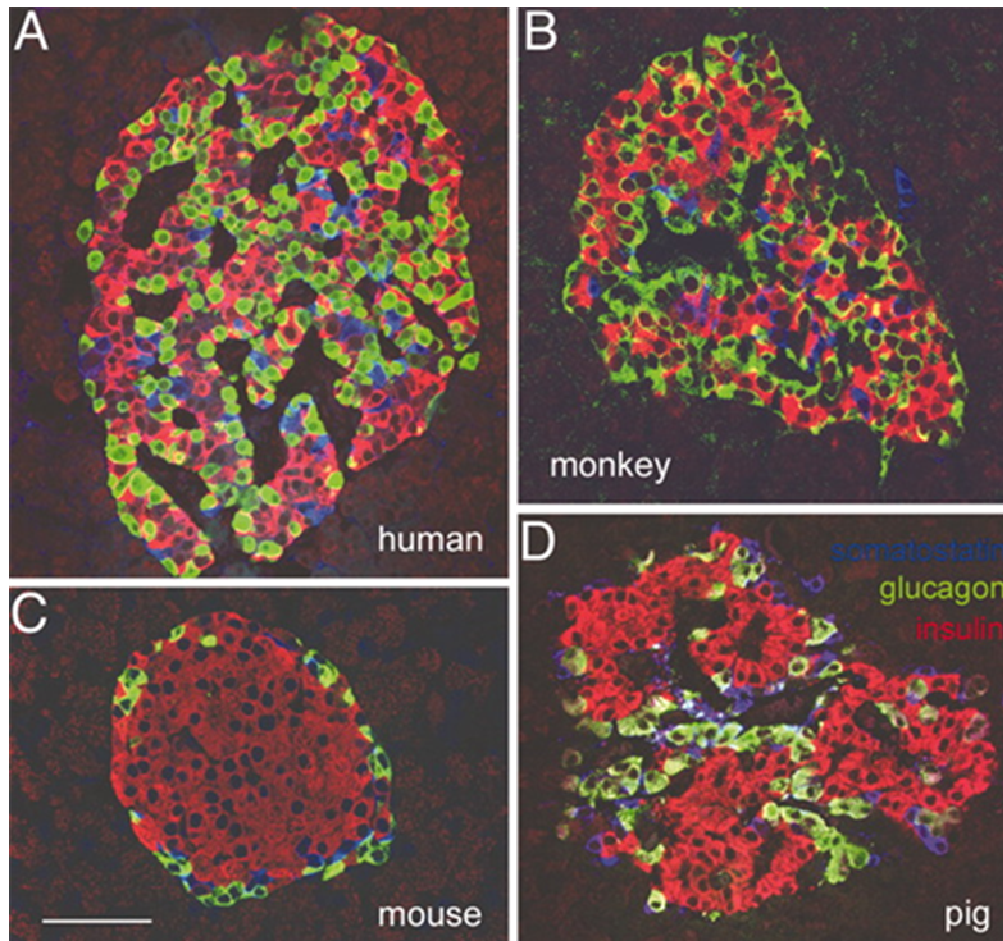


Figure 1-2. Photo of an islet of Langerhans under microscope (Courtesy: web diabetes Leeds Yorkshire)

Islet of Langerhans named after famous pathologist, Paul Langerhans, is a critical organ that is divided into million other units embedded in the pancreas. Islets are an ellipsoid cluster of cells with a diameter of $\sim 50\text{-}250\ \mu\text{m}$. Islets come of different size with medium sized islets contributing to total islet volume. But islets found in diabetes can get very large $\sim 350\ \mu\text{m}$ due to oedema and amyloid accumulation [10]. The number of islets in pancreas depends on the age, BMI, and size of pancreas.

The term “islet” was derived from their appearance as an island of cells in close proximity to capillaries. Each islet is made of 100-1000 cells and is a mix of α -, β - and δ -cells scattered throughout. Pancreatic islets are highly-vascularized micro-organs composed of diverse cell types. About $\sim 64\%$ in the human islet is made of insulin-secreting β -cells and remaining are 26% glucagon-secreting α -cells, 8% δ -cells which secrete somatostatin [11]. Human islets represent a unique cytoarchitecture [12] as

compared to rodent islets. In humans, the various cells are dispersed with no particular arrangement as opposed to rodent islets which contain a core of insulin-secreting beta-cells. Such arrangement of cells in the islet suggests paracrine effect among the various types of endocrine cells.



Cabrera O et al. PNAS 2006;103:2334-2339

Figure 1-3. Above is a representation of islets showing interspecies differences. Panels A-D represents confocal micrographs of immunostained islets from human [A], monkey [B], mouse [C] and pig [D]. Insulin was immunostained red, Glucagon stained green and somatostatin stained blue [12].

Pancreatic beta-cells are the sole source of insulin, the major regulator of blood glucose levels. Under physiological and pathological conditions, beta-cells coordinate between increased insulin secretory granule [SG] biogenesis and insulin resistance [13]. In conditions of high metabolic demands, beta-cells upregulate their insulin synthesis and secretion. Glucose promotes the release of insulin from beta-cells while increasing insulin production in order to replenish its stores. This is made effective by a concomitant increase in biosynthesis of luminal and membrane proteins that facilitate assembly and function of SGs. Secretory granules in beta cells are 300-350 nm in diameter and are found polarized in the cell facing blood capillaries. Structural separation does not stop the islet cells from communicating with each other. Each beta cell communicates with the other through paracrine actions. Electrophysiological studies also reveal beta cells to electrically synchronize with each other via gap junctions.

Insulin secretion

Over the years, pancreatic beta-cells have represented a well-characterized endocrine system for regulated secretion of insulin hormone from large dense core granules. Insulin secretion involves an intricate process, coupling various stimuli - hormones, neurotransmitters and nutrients. Signal transduction systems co-ordinate the multiple stimuli and aid in regulating secretion of insulin. Type 2 diabetes, the most prevalent form, is characterized by insulin resistance and beta-cell dysfunction. Gradual intolerance to glucose develops into diabetes where compensatory secretion of insulin is lost. Thus a fundamental understanding of molecular mechanisms governing stimulus-coupled secretion of insulin is necessary.

Overview of insulin secretion

It is widely published that glucose is a primary stimulus for secretion of insulin from pancreatic beta cells. Metabolism of glucose provides the necessary signal to activate signal transduction system within the beta cell. This pathway is nutrient-stimulated secretion which is distinct from other pathways acting via heterotrimeric G-proteins. Increase in extracellular concentration of glucose in blood creates an inflow of glucose into pancreatic beta-cell. Beta-cells are equipped with glucose-metabolizing enzymes. One such enzyme is glucokinase, which phosphorylates and increases the K_m for glucose. This increases the metabolic rate of glucose for a physiological range of glucose concentrations.

Currently the well-understood mechanism for glucose-stimulated insulin secretion [GSIS] is outlined below [14]:

- Beta-cell acts as a glucose sensor, engulfing excess glucose from the blood.
- Glucose metabolism increases ATP/ADP ratio, which in turn closes ATP-sensitive K^+ channels.
- Closure of K^+ channels depolarizes the membrane and results in opening of voltage-sensitive Ca^{2+} channels.
- Intracellular concentration of Ca^{2+} increases which sensitizes insulin-laden vesicles for fusion and release.

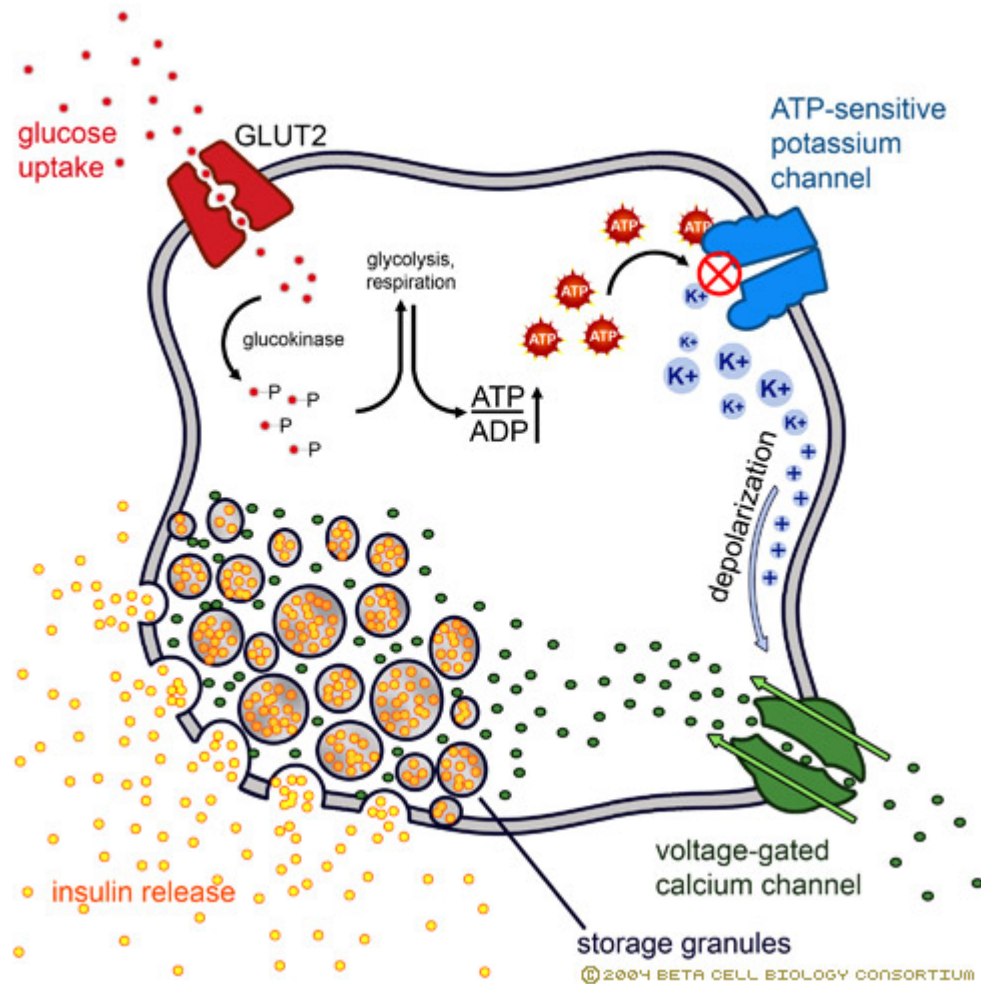


Figure 1-4. Insulin secretion in beta cells is triggered by rising blood glucose levels. Starting with the uptake of glucose by the GLUT2 transporter, the glycolytic phosphorylation of glucose causes a rise in the ATP:ADP ratio. This rise inactivates the potassium channel that depolarizes the membrane, causing the calcium channel to open up allowing calcium ions to flow inward. The ensuing rise in levels of calcium leads to the exocytotic release of insulin from their storage granules [Taken from Beta Cell Biology Consortium].

However, in recent years it has become apparent that factors other than ATP and Ca^{2+} [15] play a regulatory role in secretion of insulin. Not only nutrients, but also neurotransmitters and hormones regulate the secretory process. Non-nutrient stimulants, like acetylcholine, potentiate insulin secretion via release of Ca^{2+} from intracellular stores by activating phospholipase C [PLC] [16, 17] and generating second messengers like DAG and IP_3 , whereas neuromodulators like GLP-1 [18, 19] promote insulin release via interaction with specific cell-surface receptors. Hormones like somatostatin inhibit insulin secretion by lowering second messengers such as cAMP and indirectly modulating Ca^{2+} channels [20, 21]. Thus a balanced interplay between various regulatory factors helps to maintain glucose homeostasis.

Sequential events of exocytosis

Insulin is a peptide hormone packaged into large dense core vesicle [secretory granules] and released via exocytosis from pancreatic beta-cell. Exocytotic processes are mediated by protein components of secretory machinery which are activated in response to a stimulus. Exocytosis is marked by fusion of secretory vesicles with plasma membrane and emptying out its contents, i.e. insulin, out into the extracellular space to reach target organs [22, 23]. Apart from transporting insulin, secretory granules act as a 'warehouse' for insulin, releasing it as and when required. Only a small proportion of insulin is released on stimulation, the remaining being recycled back into the cell. Thus to comprehend insulin secretion beyond metabolism of glucose, the molecular pathway must be dissected involving exocytotic machinery and its modulatory factors.

Formation of Insulin Granules

Secretory vesicles are of two types: synaptic vesicles and large dense core vesicles [LDCV]. Neurotransmitters are packed into synaptic vesicles which are subjected to rapid exo-endocytosis, refilling and a subsequent round of fusion. On the other hand, peptide hormones like insulin are packed into large dense core vesicles [LDCV]. LDCVs are not exactly synthesized de novo but gradually “bud off” from the golgi bodies as they are appropriately filled with biosynthetic cargo, proinsulin [24, 25]. Characteristic of LDCVs, proinsulin undergoes a series of maturation steps where the C-peptide is cleaved and insulin is released from proinsulin. Pancreatic beta-cell is abundant with insulin-laden vesicles but even at maximally-stimulating conditions, only a small proportion of insulin is released. This clearly reflects importance of regulation of secretion than on the biosynthetic rate.

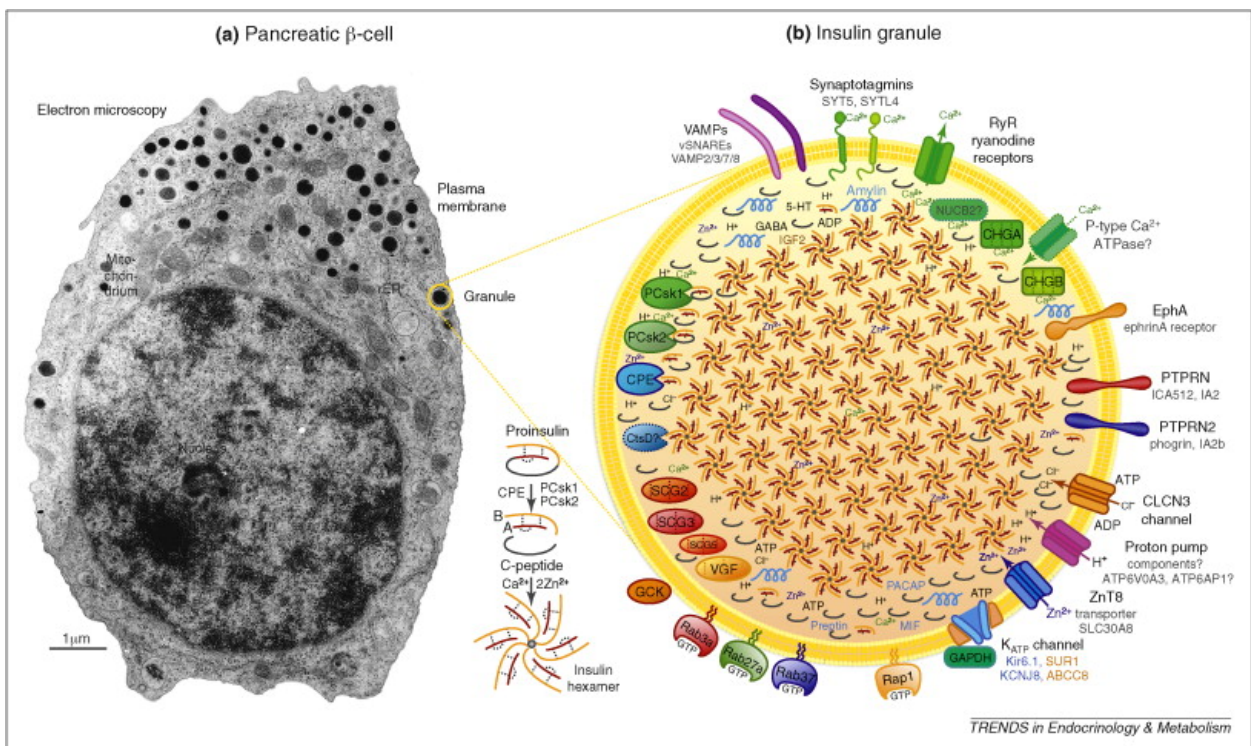


Figure 1-5. Insulin granule: Electron micrograph [Left] of beta-cell. Contains thousands of small membrane-bound vesicles loaded with insulin [Right]. These granules contain many proteins, small molecules and ions in the lumen as well as transmembrane proteins, channels and membrane-associated proteins. Insulin [INS], amylin [IAPP], insulin-like growth factor [IGF] [25].

Towards Plasma Membrane

Chromaffin cells stand as a comparable system to study exocytosis of LDCVs. Secretion of insulin granules from beta-cells mimic analogously exocytotic process in chromaffin cells [26, 27]. In resting conditions, cortical actin forms a network and acts as a barrier for the movement of insulin granules to the plasma membrane. It has been shown with the help of clostridium toxin and other pharmacologic agents that disruption of actin barrier is important for the movement of granules to access the plasma membrane [28]. Liberation of Ca^{2+} from intracellular stores aids in cytoskeletal remodeling. On the other hand, there has been evidence for involvement of molecular motors, myosin-actin system, for which ATP serves as energy sponsor activating myosin and kinases like myosin-light chain kinases [29]. With recent advances in microscopy, ultrastructural visualization has revealed vesicles docking in proximity to plasma membrane in insulin secreting beta-cells.

At the Plasma Membrane

In neuroendocrine cells, where Ca^{2+} regulated secretion has been extensively studied insertion of vesicles into plasma membrane can be divided into two distinct biochemical events: priming and fusion. Both events seem to require distinct factors. “Priming” event requires the absolute necessity for ATP, sub-micro concentrations of Ca^{2+} and alterations in lipids by phosphatidyl transfer protein I [PTP I] and

phosphatidylinositol-4-phosphate-5-kinase [PI4P5K] [30, 31]. “Fusion” step is characterized by the requirement of Ca^{2+} . Since then the importance of PI4P5K in secretion of insulin has been emphasized.

Biphasic Insulin Secretion

Priming and fusion are functional events which underlie a biphasic secretion as observed in many cell types. Like many other cells, beta-cells also display an initial rapid phase of secretion [priming] followed by more a sustainable phase [fusion]. In type II diabetes, a complete loss of first phase accompanied by reduction in second phase is observed [31]. Several studies over the past years have demonstrated the availability of a readily releasable pool [RRP] of insulin granules that are immediately available for release upon stimulation of beta-cells. The RRP is believed to be a subset of already docked granules. ATP- and time-dependent priming of newly supplied granules constitutes the second phase of secretion. The rate limiting step controlling the rate of release in the second phase is the continued recruitment and priming of new granules for release.

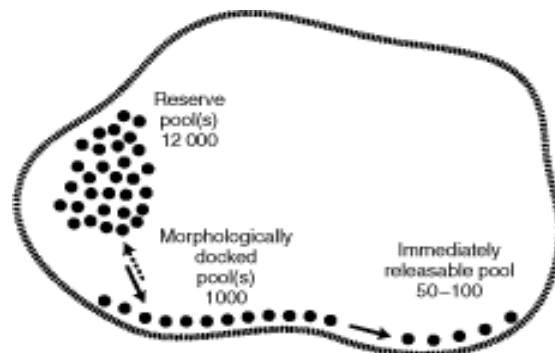


Figure 1-6. Schematic representing Reserve, Docked and Readily releasable granule pools in mouse pancreatic beta-cell [35].

As per Lang, the terms docking, priming and readily releasable pool are functional terms designed to indicate their working description [36]. Docked vesicles indicate a morphological description, primed vesicles are biochemically defined for their ability to respond to Ca^{2+} and ATP and readily releasable pool was defined as an electrophysiological description. Though each term was defined for operational purpose, relation between them is yet to be established.

Modern theory: Kiss and run

For years, the mechanism by which insulin-laden dense core SGs approach and fuse with plasma membrane have gained interest. The defects in these processes maybe one of the contributing factors to T2DM. The release of insulin through exocytosis follows similar steps as that of other peptide hormones and neurotransmitters, each involving Ca^{2+} -regulated fusion of secretory vesicles at plasma membrane. But there are several aspects which are unique to GSIS. What happens to the dense-core vesicle at the plasma membrane? Seminal work by Rothman and many others have identified a “minimal machinery” for exocytosis [37]. It comprises of soluble NH₂-ethylmaleimide-sensitive fusion protein attachment protein receptors [SNAREs].

SNARE

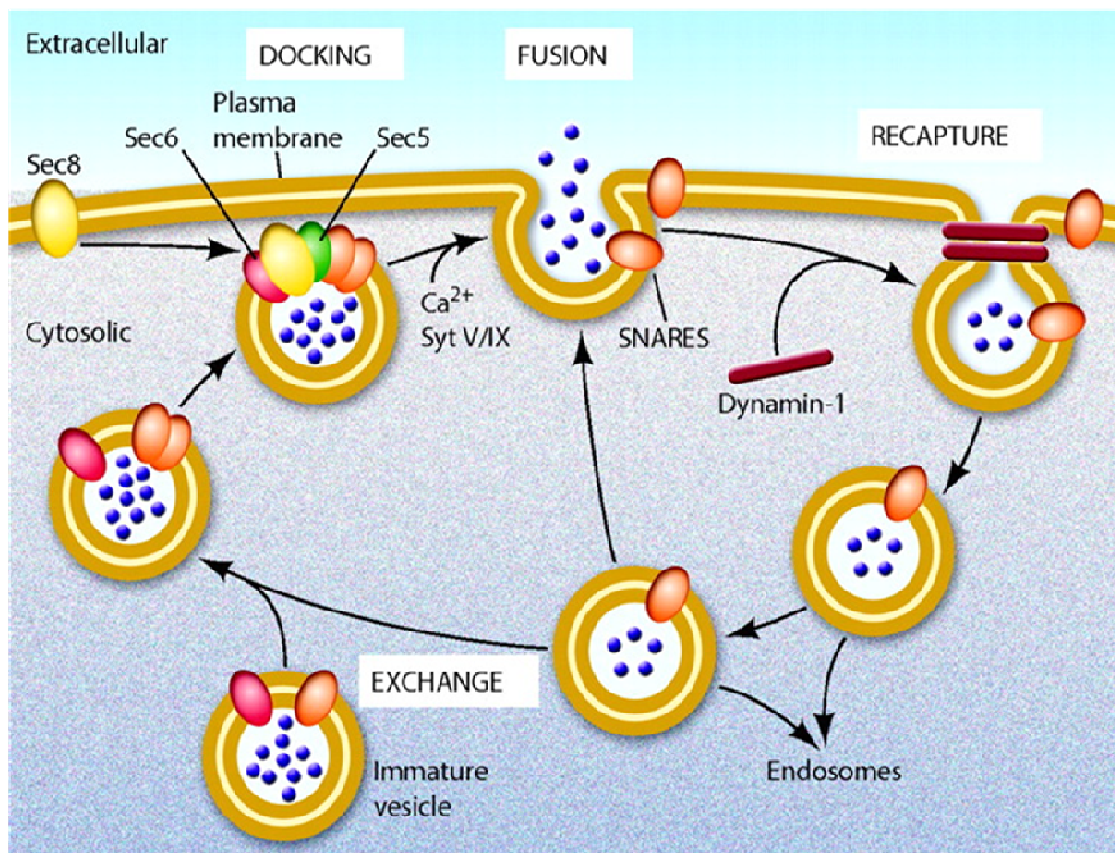
Initial studies, identified SNARE proteins in neurons and was assumed they were neuron-specific. But studies done in pancreatic beta-cells found SNARE proteins to be generally associated with exocytotic processes. Later studies characterized many of the

SNARE proteins like v-SNAREs [synaptobrevin/VAMP-2] on the vesicles and t-SNAREs [syntaxin, SNAP25] on the plasma membrane in beta-cells [38]. They play an essential role in Ca^{2+} regulated secretion. In addition to these proteins, there are Munc18 and NSF that aid in Ca^{2+} regulated exocytosis. Interaction of these proteins to form a “SNARE TUBE” serves to provide membrane selectivity and thermodynamic driving force for membrane fusion. Earlier studies by Yang and Gillis [39] in INS-1 cells demonstrated the existence of two pools of vesicles. One pool was sensitive to high Ca^{2+} and may respond only to large increases in Ca^{2+} and which are situated adjacent to Ca^{2+} channels at plasma membrane while the other pool of vesicles are remotely located and are able to fuse with the membrane at lower Ca^{2+} levels.

Vesicle docking

The process whereby remotely located vesicles finally interact with the plasma membrane with varying dependency on MgATP and phospholipids including phosphatidylinositol 4, 5 bisphosphate [PIP2] is termed as “docking”. The recent emergence of the involvement of “exocyst” complex in docking of secretory granules in neurons seems to hold true in beta-cells also [40]. The approach of vesicles to the plasma membrane requires formation of Sec6-Sec8 (exocyst) complex which is denoted as DOCKING in the figure [41]. Fusion of docked vesicles requires the elevation of Ca^{2+} sensors such as synaptotagmins. This fusion leads to a partial release of vesicle’s content [insulin] which is released as dimers with Zn^{2+} through a ~4 nm fusion pore. In the meanwhile, low molecular weight molecules such as ATP and lipid components of vesicle membrane rapidly associate with lipids of plasma membrane. This event where the membrane is captured is termed as “cavcapture” [42]. A large GTPase dynamin-1

terminates this cavicapture by pinching the vesicle membrane away from the plasma membrane. This marks the endocytic phase of vesicle trafficking. Electron microscopy in the past implied that the cargo from vesicles was emptied via an expanding pore that eventually flattened itself into the membrane [43]. Such mechanism is a result of strong stimulation as seen during repetitive membrane depolarizations measured using patch clamp technique [44]. While one set of studies suggest granule membrane to undergo complete fusion with the plasma membrane, [45-48] alternative studies suggest for a transient fusion pore opening through which cargo diffuse out followed by recycling of vesicles. This process is termed as “kiss-and-run” where the granule membrane content mix or stay separate from the plasma membrane [49-51].



Rutter G A . Hill E V Physiology 2006:21:189-196

Figure 1-7. An exocytotic-endocytotic process in beta-cells [40].

Apart from SNAREs, other important regulators of granule trafficking include guanine nucleotides, small G-proteins of Rab, Arf and Rho family and dynamin GTPases.

Guanine Nucleotides

In many systems like chromaffin cell, mast cells and pancreatic beta-cells, exocytosis can be triggered independent of Ca^{2+} . The induction of exocytosis was carried out by intracellular application of GTP γ S, a non-hydrolyzable analog of GTP. Gomperts review gave rise to the term G_E , G-protein for exocytosis, which affected exocytosis without any involvement of second messengers [52]. Wollheim's group investigated the effects of guanine nucleotides on insulin-secreting RINm5F cells and islets and demonstrated a GTP-induced, Ca^{2+} independent secretion of insulin. GTP γ S stimulated a slow but persistent release of insulin and its effect was not additive with Ca^{2+} , it did not require activation of PLC or PKC or the involvement of SNARE proteins. Since both Ca^{2+} - and GTP γ S-induced exocytosis requires ATP and metabolism of glucose regulates cellular levels of both ATP and GTP, a link was established between glucose metabolism and insulin secretion [54, 55]. Kinetic studies were performed to understand the sequential events behind Ca^{2+} and GTP γ S induction of secretion and understood that these two stimuli act at different phases of fusion of a same pool of vesicles. This made clear that Ca^{2+} and GTP γ S followed a common but distinct step in the fusion process [56].

GTP-binding proteins

The vectorial movement of proteins coupling exocytosis and endocytosis in eukaryotes is mediated by a multitude of protein families. The first ever evidence that G-proteins play an important role in hormone action was proven in early '70s in a work done by Rodbell to show that cAMP generation by glucagon stimulation required GTP [57]. Two toxins, pertussis and cholera, have been instrumental in identifying nine G-proteins and their action. It soon became apparent that G-proteins not only worked as stimulators but also inhibited secretion of hormones according to subunit activated [58, 59]. Metz SA, in their JBC article, 1992, emphasized the necessity for GTP in exocytosis of insulin from intact rat islets using selective inhibitors of GTP synthesis [60]. Ever since functional roles for G-proteins in insulin secretion have been scrutinized. GTP essentially activated either heterotrimeric G-proteins or small molecular-weight G-proteins by binding to them [58, 61].

Heterotrimeric G-proteins

Heterotrimeric G-proteins have been shown to effectively link signal recognition elements, receptors, to the signal generators, effectors. Emerging evidence indicates a role for heterotrimeric G-proteins in insulin secretion. Heterotrimeric GTPases are composed of three subunits: α -subunit and $\beta\gamma$ -dimer. α -subunit is activated by replacing GDP with GTP which allows for dissociation of $\beta\gamma$ -dimer. Both α_{GTP} and $\beta\gamma$ -dimer affect a variety of effectors from ion channels to kinases. Activity of heterotrimeric GTPases is regulated by GTPase activity intrinsic to α -subunit and also by Regulator of G-protein Signaling proteins [RGS] as described by Gilman [62, 63]. The heterogeneity offered by

the possible combinations of multiple isoforms of α , β and γ makes it difficult to understand their functional parts. In beta-cells, activators of $G_{\alpha i}$ and $G_{\alpha o}$ inhibit release of insulin via the inhibition of adenylate cyclase \longrightarrow Reduced cAMP levels and PKA activation [64]. Whereas activators of isoform $G_{\alpha q}$ influence insulin secretion via PLC activation leading to generation of IP_3 and DAG. These second messengers further release Ca^{2+} from intracellular stores and activate PKC respectively. Another set of activators stimulate adenylate cyclase and activate PKA through generated cAMP [65, 66]. Of the three subunits, α -subunit was dissected with respect to insulin secretion but later studies justified the presence of $\beta\gamma$ -subunits in both Ca^{2+} and GTPS stimulated insulin release [67].

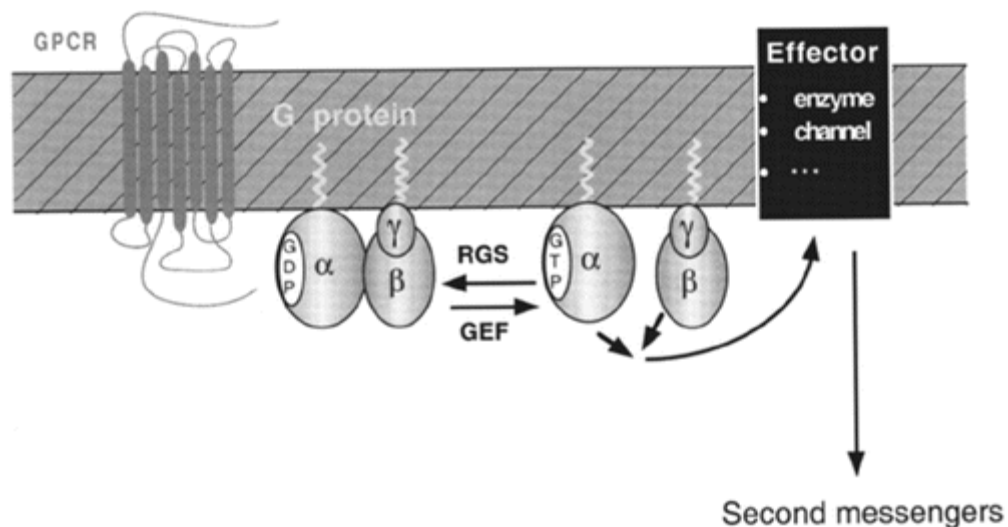


Figure 1-8. Effect of regulator of G protein signaling (RGS) proteins on the classical G protein cycle at the plasma membrane. A G protein–coupled receptor (GPCR) serves as a guanine nucleotide exchange factor (GEF) that activates the G protein by enhancing GDP dissociation from the $G\alpha$ subunit. $G\alpha$ and $G\beta\gamma$ dissociate and stimulate their respective effectors. RGS proteins serve as GTPase activating proteins that accelerate GTP hydrolysis and thereby return the $G\alpha$ subunit to its inactivated GDP-bound form, followed by reassembly of the heterotrimer [68].

Role of small monomeric G-proteins

In the classic 2-compartment model of insulin secretion, the link between small GTP-binding proteins and the K_{ATP} channel-dependent and $-$ independent pathways always remains unclear. And for the reason that guanine nucleotides have an effect on secretion of insulin the role for small G-proteins was determined. Small G-proteins have proven to play role in pairing t-SNARE with v-SNARE in Ca^{2+} -induced exocytosis. And also these proteins initiate and maintain transition of beta granules from reserve pool to readily releasable pool thus maintaining the biphasic insulin response.

Small GTP-binding proteins are fondly called as “molecular switches” for their tendency to alternate between active and inactive conformational states. GTPases of varying diversity control and modulate different cellular activities from growth to cell death. They participate in membrane traffic controlling formation, targeting and fusion of secretory vesicles. Small G-proteins are otherwise termed as Ras-like proteins with Ras being a prototype. They are essentially grouped into six major families: Ras, Rho, Rab, Arf, Sar1 and Ran family of proteins. The last four members regulate intracellular vesicle trafficking, the Ras family regulate cellular growth and the Rho family regulate cytoskeletal remodeling [69].

They all share a common sequence motif for guanine nucleotide binding which is a hallmark for all GTPases. Outside the GTP-binding motif, members of different family are related by varying degrees of functional sequences which specify a particular upstream/downstream effector. As expected with structural diversity, GTPases exhibit diverse range of functions. Members of the Arf and Sar1 family coordinate the

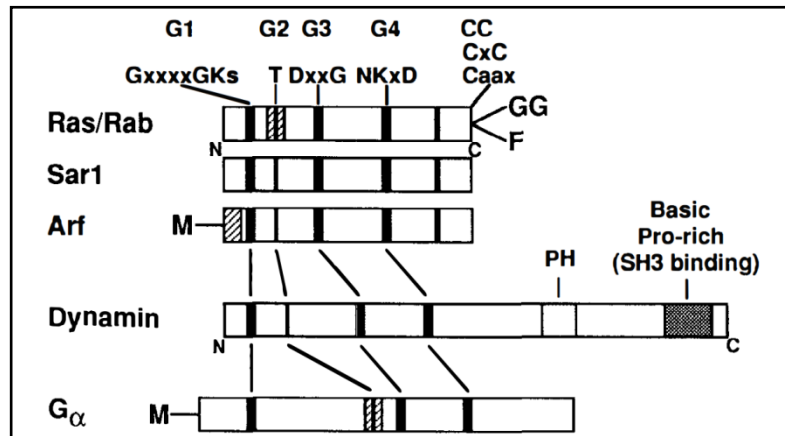


Figure 1-9. Schematic to represent both common and unique structural features of GTPases.

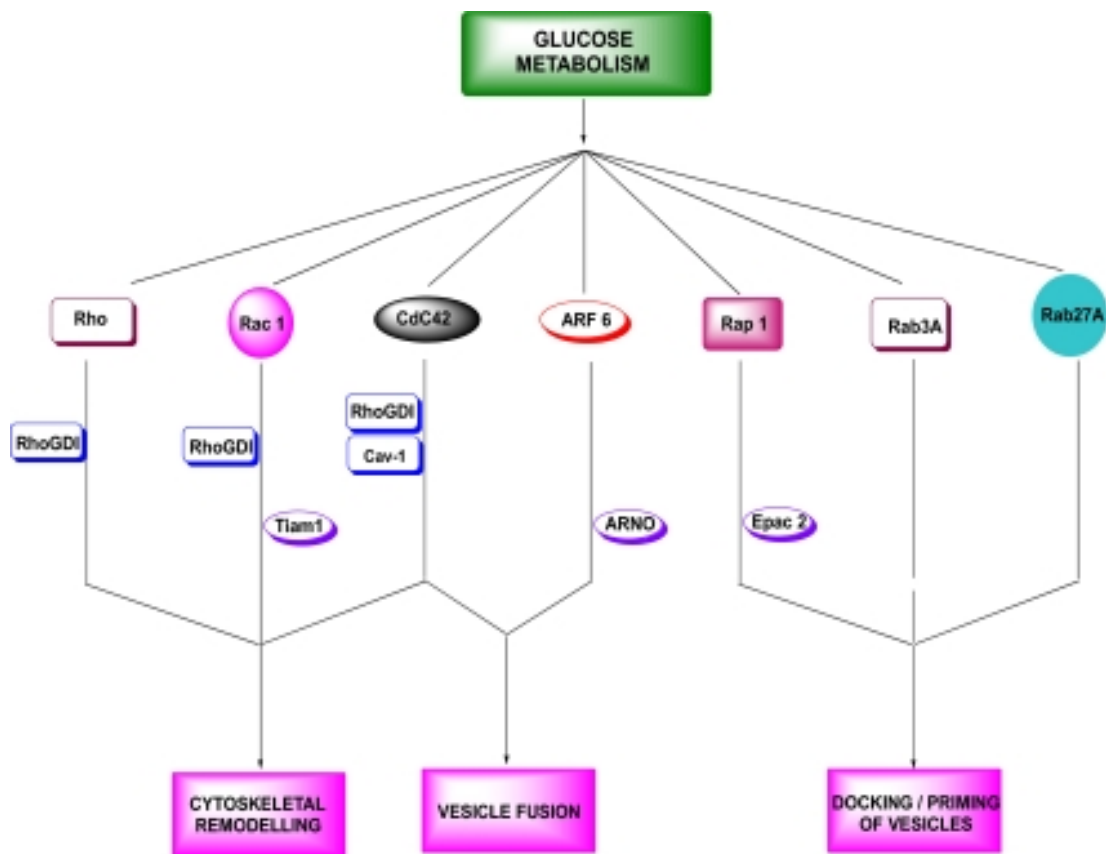
Blackbars-guanine nucleotide binding and GTP hydrolysis; **a**-aliphatic residue; **F**-farnesyl chain; **GG**-geranylgeranyl chains; **M**-myristoyl chain; **PH**-pleckstrin homology

biochemical machinery for vesicle budding, and the Rab, boasting of the largest family, controls protein-protein interactions during vesicle fusion.

Small G-proteins contain an amino and carboxy terminal, which directs the protein to a specific location and an effector domain, for binding to effector proteins [70]. Another characteristic feature of small G-proteins is the presence of accessory factors. Major accessory factors include guanine nucleotide exchange factors and GTPase activating proteins. Another accessory protein is the GDP dissociating inhibitor [GDI] which sequesters inactive protein. Some of the GEFs and GAPs are protein-specific whereas others are same for a class of proteins.

Mammalian Rho GTPases comprise of nearly 20 members among which only three are well characterized for cytoskeletal remodeling: Rho, Rac1 and Cdc42. Rho GTPases are governed by GEFs which catalyze exchange of GDP for GTP, by GAPs which activate the GTPase function and by GDIs which control the access of GTPases to GEFs and GAPs and also their access to membranes where active effectors reside

[71]. In early 90s, two Rho G-proteins Cdc42 and Rac1 were discovered to play key roles in GSIS and colocalized to insulin granule [72-75]. The activation kinetics of Cdc42 and Rac1 made clear that former was activated by glucose at an early time-point [76, 77]. Rho GTPases have also been shown to bind and be responsible for translocation of PI4P5Kinase and it has been shown that this kinase is important for preparing vesicles for fusion at the plasma membrane [78, 79].



From Kowluru A, Endocr Rev. 2010 Feb; 31(1): 52-78

Figure 1-10. Modulatory roles of various classes of small G proteins and their accessory proteins in insulin secretion. Small G proteins, such as Rac1, Cdc42, and ARF-6 (and potentially Rho) regulate cytoskeletal reorganization and vesicular fusion in the pancreatic islet. Rab3A, Rab27A, and Rap1 play an essential role in docking and priming of secretory vesicles at the exocytotic sites. Glucose-mediated activation of

these signaling proteins is under the fine control of various regulatory proteins including GDIs (e.g., Rho GDI and Cav-1) and GEFs (Tiam1, ARNO, and Epac2).

Rab GTPases have been reported to regulate different membrane trafficking pathways through their interaction with various effectors. Rab proteins are well-conserved vesicle tetherers to target membranes. Sec4 is a Rab GTPase that was implicated to play a role in secretory process [80]. Among the Rab proteins, Rab3A [81] and Rab27A have been associated with pancreatic beta cells located on insulin granules [82, 83]. Kasai et al delineated the role for Rab in glucose signaling by studying intact and Rab-defective beta cells. They quantitated insulin secretion in *ashen* mice [mutated for Rab27a] which are known for defects in pigment granule transport. These animals were characterized by impeded GSIS, reduced number of docked granules and abnormal glucose-induced replenishment of granules in RRP [84]. Granuphilin, an effector of Rab, showed diminished interaction with syntaxin-1a in *ashen* islets. This suggests an impaired SNARE complex formation, the reason for abnormal granule trafficking [84].

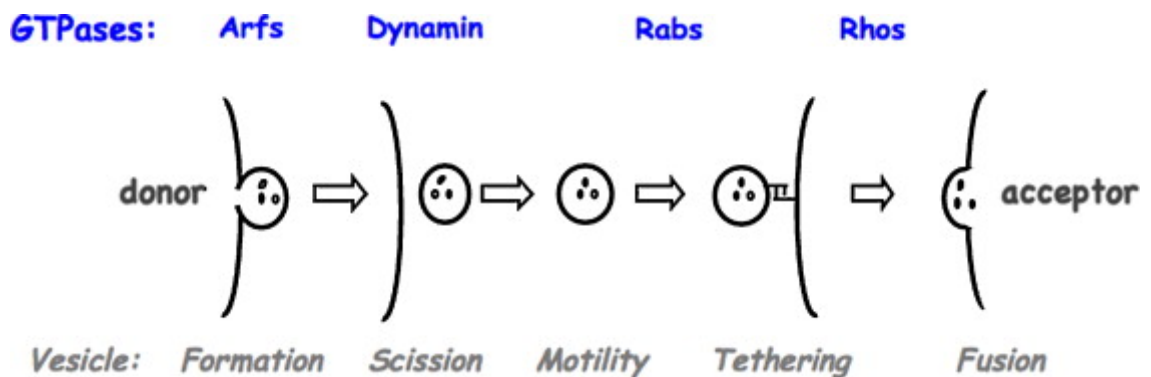


Figure 1-11. Schematic representation of various small G-proteins that regulate vesicle transport as highlighted [85].

Two other members of Ras-like GTPases, Rap1 and RalA, have been closely studied in beta cells with Rap1 being the first to be identified. Leiser showed that stimulus-induced carboxymethylation of Rap1 synced well with increased insulin secretion [86]. A GEF identified for Rap1 is Epac2 which mediates activation of Rap1 by cAMP in beta-cells. It is well-known that cAMP is a potentiator of GSIS and studies from islets of *Epac2*^{-/-} mice proved that Epac2 is a selective agonist of cAMP-mediated fusion events. Another similar protein is RalA which mediates interaction with exocyst complex and thus aids in granule tethering and docking at plasma membrane [87]. Knockdown of RalA from INS-1 beta cells caused a reduction in depolarization-induced secretion of insulin and also a reduction in size of RRP.

Arfs [ADP-ribosylation factors] have been widely implicated in vesicular organization. Arf was originally discovered as a cofactor for ADP-ribosylation of heterotrimeric G protein Gs [88]. Arf family is a highly conserved family from yeast to humans consisting of six members: Arf 1-6. Members of the family are classified into three classes based on sequence homology: Class 1, Arf1-3; Class 2, Arf 4-5; Class 3, Arf6 [89]. They differ from other members of Ras superfamily by the absence of a carboxyl-terminal prenylation motif. Arfs are posttranslationally modified by the addition of myristoyl moiety on the amino-terminal glycine residue, which is essential for membrane association and function [90-21]. Arf proteins undergo similar GTP/GDP cycling with GTP-bound form conferring activity. GTP/GDP cycling is regulated by GEFs and GAPs as Arfs seems to have negligible GTPase activity [93]. With the help of X-ray crystallography, molecular structures for mammalian Arf1 and Arf6 were determined. In Arfs, GTP binds to regions called “switch” regions which as its name implies aids in

switching Arf from inactive to active conformation. Substrate specificity for Arf1 and Arf6 is differentiated by the GTP-bound switch region. Compartment localization also determines the Arf that is activated. Genetic studies performed in yeast revealed a role for Arf in vesicular transport [94]. Their participation in membrane trafficking events depends on recruitment of coat proteins, activation of lipid-modifying enzymes. Arf proteins localize to golgi apparatus and secretory granules, two organelles in-charge of organizing vesicles. Arf has been best characterized as an essential part of COP-coated vesicles which mediate intra-golgi traffic. Apparently Arf function is as necessary for transport to ER as it is for intra-Golgi transport.

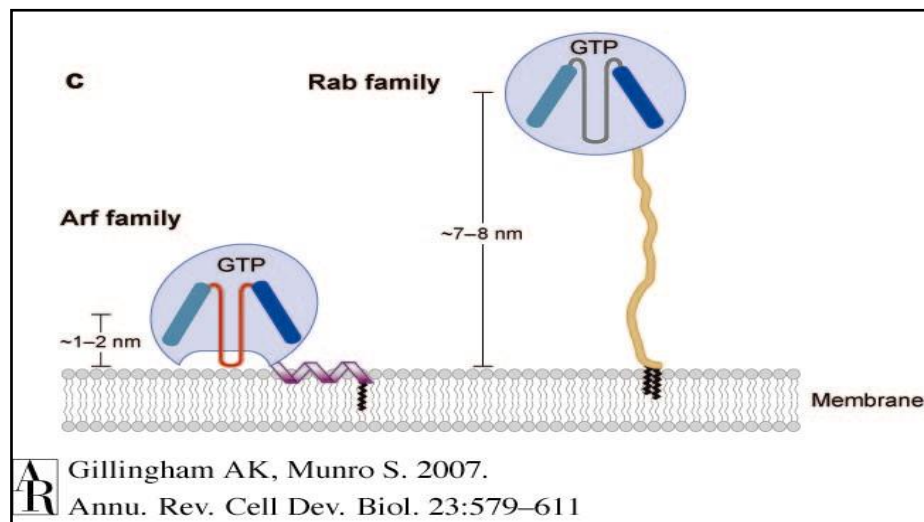


Figure 1-12. Schematic representation of typical Arf and Rab G proteins on a membrane. For the Rab family the extended hypervariable region (yellow) connecting the G protein to the lipid anchor allows bound effectors to move further from the bilayer than is the case for the Arfs [95].

Most studied Arf members are Arf1 and Arf6 which regulate Golgi-associated and granule associated functions respectively. Arf1 in the active conformation binds to Golgi membranes. A mutant of Arf1 deficient of GTP-hydrolysis causes accumulation of ER-

to-Golgi carrier vesicles in vivo. This indicates as much as GTP-binding is important, hydrolysis of bound GTP is equally necessary.

Arf6, on the other hand serves to maintain both exo- and endocytosis in secretory cells. It governs these above processes by translocating to the plasma membrane from where it can regulate vesicle traffic, cytokinesis and actin reorganization. Constitutively active mutants of Arf6 also cause accumulation of proteins in internal structures. Thus Arf6 plays an important role in spatio-temporal organization of proteins which is essential for activity.

Some of the effects are due to regulation of PLD and PIP5K. Activation of PLD generates phosphatidic acid [PA] which in turn activates PIP5K. This results in production of PI-4,5-P₂ which contribute to membrane trafficking events. There have been controversial reports implicating Arf6 in clathrin-dependent and -independent pathways [96, 97].

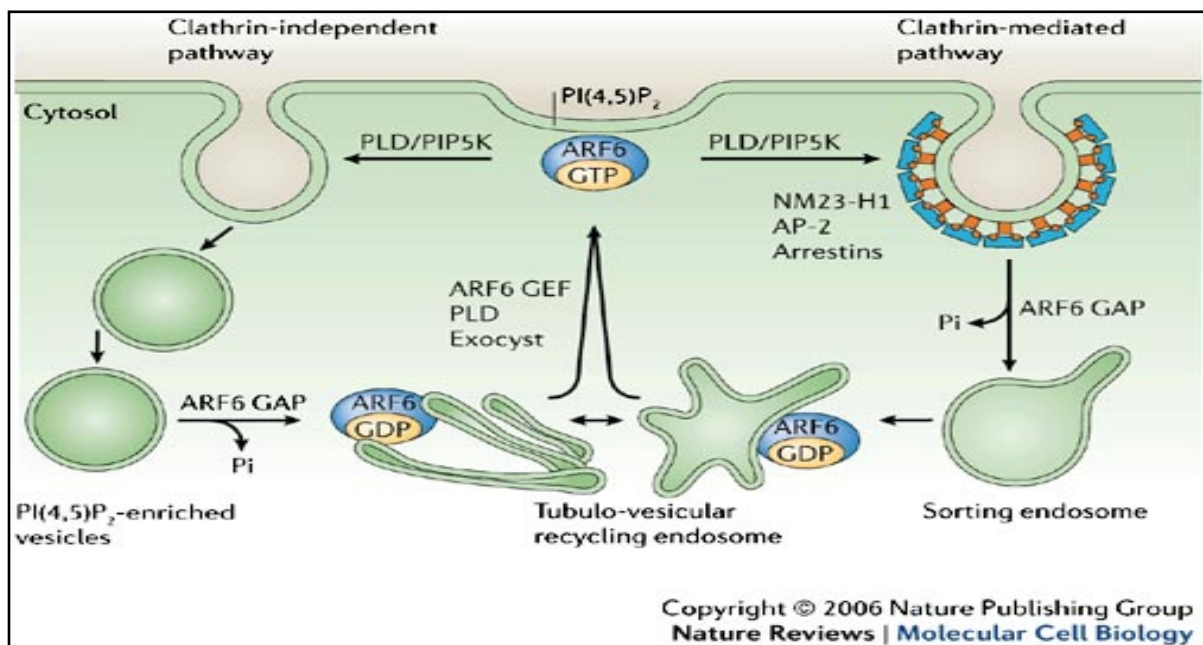


Figure 1-13. Image from D'souza-Schorey depicting a model for involvement of Arf-dependent and -independent pathways [97].

In addition to membrane traffic, Arf6 also plays an important role in actin remodeling required for ruffle formation, cell migration, cell spreading and phagocytosis. Arf6 regulates actin remodeling through the activation of Rac1 and lipid metabolism.

Modular GEFs for Arf

Small G-proteins are active in the GTP-bound form, which are catalyzed by the GEFs. In case of Arf proteins, the displacement of GDP by GTP causes displacement of N-terminal amphipathic helix and leads to interaction with membrane. From molecular analysis of various members of Arf-GEFs, it was found that they all share a catalytic domain, Sec7 domain [98]. Brefeldin A was initially used as target against Sec7 but was later found not to be effective against all Sec7 domains, especially for cytohesin family. Sec7 domain proteins also share high conservation from yeast to mammals. Along with GEF, Sec7 domain requires the presence of phospholipids to aid in guanine nucleotide exchange. How do phospholipids aid in this reaction? They serve to expose the N-terminal helix which then allows the GEF to interact with Arf protein to add on a GTP.

Sec7-domain containing proteins are represented by four subfamilies: Gea/GBF [golgi-specific brefeldin A-resistance factor], BIG [Brefeldin A-inhibited GEF], Cytohesin and EFA6. Of particular interest is the cytohesin family, which seems to play a role in activation of Arf6 for membrane trafficking events. Cytohesins gained further attention for the ability of their PH domains to bind specifically to PI(3,4,5)P3 and recruited to plasma membrane [99]. Cytohesins are again comprised of four members: cytohesin-1, ARNO, ARNO 3/GRP1 and cytohesin-4. The C-terminus of these proteins contains a

polybasic region which allows for phosphorylation by PKC. Phosphorylation of ARNO by PKC has shown to aid in cytoskeletal remodeling [100]. What then is a substrate for cytohesin? In an in vitro setting, cytohesins favor Arf1 as a substrate than Arf6 but in vivo, Arf6 is favored as a substrate [101, 102]. Overexpression of ARNO in HeLa cells induced changes in actin cytoskeletal structure as seen by the activation of Arf6 [103].

Apart from the GEF activity, cytohesins have been found to play major roles in regulating cellular gene expression in response to extracellular signals and thus pathogens exploit this to alter cellular program. Most commonly reported signaling, is the involvement of cytohesin 1 and ARNO in the signaling of ERK1/2 [104-106]. In activation of T-cell, cytohesin-1 has been found to play a role in ERK1/2 activation since a dominant-negative cytohesin-1 abolished ERK1/2 activation. In a recent report demonstrated in liver cells, insulin resistance resulted from an inhibition of cytohesins by secinH3. Blockade of cytohesin in secinH3-fed mice showed increased expression of gluconeogenic genes, reduced glycolytic and fatty acid metabolic genes and a compensatory increase in plasma insulin [107].

Dyanmins: a new league of GTPases

Dynamins [108] belong to a novel group of GTPases comprising of three members: dynamin 1-3 [109-111]. As a family they share sequence homology in an amino-terminal GTPase domain responsible for guanine nucleotide binding and GTP hydrolysis. They differ strikingly from Ras GTPases by their higher intrinsic rates of GTP-hydrolysis. While the amino terminal is responsible for GTP-binding and subsequent hydrolysis, the presence of a proline-rich region is essential for effector

binding. Each of these domains carries some significance with regards to function [112]. First evidence for dynamin in recycling of vesicles was seen in *Drosophila* where temperature-sensitive alleles exhibited rapid paralysis. This was related to the aberrant recycling of synaptic vesicles from nerve terminals [113]. In recent observations, an identical neuronal protein to dynamin, dephosphin was found to aid in stimulus-dependent dephosphorylation in nerve terminals. Dynamin is now a substrate for kinases, especially protein kinase C and casein kinase [114]. The phosphorylation cycle of dynamin is essential to regulate its GTPase activity for rapid endocytosis in nerve terminals.

This was verified in vivo in mammalian cells, using dynamin mutated for GTP binding and subsequent hydrolysis. Another mutant, with a completely truncated GTPase domain also inhibits endocytosis [115]. It also has an effector-binding domain. Thus dynamin plays an essential role in endocytosis [116], whether it plays a role in exocytosis is yet to be determined.

Chapter 2

Materials and Methods

2.1 Materials

SecinH3 was from Tocris Biosciences [Ellisville, MO]. siRNA-Arf6 consisting of pools of three to five target-specific 19-25 nt siRNAs were from Santa Cruz Biotechnology [Santa Cruz, CA]. siRNA-ARNO was from Dharmacon [Lafayette, IL]. Antisera directed against Arf6, ARNO, ICMT, phospho- and total dynamin 1 were from Santa Cruz Biotechnology [Santa Cruz, CA]. Cdc42 and Rac1 antisera were from BD Biosciences [San Jose, CA]. Cdc42 and Rac1 activation kits were from Cytoskeleton Inc., [Denver, CO]. Phospho- and total antibodies for ERK1/2 and cofilin were purchased from cell signaling. Arf6 activation assay kit and the Classic Co-IP kit were from PIERCE [Rockford, IL]. siRNA-ICMT [43907710] and scrambled siRNA [negative control; 4390843] were from Ambion. AFC was from Cayman Chemical [63270]. ECL reagent was from GE Healthcare [RPN2132]. HiPerFect transfection reagent was obtained from Qiagen [301705]. The rat insulin ELISA kit was from American Laboratory Products [80-INSRTH-E01]. DCHFDA [35845], thapsigargin [T9033] and etoposide [E1383] were from Sigma Aldrich. Alexa-fluor 488 anti-rabbit secondary antibody [A11008], PLD assay kit [A12219] and Hoechst dye [3570] was from Invitrogen molecular probes. Cell proliferation kit [MTT, 11465007001] was purchased from Roche diagnostics. All other reagents used in these studies were from Sigma Aldrich Co. [St. Louis, MO] unless stated otherwise.

2.2 Insulin-secreting INS 832/13 cells, rat islets and human islets

INS 832/13 cells were kindly provided by Dr. Chris Newgard (Duke University Medical Center, Durham, NC). The cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum supplemented with 100 IU/ml penicillin and 100 IU/ml streptomycin, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol, 11 mM glucose, and 10 mM HEPES (pH 7.4). Islets were isolated from pancreas of male Sprague-Dawley rats (Harlan Laboratories, Oxford, MI), using collagenase digestion and a ficoll gradient as we described previously [117]. All experiments were reviewed and approved by the Wayne State University Institutional Animal Care and Use Committee. Human pancreatic islet lysates were kindly provided by Dr. Karl Olson [Michigan State University, Lansing, MI].

Male [9-11 wks] ZDF and Zucker Lean control [ZLC] rats were procured from Charles River laboratories [Wilmington, MA] and maintained in a 12-h light/dark cycle with free access to water and food [Purina Diet no. 5008; Charles River Laboratories]. All animal protocols were reviewed and approved by the Wayne State University Institutional Animal Care and Use Committee. Hyperglycemia in diabetic rats was confirmed prior to sacrifice by tail vein puncture using Glucometer Elite from Bayer [Leverkusen, Germany]. Body weight of ZLC and ZDF rats were 300 ± 6 g and 396 ± 12 g respectively [n=11; p <0.05]. Islets were isolated by collagenase digestion method [117].

Human islets from normal and T2DM donors were obtained from Prodo Laboratories, Inc. [Irvine, CA]. Control islets [from a 54 year old male donor; 85-90%

purity] and diabetic islets [from a 45 year old male donor; ~60% purity] were homogenized with Tris-HCl buffer [50 mM, pH 7.4] containing sucrose [250 mM], EDTA [1 mM], DTT [1 mM], and protease inhibitor cocktail. Lysate proteins were resolved on 12% SDS-PAGE, and used for Western blot analysis.

2.3 Isolation of total particulate and soluble fractions from INS 832/13 cells and rat islets

INS 832/13 cells were homogenized in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1mM EDTA, 1mM PMSF, 1mM Na_3VO_4 , 1mM NaF and protease inhibitor cocktail) and were centrifuged at 105000 x g for 1 h to separate total particulate and soluble fractions. Proteins from individual fraction were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The blots were then probed with antibody raised against Arf6 [1:1000] or ARNO [1:1000] or ICMT [1:500 dilution] and probed with appropriate secondary antibody conjugated to horseradish peroxidase. Immune complexes were then detected using the enhanced chemiluminescence kit.

2.4 Hydrophilic and hydrophobic phase partitioning method using triton X-114

Total hydrophobic and hydrophilic phases of lysates derived from INS 832/13 cells and pancreatic islets were separated using triton X-114 according to method described earlier by us [Veluthakal]. Briefly, about 400 μg of cell [INS 832/13 cell or islet] homogenate protein, prepared in 400 μl of buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 2 mM MgCl_2 , 10 $\mu\text{g}/\text{ml}$ leupeptin, and 2 $\mu\text{g}/\text{ml}$ aprotinin), supplemented with 1 % (w/v) Triton X-114 was overlaid on 400 μl sucrose cushion 6 % (w/v) prepared in 20 mM Tris-HCl buffer (pH 7.4) containing 0.06 % (w/v) triton X-114. Following brief incubation

at 30 °C, samples were centrifuged at 300 x g for 3 min and the aqueous phase was mixed with 0.5 % (w/v) fresh triton X-114 at 4 °C. Following dissolution, the mixture was again overlaid on the same sucrose cushion, incubated for 3 min at 30 °C and centrifuged at 300 x g for 3 min. The lower hydrophobic phase was diluted to a final volume of 400 µl with homogenization buffer, while the aqueous phase was transferred into a separate tube supplemented with 2 % fresh triton X-114, incubated for 3 min at 30 °C, and centrifuged at 300 x g without sucrose cushion. The supernatant obtained thereof served as total hydrophilic phase. The relative abundance of ARNO in hydrophilic and hydrophobic phases were determined by Western blotting.

2.5 Transfection of Arf6 or ARNO mutants and siRNAs

INS 832/13 cells were subcultured at 50-60% confluency and transfected using Effectene [Qiagen, Valencia, CA], with 0.2 µg of plasmid DNA constructs against either dominant-negative of Arf6 [T27N] or ARNO [E156K] per well of a 24-well plate. Endogenous Arf6 or ARNO or ICMT expression was depleted by transfecting cells using small interfering RNA [siRNA; 100 nM] using HiPerfect transfection reagent [Qiagen, Valencia, CA]. Efficiency of mutant expression or protein knockdown was determined by Western blotting.

2.6 Insulin release studies

Arf6 or ARNO mutant or siRNA-transfected or secinH3 inhibitor-treated cells were cultured overnight in low serum and low glucose containing medium and then stimulated either with high glucose, KCl or arginine in Krebs-Ringer bicarbonate buffer [KRB, pH 7.4] for different time intervals as indicated in the text. In studies involving

KCl-induced insulin secretion, we noticed that INS 832/13 cells were not responsive to 40 mM KCl in releasing insulin. However, higher KCl concentrations [60 mM] were found to elicit robust insulin release. Therefore, in KCl-stimulated insulin secretion [KSIS] studies, cells were incubated with 60 mM KCl in an osmolarity-balanced KRB medium [79]. For arginine [L-Arg]-stimulated insulin release, a stock solution was prepared in Tris.HCl [pH 7.4], and diluted to desired concentration with KRB. The insulin released into the medium was quantitated by ELISA [117].

2.7 Quantitation of Arf6.GTP in pancreatic β -cells

Active Arf6 was quantitated by a pull-down assay. Briefly, the incubation medium was aspirated and cells were washed with ice-cold PBS. Cells were lysed with 500 μ l lysis buffer and the lysate was clarified by centrifugation at 16,000 x g at 4 °C for 15 min and incubated ~400 μ g protein with 100 μ l of glutathione resin and 100 μ g of GST-GGA3-PBD beads at 4 °C for 1 hr with gentle rocking, following which the reaction mixture was spun at 6,000 x g for 30 sec. The GST-tagged beads were washed [3x] and proteins were separated by SDS-PAGE and activated Arf6 was identified by Western blotting.

2.8 Quantitation of Cdc42 and Rac1 activation

Relative degree of activated Cdc42 and Rac1 [i.e., GTP-bound form] were determined by p21-activated kinase-p21-binding domain pull-down assay as described in [117]. Briefly, INS 832/13 cells treated with either diluent or secinH3 [50 μ M] or cells were either mock-transfected or transfected with ARNO-siRNA were cultured overnight in low serum-low glucose media. Cells were stimulated with either low [2.5 mM] or high

[20 mM] glucose for 3 or 30 min at 37 °C in the continued presence of either diluent or secinH3 with respect to inhibitor studies. The GTP-bound forms of Cdc42 and Rac1 in the pull-down samples were quantitated by Western blotting and densitometry.

2.9 Co-immunoprecipitation studies

Immunoprecipitation studies were performed using the Classic Co-IP kit as suggested by the manufacturer [118]. Briefly, β -cell lysates [500 μ g protein] were incubated with anti-ARNO for 2 hr at 4 °C followed by incubation with agarose resin for an additional 1 hr at 4 °C. Beads were washed; eluted using sample buffer and proteins were resolved by SDS page to quantify Arf6.

2.10 Immunofluorescence studies

INS 832/13 cells were plated onto coverslips and incubated with [2.5 or 20 mM] glucose for 30 or 60 min at 37 °C as indicated in text, followed by washing in PBS and fixed with 4% paraformaldehyde solution for 15 min at room temperature. They were then permeabilized with 0.2% Triton X-100 for 15 min at room temperature. After blocking with 1% BSA for 1 hr, the cells were further incubated with primary antibodies [Arf6 [1:150], ARNO [1:150], ICMT [1:150] and p-dynamin-1 [1:150] in 0.1% BSA solution for 1 hr. After extensive washes, the cells were further incubated with secondary antibodies Alexa-fluor 488 anti-mouse [1:1000], Alexa-fluor anti-goat 546 [1:1000], Alexa-fluor anti-rabbit [1:1000] or Alexa-fluor anti-sheep 546 [1:1000] in 0.1% BSA solution for 1 hr at 37 °C. Hoechst dye was used to stain for nuclei. The coverslips were then mounted on glass slides containing mounting media [DAKO corporation,

Carpinteria, CA] and visualized under a confocal LSM 510 microscope in the midplane using a 63X oil-immersion lens [118].

2.11 Quantitation of PLD activity

The Amplex Red phospholipase D [PLD] assay kit provides for a sensitive method of measuring PLD activity in vitro. An enzyme-coupled assay, PLD activity is measured by using Amplex Red reagent. First, PLD cleaves phosphatidyl choline [PC] to choline and PA. The choline generated is oxidized by choline oxidase to hydrogen peroxide. Finally, hydrogen peroxide along with horseradish peroxidase present in the reagent mix reacts with Amplex Red and generates highly fluorescent resorufin. We used the protocol for PLD enzyme activity at near neutral pH 8. Essentially treated cells were collected and lysate prepared in buffer [Tris 50 mM, pH 8.0]. Cells were broken by three freeze-thawing cycles and clarified to remove debris [unbroken cells]. Supernatant was used for protein estimation. 100 µg of lysate was made to 100 µL total volume and to which 100 µL of the prescribed reagent mix was added and incubated for 30 min at 37 °C. Fluorescence was measured in a microplate reader [Synergy Biotek instrument] at E_x 530 nm and E_m 590 nm.

2.12 Quantitation of ROS generation

INS 832/13 cells or rat islets were treated with secinH3 and stimulated with glucose for 60 min. Following incubation, medium was removed, and cells were further incubated with DCHFDA [10 µM] at 37 °C for 30 min in PBS. DCHFDA is a non-polar compound, diffuses rapidly into the cells and hydrolyzes cellular esterases into polar compound, 2', 7'-dichlorofluorescein. In the presence of ROS, 2', 7'-dichlorofluorescein

oxidizes readily to form fluorescent compound dichlorofluorescein. After 30 min, cells were washed with ice-cold PBS and harvested. Protein estimation was done and equal amounts of protein were added into 96-well black bottomed plates. Fluorescence was measured at E_m 485 nm and E_x at 535 nm using luminescence spectrophotometer [Perkin Elmer].

2.13 Preparation of cell lysates for phosphoprotein analysis

INS 832/13 cells or isolated islets were lysed in 50 mM Tris buffer pH 8.0 containing 10 mM NaCl, 1% NP-40, 5% deoxycholic acid, 0.1% SDS, 1mM EDTA, 1mM PMSF, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 5 mM EGTA, 5 mM EDTA, 10 mM NaF and 1 mM sodium orthovanadate. After incubation for 10 min in lysis buffer on ice, samples were clarified to remove debris and supernatant collected. Protein estimation was done using Pierce BCA 660 nm protein assay. Equal amounts of protein was loaded and separated by SDS-PAGE. Resolved proteins were transferred onto nitrocellulose membrane and probed for phosphoproteins as indicated in text.

2.14 Caspase-3 activity

Activation of caspase-3 was assessed in cells either transfected with ICMT siRNA or in cells treated with AFC. Cells were harvested and homogenized in sample buffer (0.5 % Nonidet P-40, 20 mM HEPES, pH 7.4, 100 mM NaCl and 20 mM DTT and PIC). And ~30 µg of proteins were resolved by SDS-PAGE (12%) and immunoprobed for caspase-3. Activation of caspase-3 is evidenced by the presence of a hydrolytic product (~17 kDa). Etoposide (60 µM, 6 h) was used as a standard for apoptotic cell death.

2.15 Cell viability assay

INS 832/13 cells were either treated with AFC (100 μ M, 1 h) or transfected with ICMT -specific or scrambled siRNA as described above. Cell viability was determined by incubating AFC treated or ICMT-siRNA transfected cells with 10 μ L of stock MTT [4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide] for 4 h at 37°C. Following dissolution of formazan crystals in solubilization solution, the absorbance was measured at 570 nm using ELISA plate reader.

2.16 ICMT expression profile

INS 832/13 cells were plated in six-well plates, grown to 70 % confluence and treated with low glucose (2.5 mM), high glucose (50 mM), palmitic acid (300 μ M), palmitic acid plus high glucose for 48 h or thapsigargin (0.5 μ M) for 9 h. Cells were then harvested and centrifuged. The pellet was resuspended in buffer solution [0.5 % Nonidet P-40, 20 mM HEPES, pH 7.4, 100 mM NaCl, 20 mM DTT and protease inhibitor cocktail]. Equal amount of proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The blots were then probed with antibody raised against ICMT [1:500 dilution] and with rabbit secondary antibody conjugated to horseradish peroxidase. Immune complexes were then detected using the enhanced chemiluminescence kit.

2.17 Statistical analyses

The statistical significance of the difference between the experimental conditions was determined by Student's t test unless mentioned otherwise. *p* values < 0.05 were considered significant.

CHAPTER 3

ARNO/Arf6 signaling cascade is involved in nutrient-induced insulin secretion from the pancreatic beta cell.

- Portions of this work have been published [copy of the published manuscript is appended].

Jayaram B, Syed I, Kyathanahalli CN, Rhodes CJ, Kowluru A. Arf nucleotide binding site opener [ARNO] promotes sequential activation of Arf6, Cdc42 and Rac1 and insulin secretion in INS 832/13 β -cells and rat islets. *Biochem Pharmacol.* 2011 Apr 15; 81(8):1016-27. Epub 2011 Jan 26.

ARNO/Arf6 signaling cascade is involved in nutrient-induced insulin secretion from the pancreatic beta cell

Homeostasis of blood glucose is a highly regulated process maintained by various hormones, nutrients and neurotransmitters. Of significance is the contribution of peptide hormone, insulin, which coordinates signals to effectively maintain blood glucose. The primary pathway for secretion of insulin from within large dense core granules involves coupling of glucose metabolism and other factors which lead to depolarization of plasma membrane thus preparing it for fusion of granules. Influx of Ca^{2+} has always been the fort for insulin secretion. Recent years of research have identified many of the molecular players responsible for vesicular transport after influx of Ca^{2+} , but it never stops there. There are still many signaling molecules like phospholipases, kinases, phosphatases, actin-modifying agents and finally G-proteins.

Extensive studies in the area of small G-proteins have established a role in regulating various cellular functions including proliferation, survival and demise. So far,

four major classes of small G-proteins have been identified in the pancreatic β -cell. These include the Ras, Rho, Rab and ADP-ribosylation factor [Arf] family of G-proteins [69]. The Ras family is responsible for signal transduction in cellular differentiation and proliferation, Rho family regulate actin cytoskeleton and Rab family aid in priming/docking of secretory vesicles. For decades, the Arf family has been studied in relation to cellular growth and secretion in several cell types. Yet its potential in the pancreatic beta cell was untapped. Though originally identified as an ADP-ribosylator for cholera toxin, Arf has gained much importance as a critical modulator of membrane traffic in eukaryotic cells. Among the six members of the Arf family, Arf1 has been the paradigm for intracellular vesicular trafficking by regulating budding via recruitment of coat proteins. Initial evidence for involvement of Arf1 in secretory process came from yeast genetic studies where a deletion of Arf gene led to a secretory defect in *Saccharomyces cerevisiae* [119]. Another member, Arf6 is also a well documented protein for its positive modulatory roles in trafficking of secretory granules to the plasma membrane for exocytosis [120]. Regazzi and coworkers first described localization and regulation of Arfs in insulin-secreting RINm5f cells [72, 73]. More recently, Lawrence and Birnbaum have demonstrated regulatory roles for Arf6 in insulin secretion mediated by glucose, GTP γ S and membrane depolarization. They further demonstrated that Arf6 regulates insulin secretion by maintaining plasma membrane phosphatidylinositol 4, 5-biphosphate [PIP₂; Lawrence PNAS]. Existing evidence also supports a potential role for PLD in physiological insulin secretion [121-123].

Arf6 cycles between the GDP-bound [inactive] and GTP-bound [active] configurations; which are tightly regulated by two distinct classes of regulatory factors

namely the GTPase activating proteins [GAP] and the GTP/GDP exchange factors [GEF; **95; 124**]. GAPs inactivate Arf6 by promoting its conversion to the inactive GDP-bound form, while GEFs facilitate its activation. The GEF activity is a rate-defining step and involves coordination of multiple intracellular signals. In this context, many GEFs with distinct size and structure have been identified for Arf6 [**102, 125, 126**]. However, in the majority of the signaling events, only one member belonging to the cytohesin family has been closely linked to activate Arf6 [**127, 128**].

Arf6 and ARNO are present in pancreatic beta cells: subcellular view

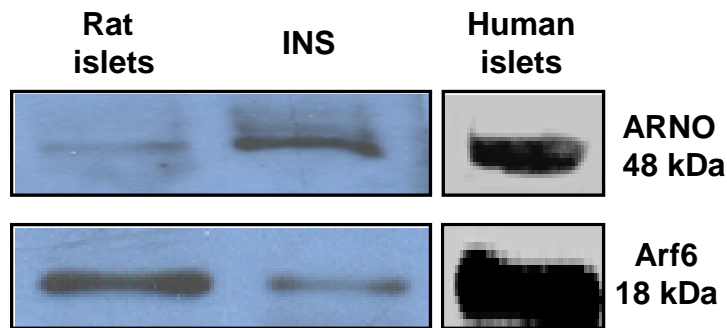
Thus the first goal of this project was aimed at establishing a positive involvement for Arf6 and its exchange factor ARNO in regulated insulin secretion. Galas MC demonstrated a potential role for Arf6 associated with secretory granule in chromaffin cells [**120**]. As an initial step, we immune-characterized Arf6/ARNO in pancreatic beta cells: INS 832/13 cells, rat and human islets. **Figure 3-1 A** clearly shows the presence of Arf6 and ARNO in the various pancreatic beta cells. It is well-reported that small G-proteins, including Rho family i.e Cdc42 and Rac1, are cytosolic in distribution and translocate to membranous sites on activation [**129**]. Unlike most smgs, Arf6 was predominantly localized to membranous fraction. Data in **Figure 3-1 B** demonstrates cytosol/membrane abundance of Arf6 and ARNO in clonal pancreatic beta cells and rat islets. As reported in other neuroendocrine cells, Arf6 was abundant in membranous fraction and ARNO in cytosol.

As the total membrane fraction derived from the single step centrifugation technique would comprise of membranes derived from various intracellular organelles

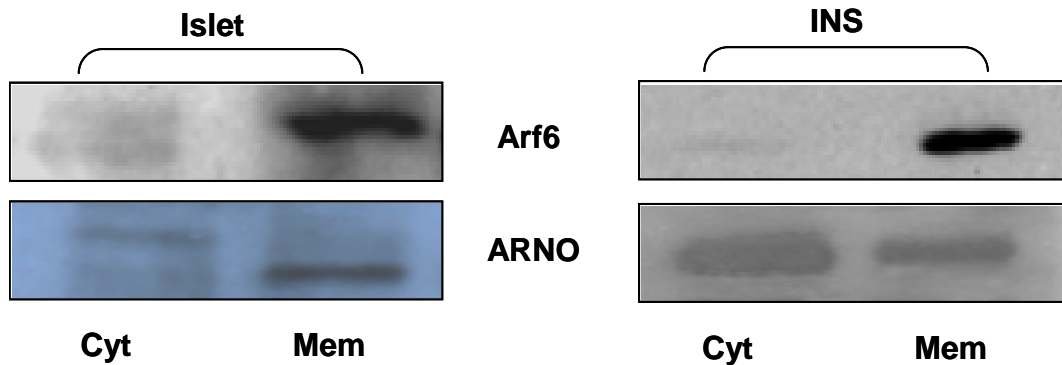
[secretory granules, mitochondria, microsomes], we studied the intracellular distribution of both Arf6 and ARNO via differential centrifugation. Subcellular fractionation in cultured chromaffin cells revealed Arf6 to be bound to secretory granule membrane.

Figure 3-1 C indicates a similar pattern in INS 832/13 cells.

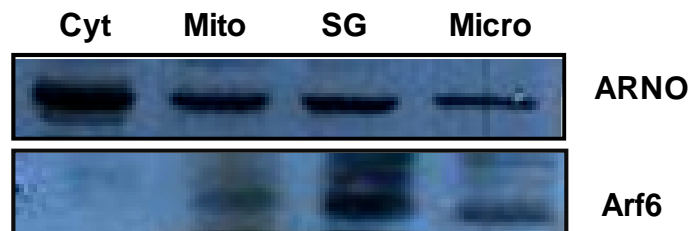
[A]



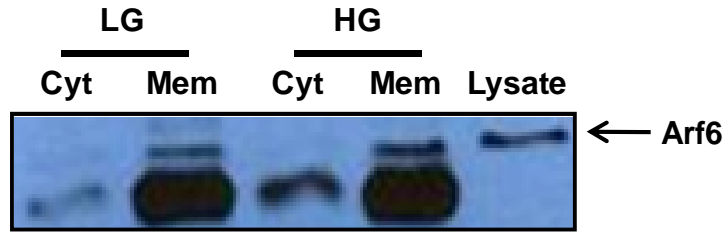
[B]



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[E]

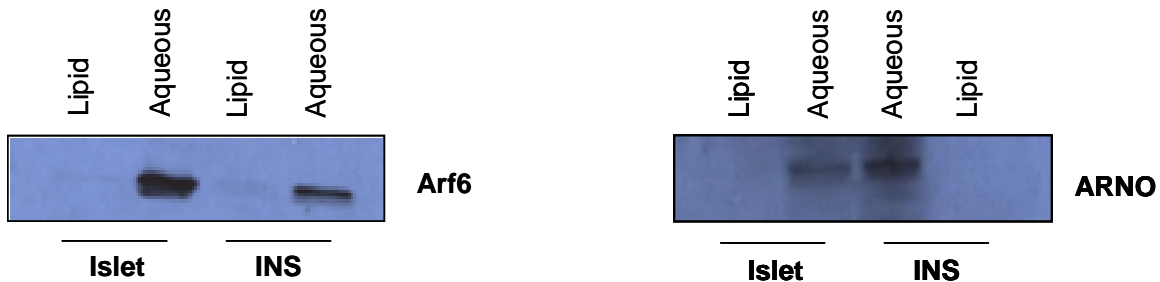


Figure 3-1: Expression of ARNO in INS 832/13 cells, rat islets and human islets

Panel A: Lysates from rat islets, human islets and INS 832/13 cells were separated by SDS-PAGE and probed for Arf6 [18 kDa] and ARNO [48 kDa].

Panel B: Lysates from rat islets and INS 832/13 cells were separated into cytosolic [Cyt] and membranous [Mem] fraction by a single-step centrifugation. Proteins were separated by SDS-PAGE and probed for Arf6 and ARNO.

Panel C: Lysates from INS 832/13 cells were differentially centrifuged into various subcellular fractions: Cytosol [Cyt], mitochondria [Mito], secretory granules [SG] and microsomes [Micro] and proteins were separated by SDS-PAGE. Blot was probed for Arf6 and ARNO.

Panel D: INS 832/13 cells were serum-starved and further stimulated with low [2.5 mM] glucose and high [20 mM] glucose for 30 min. Lysates were separated into cytosolic and membranous fractions by single-step centrifugation and proteins were separated by SDS-PAGE. Blot was probed for Arf6.

Panel E: Hydrophilic [Aqueous] and hydrophobic [Lipid] phases of the homogenates from rat islets and INS 832/13 cells were isolated using triton X-114 partition technique [see Methods for additional details]. Proteins were separated by SDS-PAGE and probed for Arf6 and ARNO. A representative blot from three independent experiments is shown here.

Among the various cellular organelles, Arf6 is found to be predominantly associated with secretory granule and ARNO in the cytosolic fraction. This adds to the fact that Arf6 might regulate stimulated-insulin secretion through priming/docking of granules. Even though Arf6 is abundant in membranous fraction, some amount of free Arf6 is found in cytosol. Bands seen in cytosol/membrane fraction reveal the presence of two bands for Arf6. This could be explained by a possible post-translational modification of Arf6 protein which aids it to bind to granule membranes. And in evidence to this in chromaffin cells, granule-bound Arf6 displayed two spots on a 2D gel electrophoresis, the two spots different only in isoelectric points but of same molecular weight [120]. Sucrose density gradient centrifugation performed in chromaffin cells to separate plasma membrane and secretory granules clearly showed the translocation of Arf6 from granule fraction to plasma membrane upon stimulation. INS 832/13 cells were stimulated with insulinotropic concentrations of glucose and lysates were separated into cytosol and membrane by single-step centrifugation. Data in **Figure 3-1 D** clearly indicates an increased amount of Arf6 localized to membrane fraction upon glucose stimulation as compared to control. In another approach, to determine whether Arf6 and ARNO preferred the hydrophilic/hydrophobic compartment in a phase partitioning assay, we used Triton X-114. As a characteristic property of nonionic detergents, Triton X-114 is homogeneous at 0 °C but separates into an aqueous and detergent phase at greater than 20 °C. The assay makes use of the principle that integral membrane proteins interact directly with the lipid bilayer by a hydrophobic domain. Triton X-114

essentially mimics the lipid bilayer thus causing integral membrane proteins to separate into the hydrophobic phase. Phase partitioning experiment clearly indicates that both Arf6 and ARNO are loosely-bound and not integral membrane proteins [Figure 3-1 E].

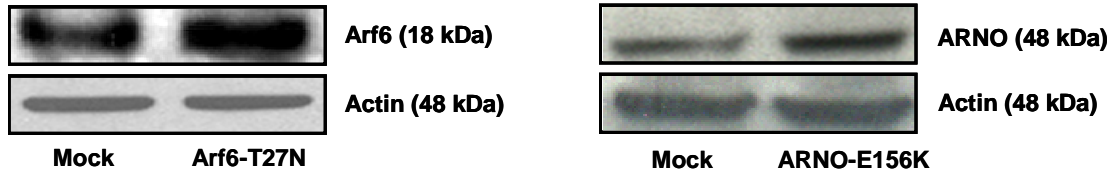
Expression of a dominant negative mutant of Arf6 and ARNO inhibits GSIS

Localization studies intrigued us to believe that Arf6 was an essential component of catecholamine-secretion as demonstrated in chromaffin cells. Furthermore, a synthetic myristoylated peptide of Arf6 blocked catecholamine secretion and PLD activation. These blocking peptides demonstrated the fact that myristoylation, a post-translational modification, was essential for regulated-secretion, but it does not exhibit any functional evidence for involvement of GTP-bound Arf6 in secretion [120]. Therefore to verify the role for Arf6 in GSIS, we used the more conventional GTP-binding deficient mutant, Arf6-T27N. The dominant negative Arf6 [DN-Arf6] mutant lacks the inherent ability to bind GTP which switches the protein to the active conformation. Lawrence and Birnbaum have demonstrated earlier that DN-Arf6 blocks both depolarization- and GTP γ S-induced secretion in MIN6 cells [79]. As a logical step to validate our hypothesis, we tested the effect of DN-Arf6 or DN-ARNO in GSIS. **Figure 3-2 A** indicates expression levels of Arf6 or ARNO mutants in INS 832/13 cells either mock-transfected or transfected with plasmid mutants as indicated. Cells overexpressing either DN-Arf6 or DN-ARNO were assayed for insulin release in the presence of 2.5 or 20 mM glucose. Data in **Figure 3-2 B** showed a marked reduction in GSIS in cells expressing Arf6-T27N. Likewise, overexpression of ARNO-E156K, a mutant lacking the GEF function, significantly inhibited GSIS in these cells [Figure 3-2

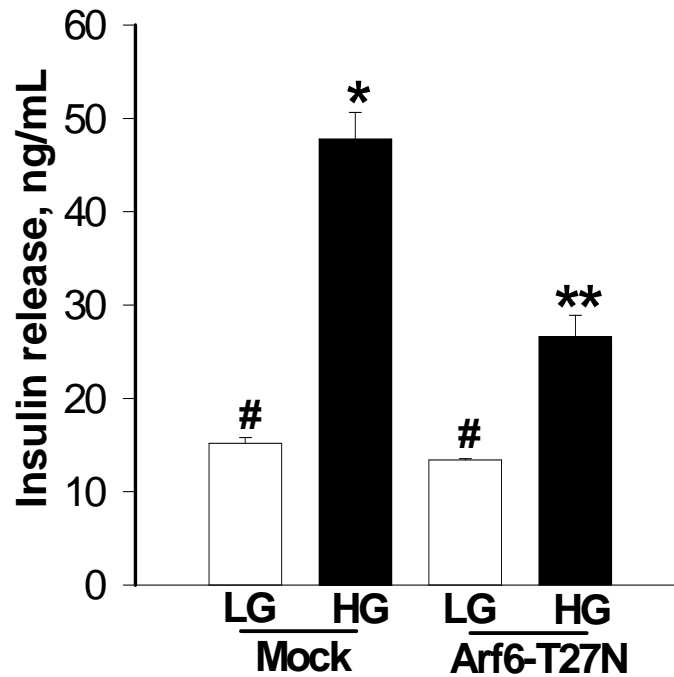
C]. Together, these data suggested key roles for Arf6 and ARNO in signaling events leading to GSIS.

A. Overexpression of dominant negative mutants of Arf6 or ARNO

[A]



[B]



[C]

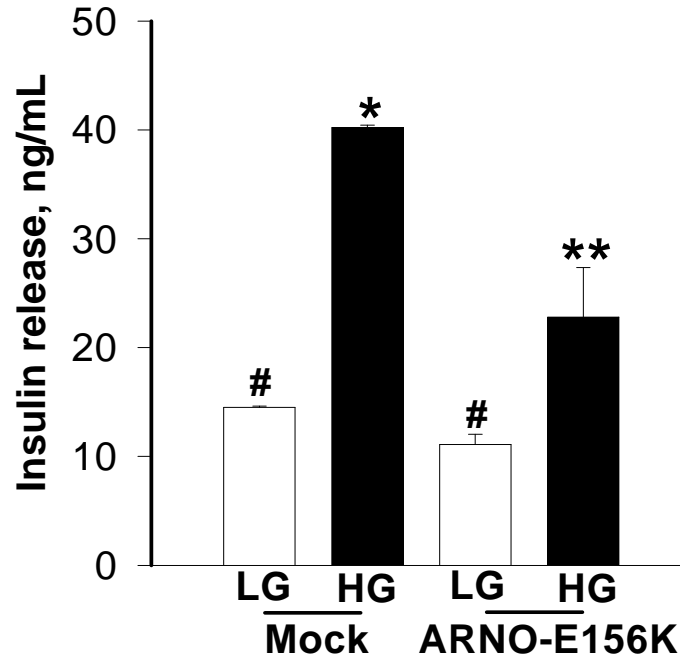


Figure 3-2: Overexpression of inactive mutants markedly inhibits glucose-induced insulin secretion in INS 832/13 cells

Panel A: INS 832/13 cells were mock-transfected or with either dn Arf6/ARNO (see Methods). Overexpression of Arf6/ARNO was verified by Western blotting. A representative blot from three independent studies yielding similar results is provided here.

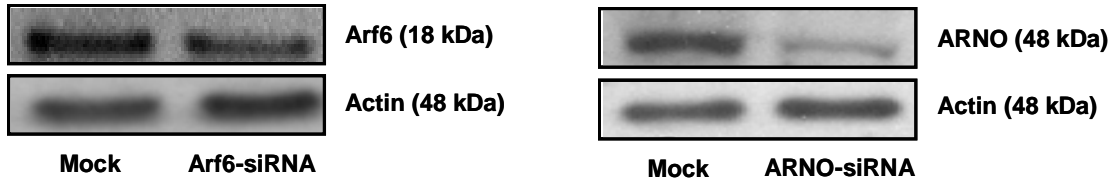
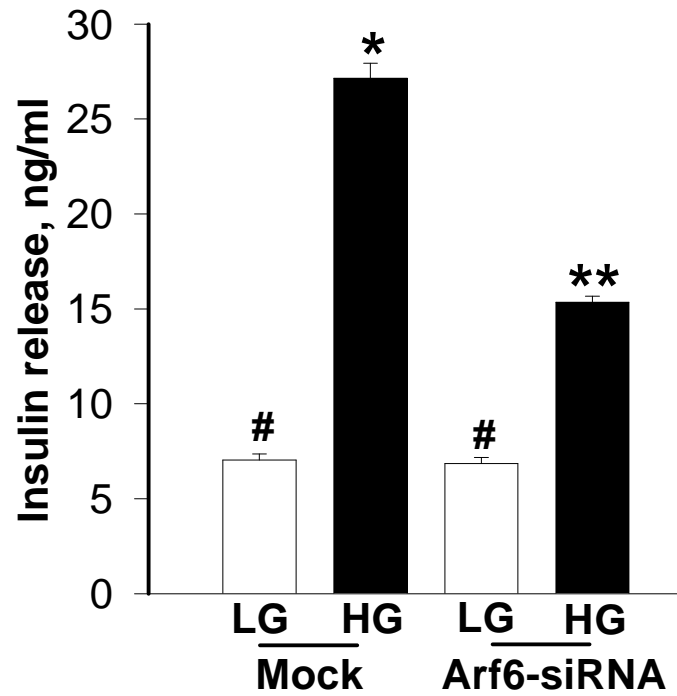
Panel B: INS 832/13 cells were transfected with dominant negative Arf6 [T27N] at a final concentration of 0.2 μ g of DNA and cultured for 48 hr. Following this, cells were stimulated with either low [2.5 mM] or high [20 mM] glucose in KRB for 30 min at 37°C. Insulin released into the media was quantitated and expressed as ng/mL. Data are mean \pm SEM from three independent experiments. * represents $p < 0.05$ vs. mock low glucose; ** $p < 0.05$ vs. mock transfected cells treated with high glucose, and data points with similar symbol did not differ significantly.

Panel C: INS 832/13 cells were transfected with dominant negative ARNO [E156K] at a final concentration of 0.2 μ g of DNA and cultured for 48 hr following which cells were stimulated with either low [2.5 mM] or high [20 mM] glucose for 30 min at 37°C. Insulin released into the medium was quantitated and expressed as ng/mL. Data are mean \pm SEM from three independent experiments. * represents $p < 0.05$ vs. mock transfected low glucose and ** $p < 0.05$ vs. mock transfected high glucose, and data points with similar symbol do not differ significantly.

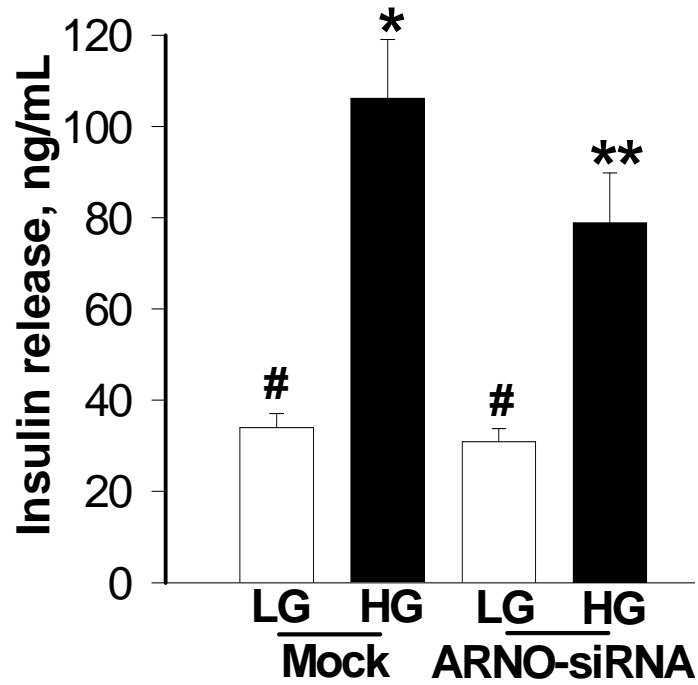
Silencing of endogenous Arf6 or ARNO attenuates insulin release by various insulin secretagogues in INS 832/13 cells

Next, we verified if knockdown of endogenous Arf6 and ARNO by using siRNA affects insulin secretion elicited by high glucose. We observed 50-60% reduction in the expression of Arf6 or ARNO by Western blotting analysis **[Figure 3-3 A]**. Under these conditions, GSIS was significantly inhibited in Arf6- and ARNO-knocked-down cells **[Figure 3-3 B and C]**. Together, data in Figure 3-2 and 3-3 implicated Arf6/ARNO signaling axis in GSIS.

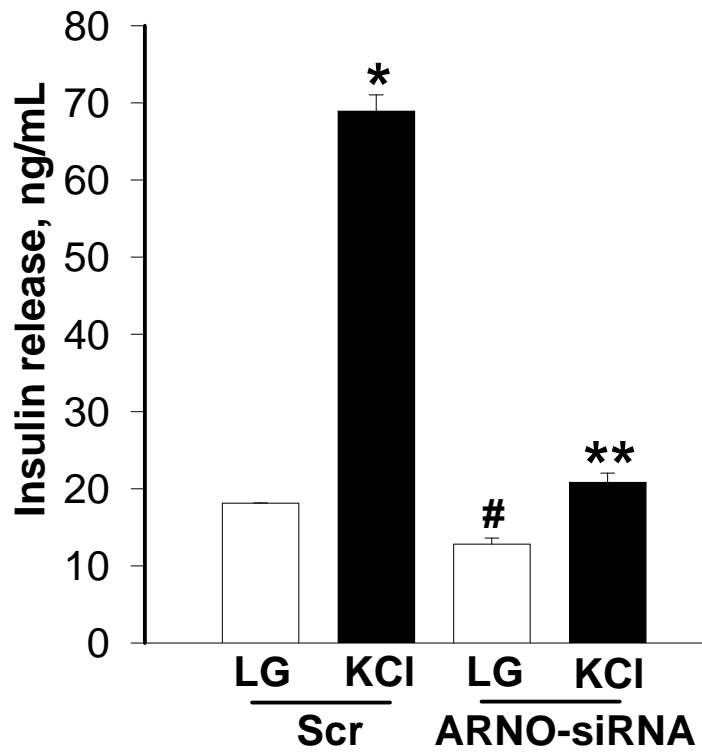
In order to test the possibility that inhibition of GSIS by deficient Arf6/ARNO function is due to a block in coupling of glucose metabolism to secretion, we tested the effect of siRNA-Arf6/-ARNO in depolarization-induced insulin secretion. Since work has been done in MIN6 cells, demonstrating the involvement of Arf6 in depolarization-induced secretion, we chose to study the effect of siRNA-ARNO in depolarization induced by high K^+ . The next series of studies, essentially determined potential roles of Arf6 or ARNO in insulin secretion elicited by a membrane-depolarizing concentration of KCl or arginine. In case of arginine, depolarization is thought to result in some part from the entry of the positively charged amino acid per se but with a clear glucose dependency and not by closure of K_{ATP} channels **[130]**. To address this, INS 832/13 cells were transfected with either siRNA-Arf6 or siRNA-ARNO, and insulin secretion in the presence of KCl [60 mM; **Figure 3-3 D**] or arginine [20 mM + 1 mM glucose; **Figure 3-3 E and F**] was quantitated. Data depicted in **Figure 3-3 [Panels D-E]** demonstrated that insulin secretion elicited by KCl or arginine was inhibited in cells in which endogenous Arf6 or ARNO was knocked-down.

A. Knockdown of endogenous expression of Arf6 or ARNO with siRNA**[A]****[B]**

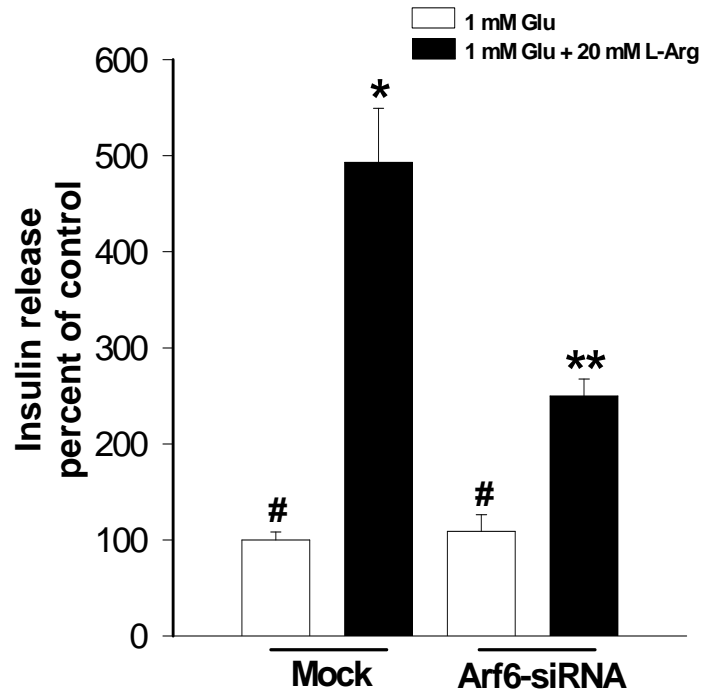
[C]



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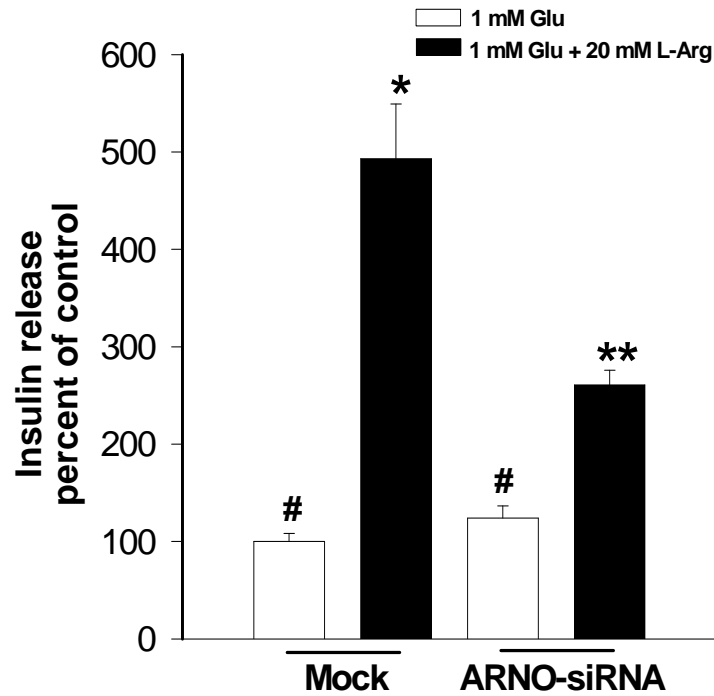


Figure 3-3: siRNA-Arf6/-ARNO markedly inhibits glucose-induced insulin secretion in INS 832/13 cells

Panel A: INS 832/13 cells were mock-transfected or transfected either with Arf6-siRNA/ARNO siRNA as described in the Methods section. Expression of Arf6/ARNO was verified by Western blotting. A representative blot from three independent studies yielding similar results is provided here.

Panel B: INS 832/13 cells were either mock-transfected or transfected with Arf6-siRNA at a final concentration of 100 nM. After 48 hr culture in regular medium, cells were stimulated with low [2.5 mM] or high [20 mM] glucose for 30 min. Insulin released into the medium was quantitated and expressed as ng/mL. Data are mean \pm SEM from three independent experiments. * represents $p < 0.05$ vs. mock low glucose; ** $p < 0.05$ vs. mock transfected cells treated with high glucose, and data points with similar symbol did not differ significantly.

Panel C: INS 832/13 cells were either mock-transfected or transfected with ARNO-siRNA at a final concentration of 100 nM. After 48 hr culture in regular medium, cells were stimulated with low [2.5 mM] or high [20 mM] glucose for 30 min. Insulin released into the medium was quantitated and expressed as ng/mL. Data are mean \pm SEM from five independent experiments. *represents $p < 0.05$ vs. mock transfected low glucose; ** $p < 0.05$ vs. mock transfected high glucose. Insulin release values between mock low glucose or siRNA transfected low glucose did not differ significantly.

Panel D: INS 832/13 cells were either mock-transfected or transfected with ARNO-siRNA at a final concentration of 100 nM. After 48 hr culture in regular medium, cells were stimulated with low [2.5 mM] or K^+ [60 mM] for 60 min. Insulin released into the medium was quantitated and expressed as ng/mL. Data are mean \pm SEM from three independent experiments. *represents $p < 0.05$ vs. mock-transfected low glucose; ** $p < 0.05$ vs. mock transfected K^+ . Insulin release values between mock low glucose or siRNA transfected low glucose did not differ significantly.

Panel E & F: INS 832/13 cells were mock-transfected or transfected either with Arf6-siRNA/ ARNO-siRNA at a final concentration of 100 nM. After 48 hr culture in regular medium, cells were stimulated with 1 mM glucose [Glu] or 1 mM glucose + 20 mM L-arginine [L-Arg] for 15 min. Insulin released into the medium was quantitated and expressed as percent of control. Data are mean \pm SEM from replicates. * represents $p < 0.05$ vs. mock-transfected 1 mM glucose; ** $p < 0.05$ vs. mock-transfected 1 mM Glu + 20 mM L-Arg. Insulin release values between mock 1 mM Glu or siRNA transfected 1 mM Glu did not differ significantly.

Together, data described in Figure 3-3 implicate regulatory roles for ARNO/Arf6 in insulin secretion elicited by a variety of secretagogues.

Stimulation with secretagogues changes Arf6 to active state

Like every other small GTPases, the guanine nucleotide-bound state regulates Arf6 function [131, 132]. With no exception, GTP-bound Arf6 confers “activity” and enables it to interact with effectors. To further understand the role of Arf6 in stimulus-coupled secretion, we examined whether insulinotropic concentrations of glucose/K⁺ activates Arf6 in the β -cell. This was accomplished by using a recently developed GST-GGA3 pull-down assay, which utilizes GGA proteins to capture activated forms of Arf6 by interacting with the Arf-binding domain [133]. Efficiency and specificity of the activation assay was confirmed by the ability of GTP γ S to stimulate Arf6 activation in broken cell preparations [Figure 3-4 A]. A time-course study for Arf6 activation by glucose/K⁺ [Figure 3-4 B and C] suggested that glucose-induced activation was seen as early as 1 min and reached optimum at 3 min time point. Even though, we noticed a reduction in activated Arf6 at 5 min time point, Arf6 remained active [Arf6.GTP] above basal till 30 min [additional data not shown]. Together, these data suggest that Arf6 activation might represent one of the early signaling steps leading to GSIS.

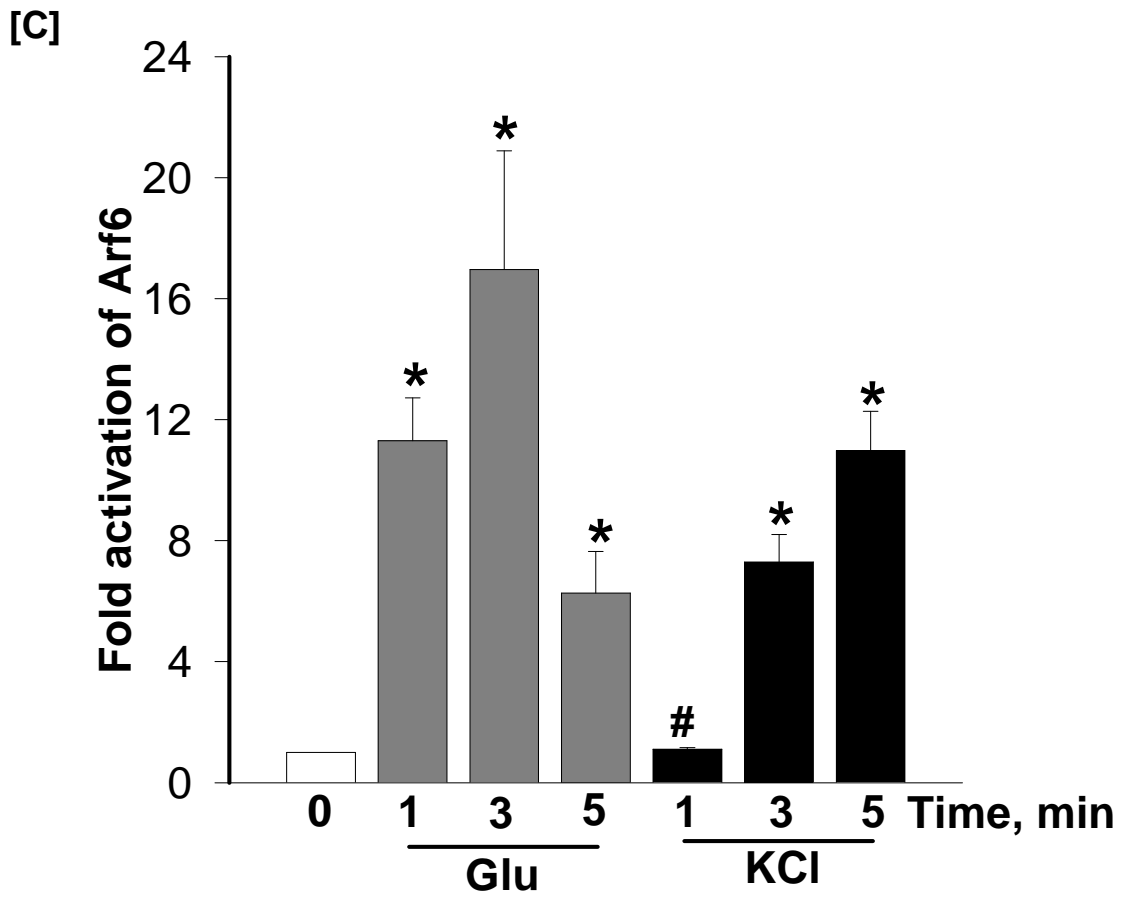
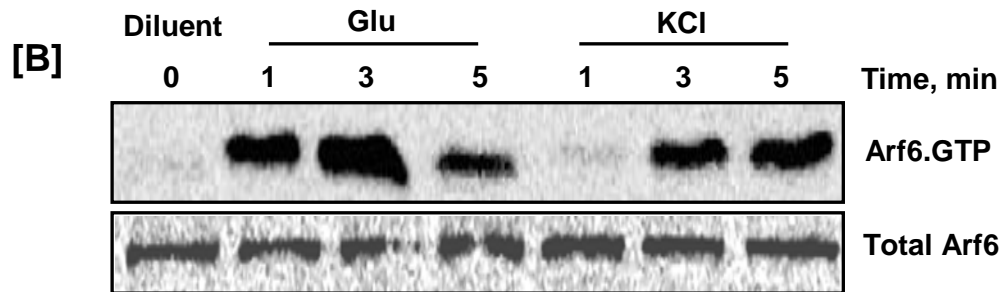
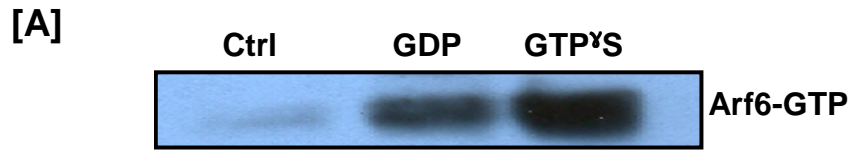


Figure 3-4: Time-dependent activation of Arf6 by glucose in pancreatic β -cells

Panel A: Lysates from INS 832/13 cells were incubated with either GDP or GTP \square S. Cell lysates were used for detecting activated Arf6 [Arf6.GTP] by GST-GGA3-PBD pull down assay [see Methods]. Total Arf6 was used as the loading control and a representative blot from three independent experiments is shown.

Panel B: INS 832/13 cells were incubated with KRB for 1 hr and either left unstimulated [diluent] or stimulated with high glucose [20 mM] or K⁺ [60 mM] for different time points as indicated. Cell lysates were used for detecting activated Arf6 [Arf6.GTP] by GST-GGA3-PBD pull down assay [see Methods]. Total Arf6 was used as the loading control and a representative blot from three independent experiments is shown.

Panel C: Densitometric quantitation of Arf6 activation depicted in **Panel B** is shown here. * represents $p < 0.05$ vs. diluent. Statistical analysis was performed using One-way ANOVA, All pairwise multiple comparison method (Dunnetts').

SecinH3 markedly attenuates GSIS in INS 832/13 cells and rat islets

Recently, Hafner et al., reported a small molecule inhibitor, secinH3, which selectively blocks ARNO-mediated activation of Arf6 [107]. The inhibitor was tested for its antagonistic potential against other members of Arf family and Rho GTPases [134]. SecinH3 showed a higher affinity towards ARNO and in particular inhibited the ARNO-catalyzed activation of Arf6. Previous studies have utilized secinH3 to determine the regulatory roles for ARNO/Arf6 signaling in cellular signal transduction [135, 136]. As a logical extension to the above studies, we evaluated the potential impact of pharmacological inhibition of ARNO on GSIS in INS 832/13 cells and in normal rat islets. It should be noted that inhibitory effects of secinH3 on GSIS [Figure 3-5] or Arf6 activation [Figure 3-6] were not due to its cytotoxic effects since we noticed no significant effects of this inhibitor on the metabolic cell viability of β -cells under these conditions [Figure 3-5 C]. Data in Fig 3-5 demonstrated complete inhibition of GSIS by secinH3 in INS 832/13 cells [Panel A] and normal rat islets [Panel B]. Under these

conditions secinH3 failed to increase basal secretion in either INS 832/13 cells or rat islets [Figure 3-5 A and B]. Together, these pharmacological data confirm the above molecular biological data to support our hypothesis that ARNO/Arf6 signaling cascade plays a positive modulatory role in GSIS.

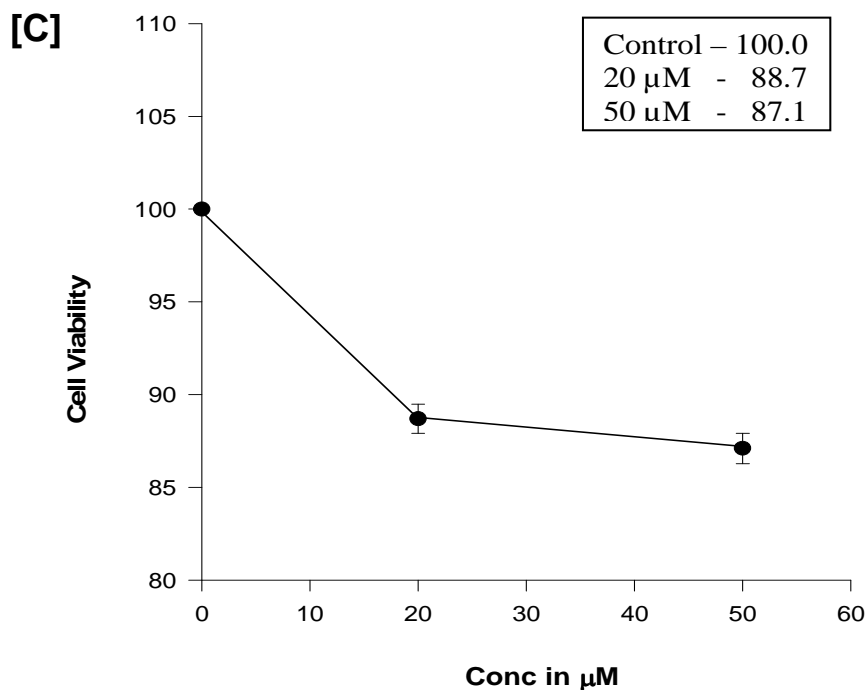
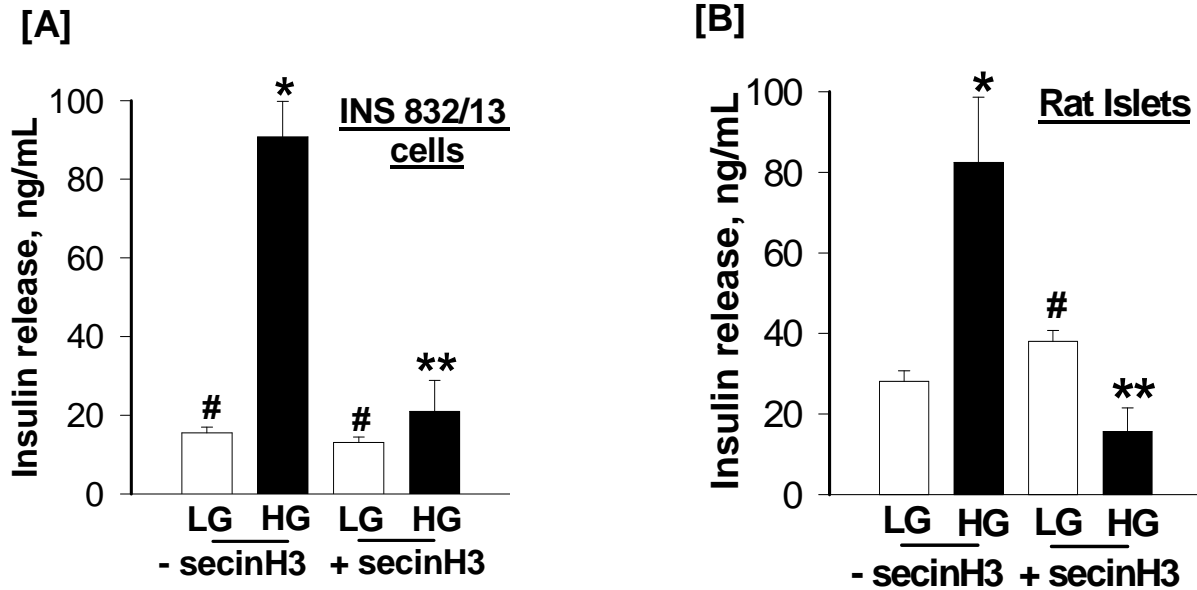


Figure 3-5: SecinH3, a selective inhibitor of ARNO attenuates GSIS in INS 832/13 cells and normal rat islets

Panel A: INS 832/13 cells were incubated in low serum-low glucose overnight in the continuous presence of 50 μ M secinH3 or diluent and stimulated with either low [LG, 2.5 mM] or high glucose [HG, 20 mM] for 30 min in KRB. Insulin released into the medium was quantitated by ELISA and expressed as ng/mL. Data are mean \pm SEM from four independent experiments. * represents $p < 0.05$ vs. low glucose without secinH3; ** $p < 0.05$ vs. high glucose without secinH3 and data points with similar symbol do not differ statistically.

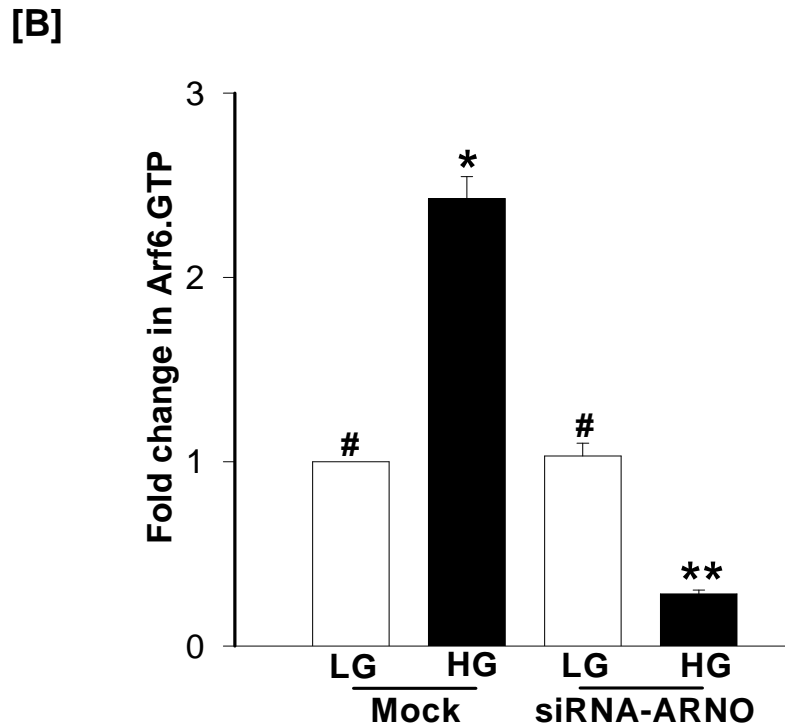
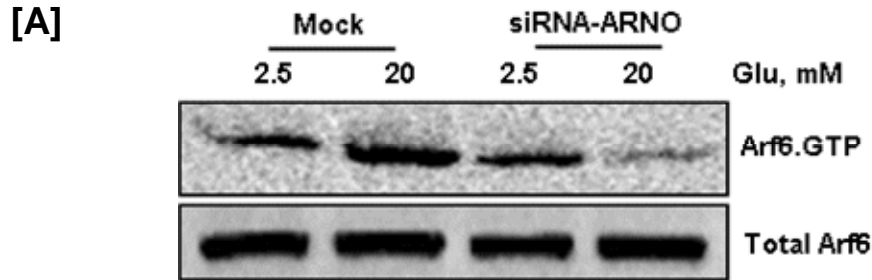
Panel B: Normal rat islets were incubated in low serum-low glucose overnight in the continuous presence of either 50 μ M secinH3 or diluent and stimulated with either low [LG, 2.5 mM] or high glucose [HG, 20 mM] for 30 min in KRB. Insulin released into the medium was quantitated by ELISA. Data are expressed as ng/mL insulin released and are mean \pm SEM from four independent experiments. * represent $p < 0.05$ vs. low glucose with secinH3 and without secinH3; and ** $p < 0.05$ vs. high glucose without secinH3. Data points with similar symbol do not differ significantly.

Panel C: INS 832/13 cells were incubated in the presence or absence of secinH3 [20 or 50 μ M] for 15 hr. Metabolic cell viability was determined using MTT assay [methods]. The data indicated a modest inhibition of $\sim 13\%$ in cells treated with maximum concentration of inhibitor. Data are expressed as percent of control [mean \pm SEM].

ARNO activates Arf6 in pancreatic beta cells

The conversion of the GDP-bound inactive forms of G-proteins to their GTP-bound active state is mediated by GEFs. ARNO is one of the four related proteins in the cytohesin family of Arf-GEFS. They share a $\sim 70\%$ identity with one another. To investigate the possibility that ARNO plays a role in glucose-mediated activation of Arf6, we undertook the following two complementary methods. In the first, glucose-induced activation of Arf6 was examined in cells in which ARNO expression was reduced by siRNA-ARNO. Data in **Figure 3-6 A and B** indicated complete inhibition of glucose-induced activation of Arf6 under these conditions. This was further verified by a second approach involving pharmacological inhibition of ARNO/Arf6 signaling by secinH3 [107]. Data in **Figure 3-6 C and D** suggested a complete inhibition of glucose-induced

activation of Arf6 by secinH3. Taken together, these data indicate that glucose-induced activation of Arf6 requires the intermediacy of ARNO.



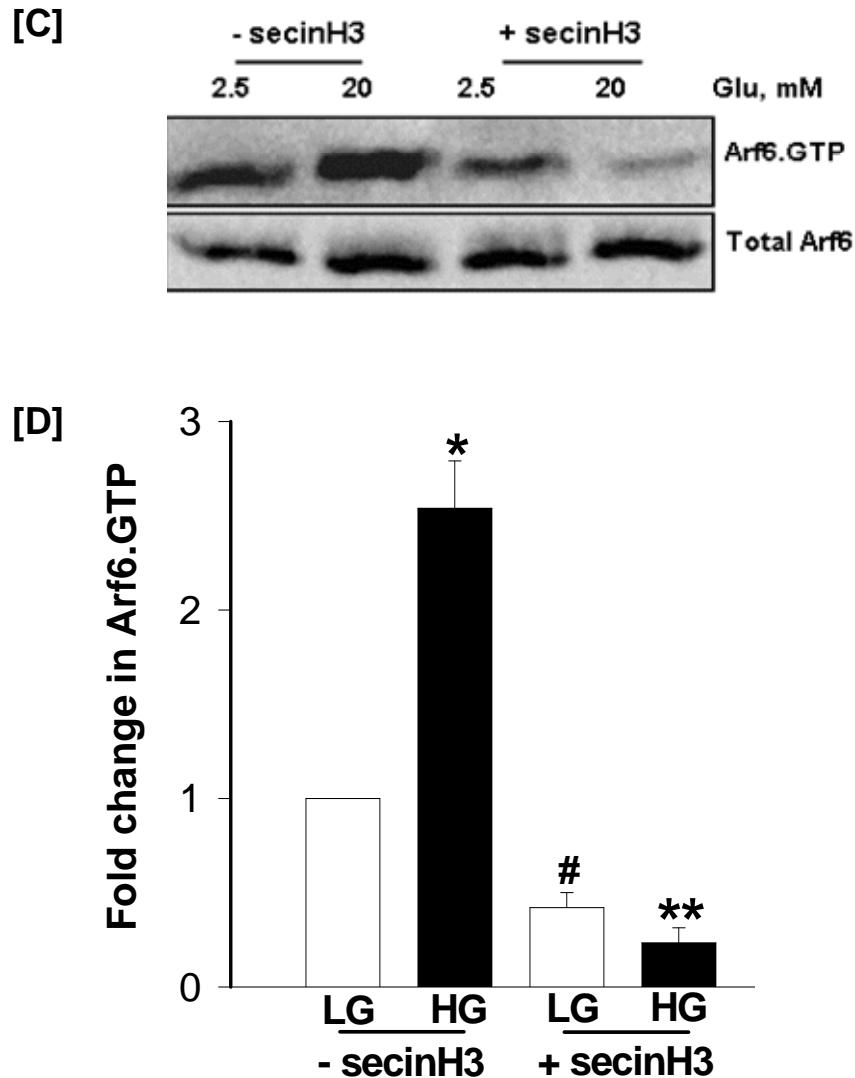


Figure 3-6: Molecular biological or pharmacological inhibition of ARNO attenuates glucose-induced activation of Arf6 in INS 832/13 pancreatic β -cells

Panel A: INS 832/13 cells were either mock-transfected or transfected with siRNA-ARNO and cultured for 48 h following which cells were stimulated in the presence of either low glucose [LG, 2.5 mM] or high glucose [HG, 20 mM] for 30 min at 37°C. The relative amounts of activated Arf6 [i.e, Arf6.GTP] were determined by pull down assay. Total Arf6 from cell lysates was used as the loading control and a representative blot from three independent experiments is shown.

Panel B: Data shown in the **panel A** were analyzed densitometrically and expressed as fold change in Arf6.GTP over basal and are mean \pm SEM of three independent experiments. * represents $p < 0.05$ vs. mock transfected low glucose; ** $p < 0.05$

vs. mock transfected high glucose, and data points with similar symbol do not differ statistically.

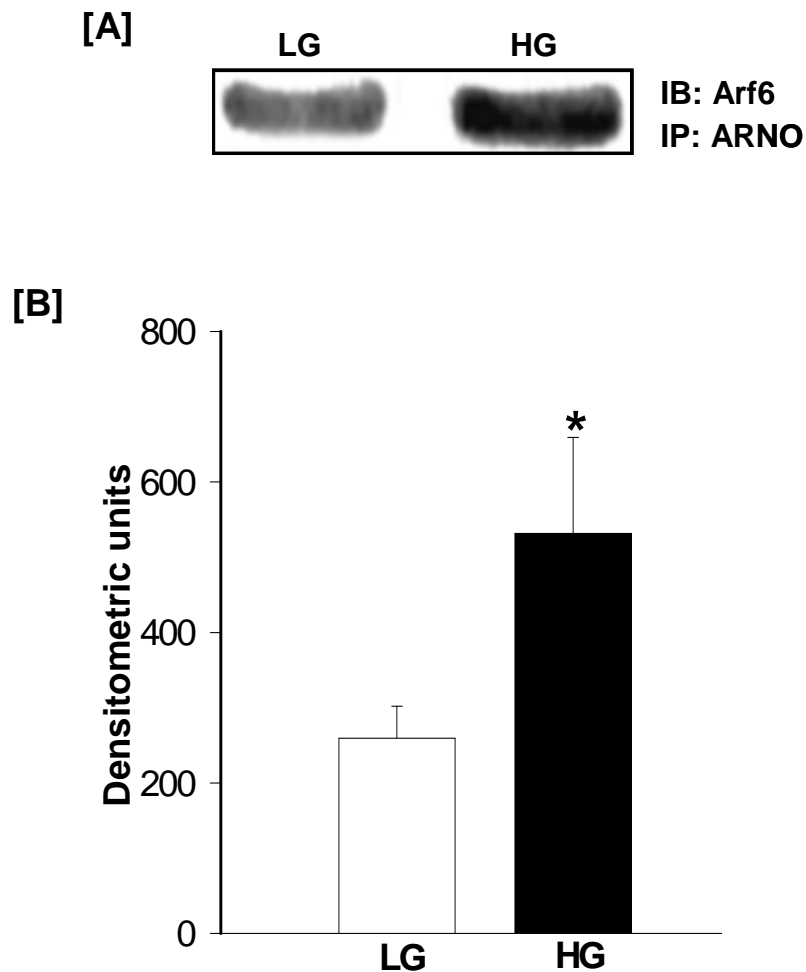
Panel C: INS 832/13 cells were incubated overnight in the presence or absence of secinH3 [50 μ M] and stimulated with either low glucose [LG, 2.5 mM] or high glucose [HG, 20 mM] in the continuous presence or absence of secinH3 [50 μ M] for 30 min. Relative degrees of Arf6 activation was quantitated as described in **Panel A**. Total Arf6 from cell lysates was used as the loading control and a representative blot from three independent experiments is shown.

Panel D: Data shown in the **Panel C** are analyzed densitometrically and expressed as fold change in Arf6.GTP over basal. Data are mean \pm SEM from three independent experiments. * and # represents $p < 0.05$ vs. low glucose without secinH3; and ** $p < 0.05$ vs. high glucose without secinH3.

Glucose promotes association between Arf6 and ARNO in pancreatic β -cells

Then, I examined the ability of ARNO to interact physically with Arf6 in response to glucose. To investigate this interaction, I utilized co-immunoprecipitation and immunofluorescence approaches on β -cells exposed to an insulinotropic concentration of glucose. In our system, activation of ARNO was evidenced by a substantial increase in the amount of Arf6 coupled to ARNO in pancreatic beta cells when stimulated with glucose. Data shown in **Figure 3-7 A** indicate detectable levels of Arf6 in ARNO immunoprecipitates suggesting that these two proteins stay complexed under basal conditions. Moreover, incubation of these cells with stimulatory glucose resulted in a significant increase [\sim 2-fold] in the amount of Arf6 in the ARNO immunoprecipitates [**Figure 3-7 B**]. Together, these data suggest that glucose promotes physical association between ARNO and Arf6 in insulin-secreting cells. We verified these findings *via* a complementary immunofluorescence approach. Data in **Figure 3-7 C** suggested that both Arf6 [green] and ARNO [red] remain diffused throughout the cell under basal conditions [2.5 mM glucose]. Merged images of subpanels **a and b** in

Figure 3-7 C [i.e., subpanel c] further suggested that the two proteins remain localized in the cytosolic compartment. However, exposure of these cells to a stimulatory concentration of glucose [20 mM] led to a significant association of these proteins as evidenced in the merged images of subpanels d and e of Figure 3-7 [i.e., sub-panel f]. Together, these findings [Figure 3-7] provide evidence for increased association of Arf6 and ARNO in the presence of glucose leading to the activation of ARNO/Arf6 signaling pathway culminating in insulin secretion.



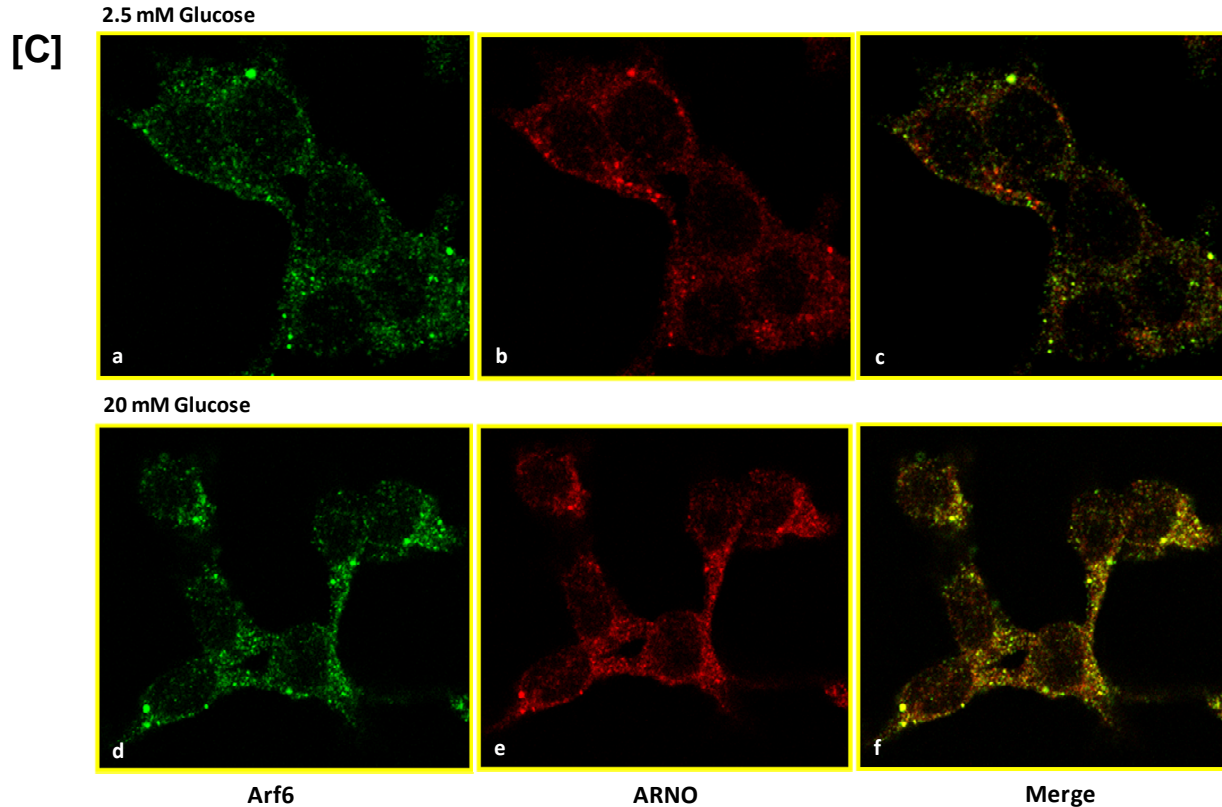


Figure 3-7: Glucose promotes association between Arf6 and ARNO in INS 832/13 cells

Panel A: Coimmunoprecipitation studies: Herein, INS 832/13 cells were incubated in the presence of low glucose [LG, 2.5 mM] or high glucose [HG, 20 mM] for 30 min at 37°C. ARNO was immunoprecipitated in the lysates using a specific antibody as described in Methods. The immunoprecipitates were separated by SDS-PAGE and probed for Arf6. A representative blot from three studies is shown.

Panel B: Data from multiple studies shown in **Panel A** are analyzed densitometrically and expressed as densitometric units and are mean \pm SEM. * represents $p < 0.05$ vs. low glucose.

Panel C: Immunofluorescence studies using confocal microscopy: INS 832/13 cells were cultured on coverslips and cultured overnight prior to the incubation with either 2.5 mM or 20 mM glucose for 30 min at 37°C. The cells were fixed in 4% paraformaldehyde solution in PBS for 15 min and permeabilized using 0.2% triton X-100 for 15 min. Fixed cells were examined for Arf6 [stained in green] and ARNO [stained in red] as described under Methods.

Chapter 4

Potential down-stream signaling events such as activation of effector proteins involved in ARNO/Arf6 signaling and posttranslational modification of small G-proteins in pancreatic beta- cells

- Portions of this work have been published, or have been submitted for publication [copies of the published/submitted manuscripts are appended].

Jayaram B, Syed I, Kyathanahalli CN, Rhodes CJ, Kowluru A. Arf nucleotide binding site opener [ARNO] promotes sequential activation of Arf6, Cdc42 and Rac1 and insulin secretion in INS 832/13 β -cells and rat islets. *Biochem Pharmacol.* 2011 Apr 15; 81(8):1016-27. Epub 2011 Jan 26.

Jayaram B, Syed I, Singh A, Subasinghe W, Kyathanahalli CN, Kowluru A. Isoprenylcysteine carboxyl methyltransferase facilitates glucose-induced Rac1 activation, ROS generation and insulin secretion in INS 832/13 β -cells. *Islets.* 2011; 3(2):48-57. Epub 2011 Mar 1.

Active Arf6 regulates putative downstream effectors in the events leading to insulin release

Arf6 GTPase has a dual role in cells, regulating membrane traffic and organizing cortical actin. Many of its downstream effectors include lipid-modifying enzymes, actin remodelers, kinases and also other small G-proteins. As such, I investigated a number of potential effectors either using siRNA-ARNO or secinH3 inhibitor.

Nm23H1 may act as local supplier of GTP for Arf6

In cancer biology, nm23H1 is termed as “tumor metastasis suppressor” and is an important modulator of tumor invasion. An article by Palacios F in Nature Cell biology demonstrated a cross-talk between Arf6 and nm23H1 in epithelial cells during adherens junction disassembly caught our attention [137]. Second, there were intriguing observations which suggested that NDP kinase can act as a local GEF, for GTPases [138]. So I investigated whether nm23H1 had any effect on levels of Arf6-GTP as Arf6 seemed to be constitutively active for almost 30 min [Chapter 3]. I investigated this by silencing nm23H1 using siRNA and studied the levels of Arf6-GTP upon glucose stimulation by pull-down assay. Interestingly, cells transfected with nm23H1-siRNA (si-nm23H1) showed a reduced level of Arf6-GTP [Figure 4-1]. This was consistent with our hypothesis that nm23H1 might act as a localized GEF for Arf6, thus providing a constant supply of GTP at the plasma membrane where active Arf6 is required to regulate downstream effectors.

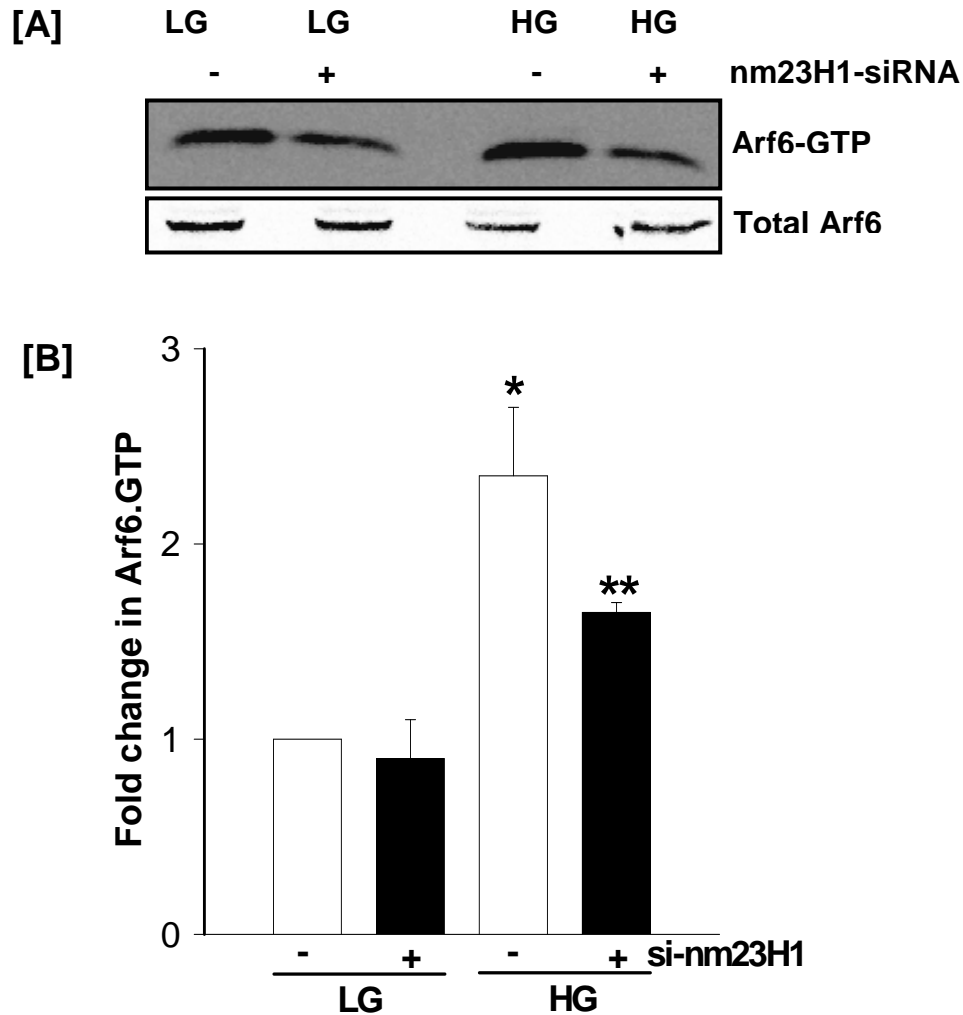


Figure 4-1: Molecular biological inhibition of nm23H1 attenuates glucose-induced activation of Arf6 in INS 832/13 pancreatic β -cells

Panel A: INS 832/13 cells were either mock-transfected or transfected with siRNA-nm23H1 (i.e., si-nm23H1) and cultured for 48 h following which cells were stimulated in the presence of either low glucose [LG, 2.5 mM] or high glucose [HG, 20 mM] for 30 min at 37°C. The relative amounts of activated Arf6 [i.e., Arf6.GTP] were determined by pull down assay. Total Arf6 from cell lysates was used as the loading control and a representative blot from three independent experiments is shown.

Panel B: Data shown in the **panel A** were analyzed densitometrically and expressed as fold change in Arf6.GTP over basal and are mean \pm SD from two independent experiments. * represents $p < 0.05$ vs. mock transfected low glucose; ** $p < 0.05$ vs. mock transfected high glucose.

Arf6/ARNO affects the activation of Rho family of GTPases

Several recent studies, including our own, have implicated Rho subfamily of small G-proteins [e.g., Cdc42 and Rac1] in cytoskeletal remodeling leading to the translocation of insulin-laden secretory granules to the plasma membrane for fusion and exocytotic secretion of insulin [139, 140]. In this context, recent evidence appears to suggest a significant cross-talk between Arf6/ARNO and Rac1 in the regulation of cell function in multiple cell types [141-146]. Therefore, I wondered if Arf6/ARNO signaling axis regulates glucose-induced Rac1 activation in the pancreatic β -cell. We addressed this question by quantitating glucose-induced Rac1 activation in INS 832/13 cells in which ARNO function is compromised *via* pharmacological [e.g., secinH3] or molecular biological [e.g., siRNA-ARNO] approaches. As expected, we noticed a significant Rac1 activation in control cells exposed to glucose [Figure 4-2 A]. Interestingly, siRNA-mediated knockdown of ARNO increased Rac1 activation under basal glucose conditions. However, glucose-induced activation of Rac1 was completely inhibited in ARNO-depleted β -cells [Figure 4-2 A and B]. Likewise, pharmacological inhibition of ARNO/Arf6 signaling axis with secinH3 abolished glucose-induced activation in these cells [Figure 4-2 C and D] suggesting that ARNO/Arf6 signaling step might be regulate Rac1 activation in the cascade of events leading to GSIS. These data, which are compatible with our original proposal [117] also fit into the time-frame for glucose-induced activation of these proteins. We noticed in this study that Arf6 activation is seen as early as 1 min while glucose-induced activation of Rac1 is maximal at 15-20 min [139, 140].

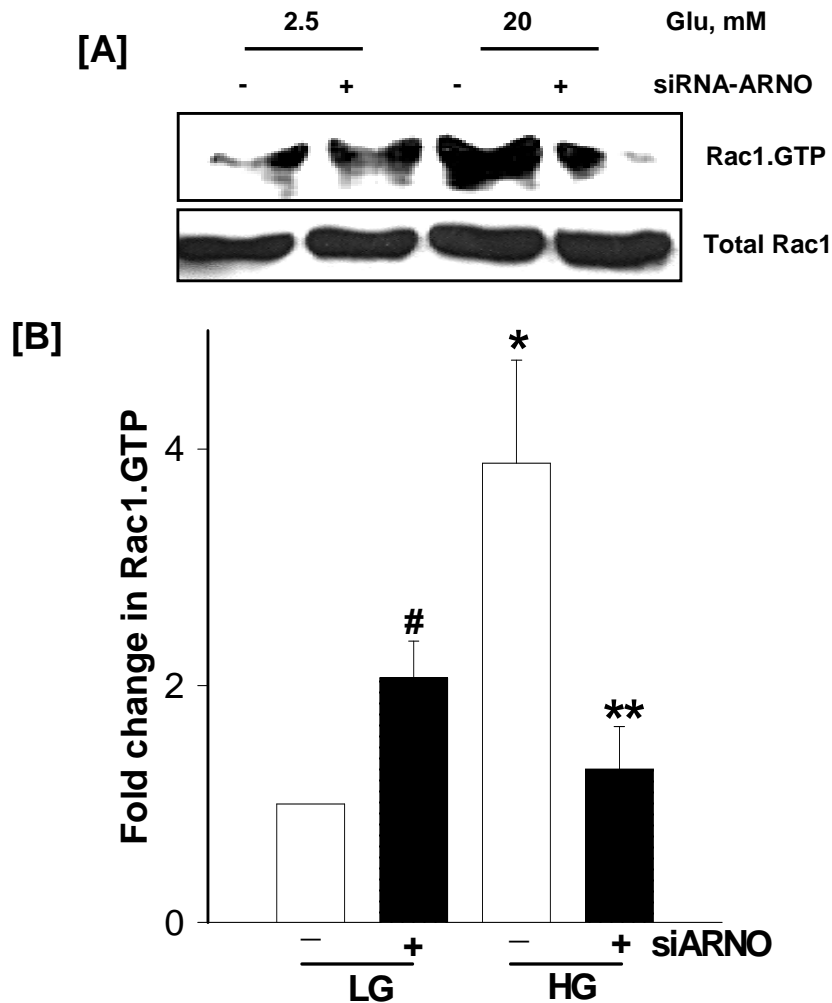
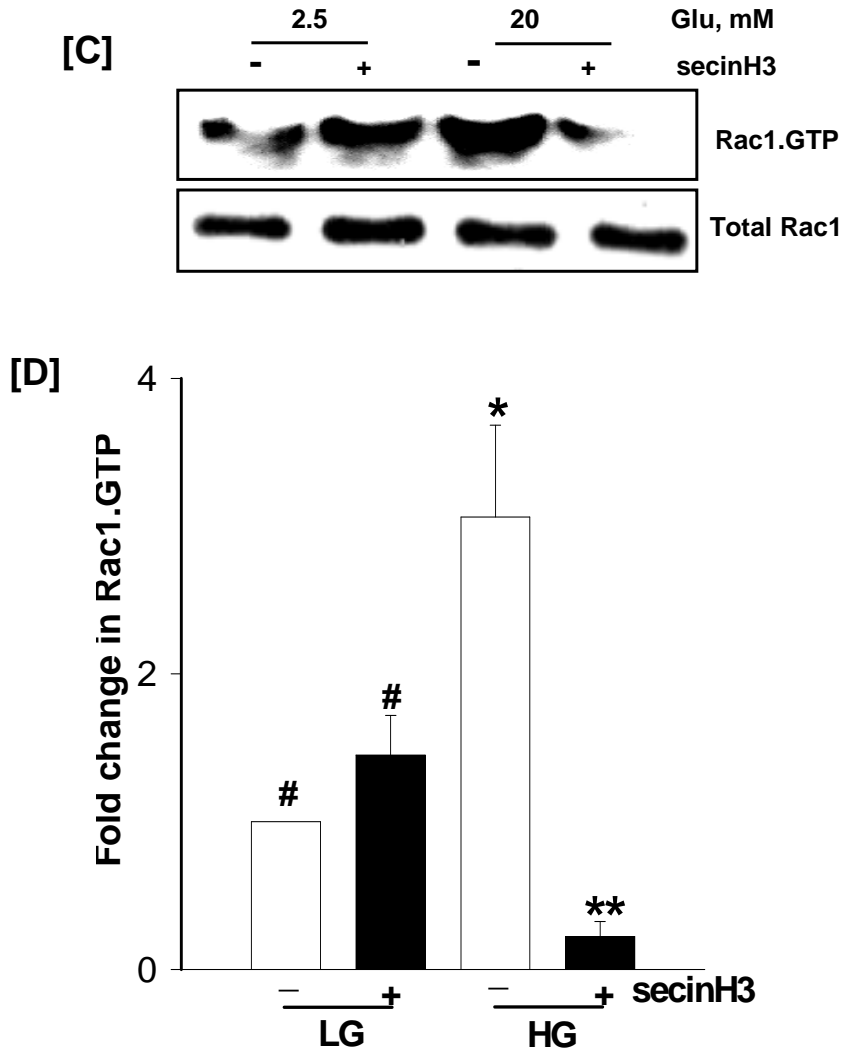


Figure 4-2: Molecular biological or pharmacological inhibition of ARNO function attenuates glucose-induced Rac1 in INS 832/13 cells

Panel A: INS 832/13 cells were either mock-transfected or transfected with siRNA-ARNO at a final concentration of 100 nM and after 48 hr culture, cells were stimulated with either low glucose [LG, 2.5 mM] or high glucose [HG, 20 mM] for 30 min at 37°C. The relative amounts of activated Rac1 [i.e, Rac1.GTP] were quantitated by PAK-PBD pull down [see Methods for additional details]. Total Rac1 from cell lysates was used as the loading control. A representative blot from three independent experiments is shown here.

Panel B: Data from Panel A were analyzed densitometrically and expressed as fold change in Rac1.GTP over basal. Data are mean \pm SEM of five independent experiments. * and # represent $p < 0.05$ vs. low glucose without siRNA-ARNO; and ** $p < 0.05$ vs. high glucose without siRNA-ARNO.



Panel C: INS 832/13 cells were cultured overnight in the presence or absence of secinH3 [50 μ M] and further stimulated with low glucose [LG, 2.5 mM] and high glucose [HG, 20 mM] for 30 min in the continuous presence or absence of secinH3. The relative amounts of activated Rac1 [i.e., Rac1.GTP] were determined by PAK-PBD pull down assay as described in **Panel A**. Total Rac1 from cell lysates was used as the loading control.

Panel D: Data were analyzed densitometrically and expressed as fold change in Rac1.GTP over basal and are mean \pm SEM of three independent experiments. * represents $p < 0.05$ vs. low glucose without secinH3; ** $p < 0.05$ vs. high glucose without secinH3, and data points with similar symbol do not differ statistically.

Along these lines, earlier studies from our laboratory have suggested that the carboxymethylation of Cdc42 is stimulated by glucose within 1 min of exposure [75]. More recent and comprehensive investigations by Thurmond's group [76, 139] have reported glucose-induced activation of Cdc42 within 3 min of exposure. They also demonstrated that Cdc42 activation is upstream to Rac1 activation in the cascade of events leading to GSIS [139]. Therefore, we examined if inhibition of ARNO/Arf6 signaling step affects Cdc42 activation. Our findings suggested ~50% inhibition of glucose-induced activation of Cdc42 in INS 832/13 cells following inhibition of ARNO/Arf6 either by siRNA-ARNO or by secinH3 [Figure 4-3 A-D]. Together, our findings are suggestive of sequential activation of Arf6, Cdc42 and Rac1 by ARNO in glucose-stimulated β -cell culminating in insulin secretion.

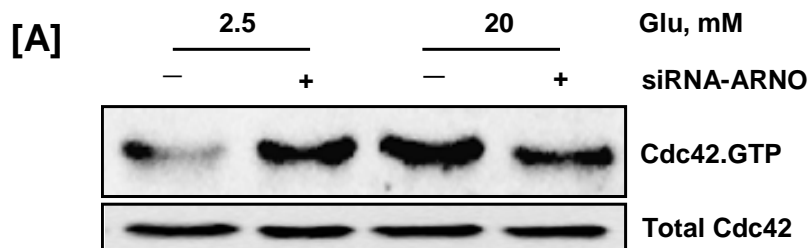
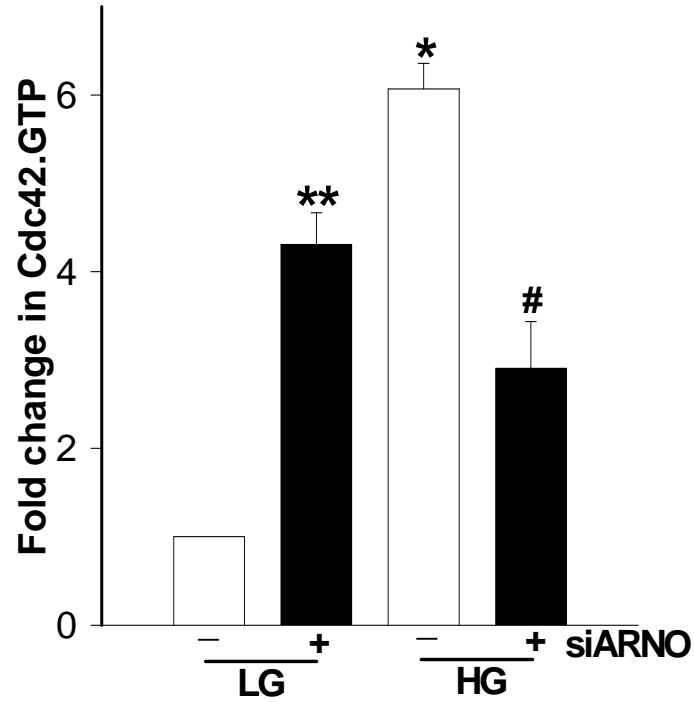


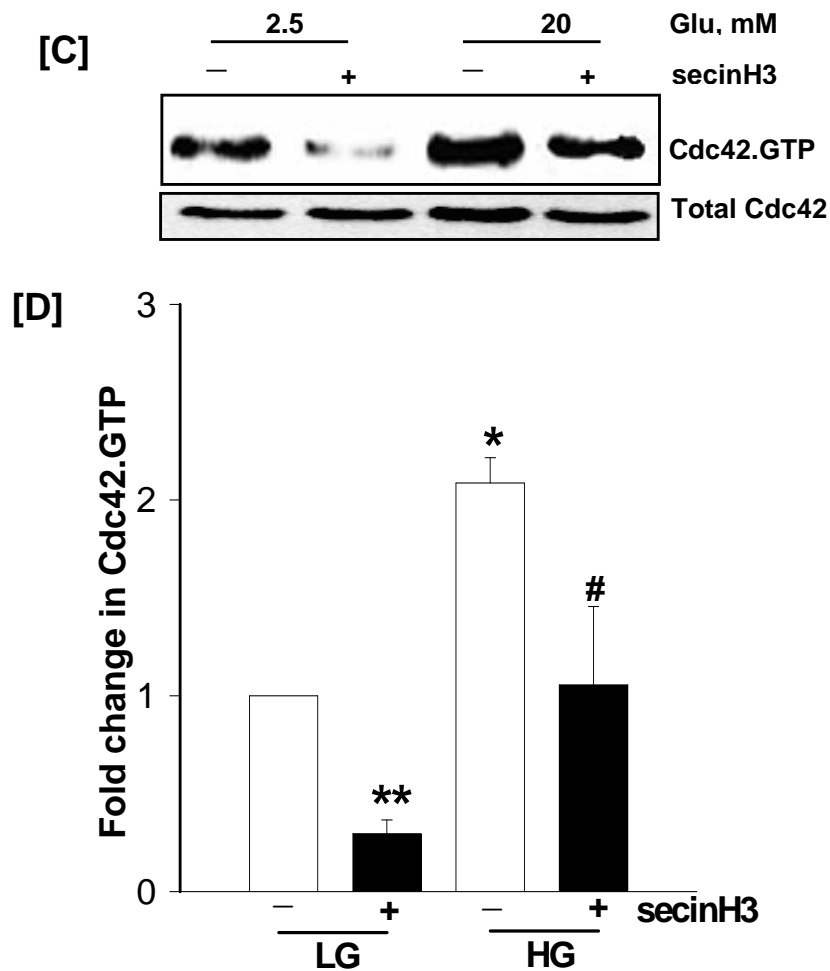
Figure 4-3: Molecular biological or pharmacological inhibition of ARNO function attenuates glucose-induced Cdc42 activation in INS 832/13 cells

Panel A: INS 832/13 cells were either mock-transfected or transfected with siRNA-ARNO (i.e., si-ARNO) at a final concentration of 100 nM and after 48 hr culture, cells were stimulated with either low glucose [LG, 2.5 mM] or high glucose [HG, 20 mM] for 30 min at 37°C. The relative amounts of activated Cdc42 [i.e, Cdc42.GTP] were quantitated by PAK-PBD pull down [see Methods for additional details]. Total Cdc42 from cell lysates was used as the loading control. A representative blot from three independent experiments is shown here.

[B]



Panel B: Data from Panel A were analyzed densitometrically and expressed as fold change in Cdc42.GTP over basal. Data are mean \pm SEM of three independent experiments. * and ** represent $p < 0.05$ vs. low glucose without si-ARNO; and *** $p < 0.05$ vs. high glucose without si-ARNO.



Panel C: INS 832/13 cells were starved overnight in the presence or absence of secinH3 [50 μ M] and were stimulated with low glucose [LG, 2.5 mM] and high glucose [HG, 20 mM] for 3 min in the continuous presence or absence of secinH3. The relative amounts of activated Cdc42 [i.e., Cdc42.GTP] was determined by PAK-PBD pull down assay. Total Cdc42 from cell lysates was used as the loading control.

Panel D: Data were densitometrically analyzed and is expressed as fold change in Cdc42.GTP over basal and are mean \pm SEM of three independent experiments yielding similar results. * and ** represents $p < 0.05$ vs. low glucose without secinH3 and # $p < 0.05$ vs. high glucose without secinH3.

Phospholipase D is modulated by ARNO/Arf6

Phospholipase D [PLD] has been a key player in the regulation of golgi trafficking as well as exo-endocytotic processes. Membrane trafficking involves the input of lipid-modifying enzymes which either help to form micro-domains or supply second messengers. PLD catalyzes the breakdown of phosphatidyl choline to phosphatidic acid [PA] and choline. A role for PLD in regulating vesicular traffic is particularly compelling due its cellular location and its function as a lipid-modifier. It has been well-established to play a regulatory role in release of insulin by secretagogues. Arf6, so far, has been a well-known mediator of membrane reorganization. Earlier work has shown the interaction between Arf6 and PLD in MIN6 cells upon glucose stimulation [147]. They also demonstrated the ability of Arf6 to regulate the PLD's activity under stimulatory conditions using brefeldin A [BFA] which inhibits the guanine nucleotide exchange on the Arf6. Therefore we sought to assess if PLD activity was dependent on activation of Arf6 via its GEF, ARNO. As seen in **Figure 4-4**, INS 832/13 cells show an increased activity of PLD when stimulated with glucose, consistent with findings in chromaffin cells [148]. But in cells challenged with secinH3, an inhibitor of ARNO, cellular activity of PLD was reduced, notably more than 40% when stimulated with glucose. These data confirm a close relationship between active Arf6 and PLD activity in pancreatic beta cells.

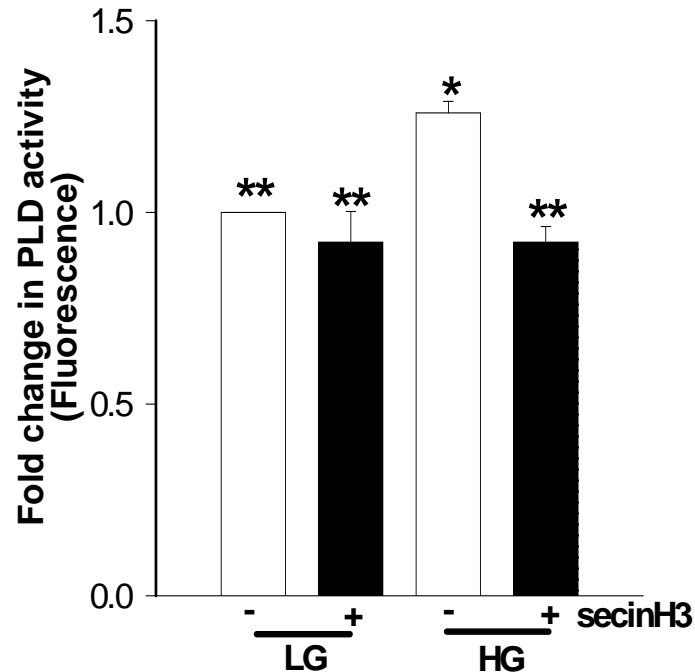


Figure 4-4: SecinH3 inhibits glucose-induced activation of PLD

INS 832/13 cells were starved in low serum-low glucose overnight \pm secinH3 [50 μ M] and stimulated with either low glucose [LG, 2.5 mM] or high glucose [HG, 20 mM] \pm secinH3 [50 μ M] for 30 min. PLD activity was determined by Amplex red PLD assay kit. 50 μ g protein was made up to 100 μ L volume with 1x reaction buffer [kit]. 100 μ L of working reagent [kit components] was added and incubated for 30 min at 37 $^{\circ}$ C. Fluorescence was measured at 530/590 nm and hydrogen peroxide was used as a positive control. Data shown above are expressed as fold change over basal and mean \pm SEM from three independent experiments. * represents $p < 0.05$ vs. low glucose without secinH3; high glucose with secinH3 and low glucose with secinH3. Similar symbols do not differ significantly.

ERK1/2 activation requires active Arf6

Cationic events following glucose metabolism activates protein kinases. In addition to calcium-dependent protein kinases, mitogen-activated protein kinases like extracellular signal-regulated kinases [ERK1/2] have been characterized in the beta-cell [149]. ERK1/2 are regulated in a manner to meet the secretory demands of the pancreatic beta-cell, integrating long- and short-term fuel sensing information. Recently published evidence from our laboratory demonstrated activation of ERK1/2 in GSIS. Data using siRNA of ERK1/2 clearly indicated that ERK1/2 activation regulates the activity of Rho GTPase, Rac1, leading to insulin release [150]. Added to this, my earlier work has demonstrated Arf6 to be a potential upstream regulator of Rac1 in GSIS from pancreatic beta-cells. Furtherance to earlier findings, I tested the effect of secinH3 on activation of ERK1/2. Data described in **Figure 4-5 A-B** demonstrated that incubation of INS 832/13 cells with a stimulatory concentration of glucose leads to a significant increase in ERK1/2 phosphorylation whereas secinH3, a known inhibitor of ARNO-mediated activation of Arf6, totally abrogated the effect of glucose on ERK1/2 activation. This clearly indicates the role for ARNO/Arf6 in modulating the activity of ERK1/2 in the events leading to insulin secretion.

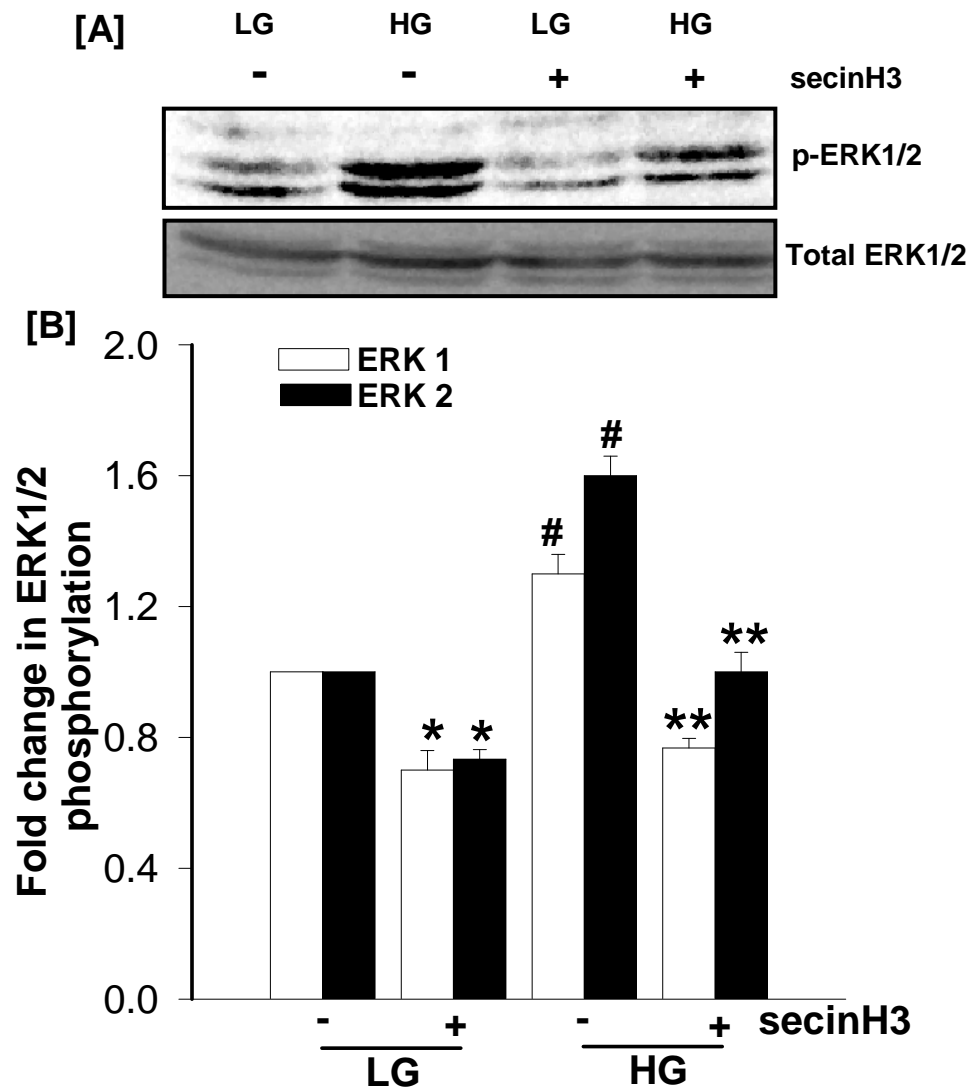


Figure 4-5: Pharmacological inhibition of ARNO function attenuates glucose-induced ERK1/2 activation in INS 832/13 cells

Panel A: INS 832/13 cells were starved overnight in the presence or absence of secinH3 [50 μ M] and were stimulated with low glucose [LG, 2.5 mM] and high glucose [HG, 20 mM] for 30 min in the continuous presence or absence of secinH3. Lysates were prepared in RIPA lysis buffer substituted with appropriate phosphatase and protease inhibitors. Quantitated amounts of proteins were loaded and probed for p-ERK 1/2 [p-44/42]. The blot was reprobed for total ERK 1/2.

Panel B: Data were densitometrically analyzed and is expressed as fold change in ratio of p-ERK 1/2 over total ERK 1/2 and are mean \pm SEM of three independent experiments yielding similar results. * represents $p < 0.05$ vs. low glucose without secinH3; ** $p < 0.05$ vs. high glucose without secinH3 and *** $p < 0.05$ vs. low glucose without secinH3.

Arf6-mediated regulation of ROS

Reactive oxygen species [ROS] are involved in pathogenesis of diabetes [151], cardiovascular [152] and neurodegenerative diseases [153]. But growing evidence has suggested ROS to play an essential role in physiological processes such as insulin secretion and signaling acting as second messengers [154]. Apart from being produced by mitochondria, ROS is also generated by NADPH oxidase which also contributes to GSIS in the pancreatic islets [155, 156]. Evidence from Collins' and Pénicaud's laboratory [157] provided strong evidence in favor of ROS as a metabolic signal in insulin secretion. Collin's group demonstrated the following: i) glucose stimulates production of ROS, ii) increased ROS stimulates insulin secretion and iii) use of antioxidants that quenched ROS production inhibited secretion of insulin [154]. Recent evidence from my lab demonstrated a role for prenylated Rac1 in glucose- and mitochondrial fuel-induced Nox-dependent ROS generation in INS 832/13 cells and rodent islets [158]. Apparently, the unidentified prenylated protein is required for glucose-induced ERK1/2 phosphorylation, ROS generation and Rac1 activation. Along this line of evidence, I wondered if Arf6 had any regulatory role over glucose-induced ROS generation. With this in mind, using a pharmacological approach, I examined whether activated Arf6 was necessary for ROS generation. Data in **Figure 4-6** demonstrated a significant reduction in glucose-induced ROS generation in INS 832/13 cells [Panel A] and in rat islets [Panel B]. Together, these findings suggested involvement of Arf6/ARNO in the signaling cascade leading to ROS generation by glucose.

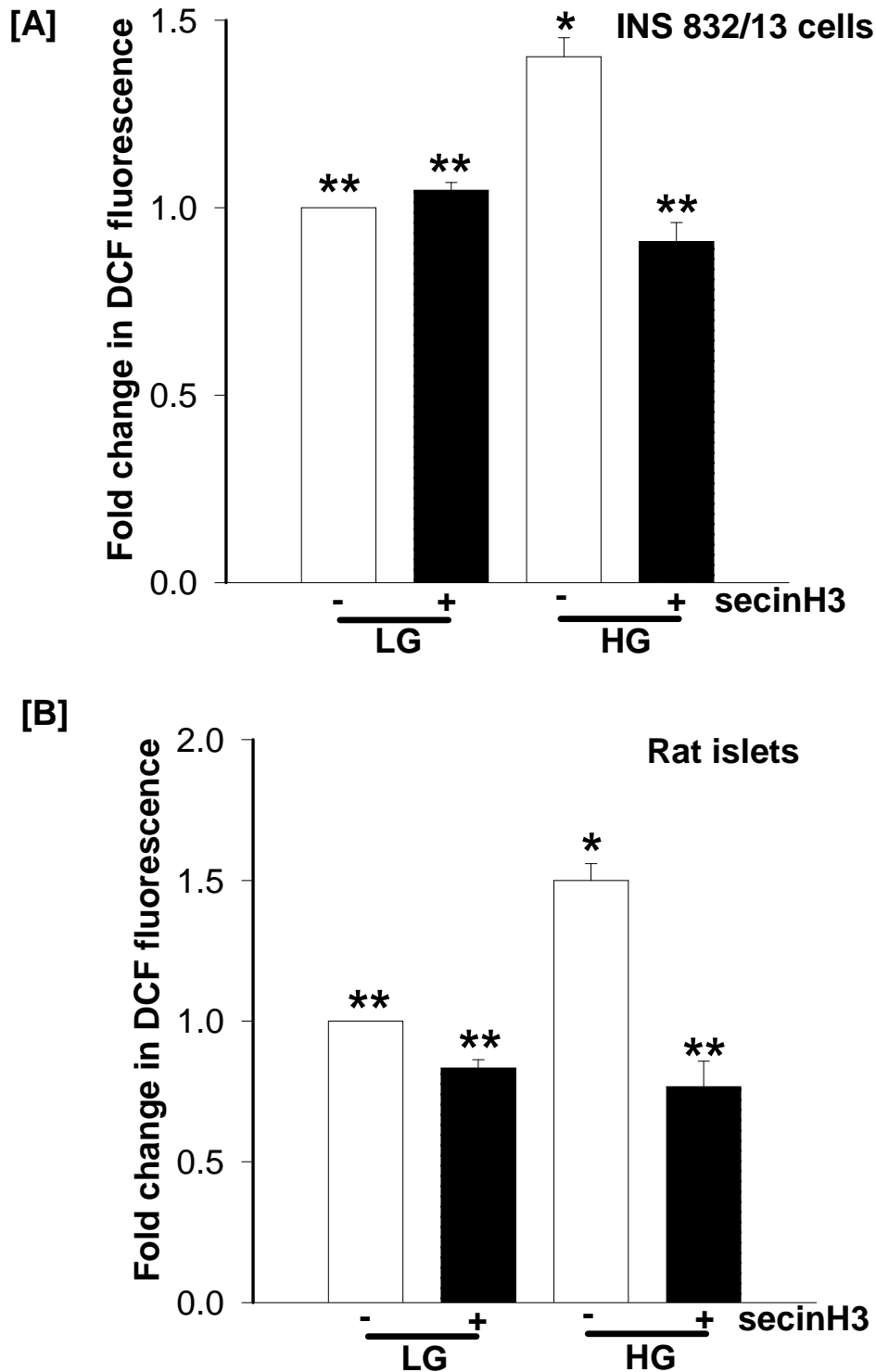


Figure 4-6: SecinH3 inhibits glucose-induced generation of ROS

Panel A and B: INS 832/13 cells [A] or rat islets [B] were starved in low serum-low glucose overnight \pm secinH3 [50 μ M] and stimulated with either low glucose [LG, 2.5 mM] or high glucose [HG, 20 mM] \pm secinH3 [50 μ M] for 60 min. At the end of stimulation, cells were incubated with DCHFDA (10 μ M; 30 min) and harvested for

quantitation of DCF fluorescence. Data shown above are expressed as fold change over basal and mean \pm SEM from three independent experiments. In Panel A, * represents $p < 0.05$ vs. low glucose without secinH3; high glucose with secinH3 and low glucose with secinH3. Similar symbols do not differ significantly. Panel B, * represents $p < 0.05$ vs. low glucose without secinH3; low glucose with secinH3 and high glucose with secinH3. Similar symbols do not differ significantly.

Regulation of NADPH Oxidase by ARNO/Arf6

At the outset, I demonstrated that generation of ROS is regulated by ARNO/Arf6 upon stimulation with glucose. ROS is generated by a family of Noxs in response to stimuli that signal through Rho GTPases, Rac [159, 160]. Supportive evidence demonstrated the localization and activation of Nox subunits in clonal beta-cells, rat islets and human islets [161, 162]. Activated Rac1 is an essential component of Nox holoenzyme complex and initiates the assembly of both cytosolic and membranous Nox subunits. Activation of the Nox complex has been associated with phosphorylation of p47^{phox}, p67^{phox} and p40^{phox} and their subsequent translocation to plasma membrane [159]. Since ARNO/Arf6 has been shown to regulate both the activation of Rac1 and generation of ROS, I wanted to determine if the regulatory pair modulated the assembly of Nox by affecting the activation and translocation of p47^{phox}, a cytosolic subunit of Nox. Therefore, I monitored the impact of secinH3 on glucose-induced phosphorylation of p47^{phox} and its recruitment to membranes in INS 832/13 cells. **Figure 4-7 A and B** shows that glucose stimulation increased the phosphorylation of p47^{phox} which was reduced in secinH3-treated cells to near basal levels. In **Figure 4-8 A and B**, secinH3 treated cells showed reduced translocation of p47^{phox} to membrane compared to control cells when stimulated with glucose. Collectively, the above evidence demonstrates ARNO to regulate the activation and assembly of Nox enzyme complex in pancreatic beta cells leading to insulin secretion.

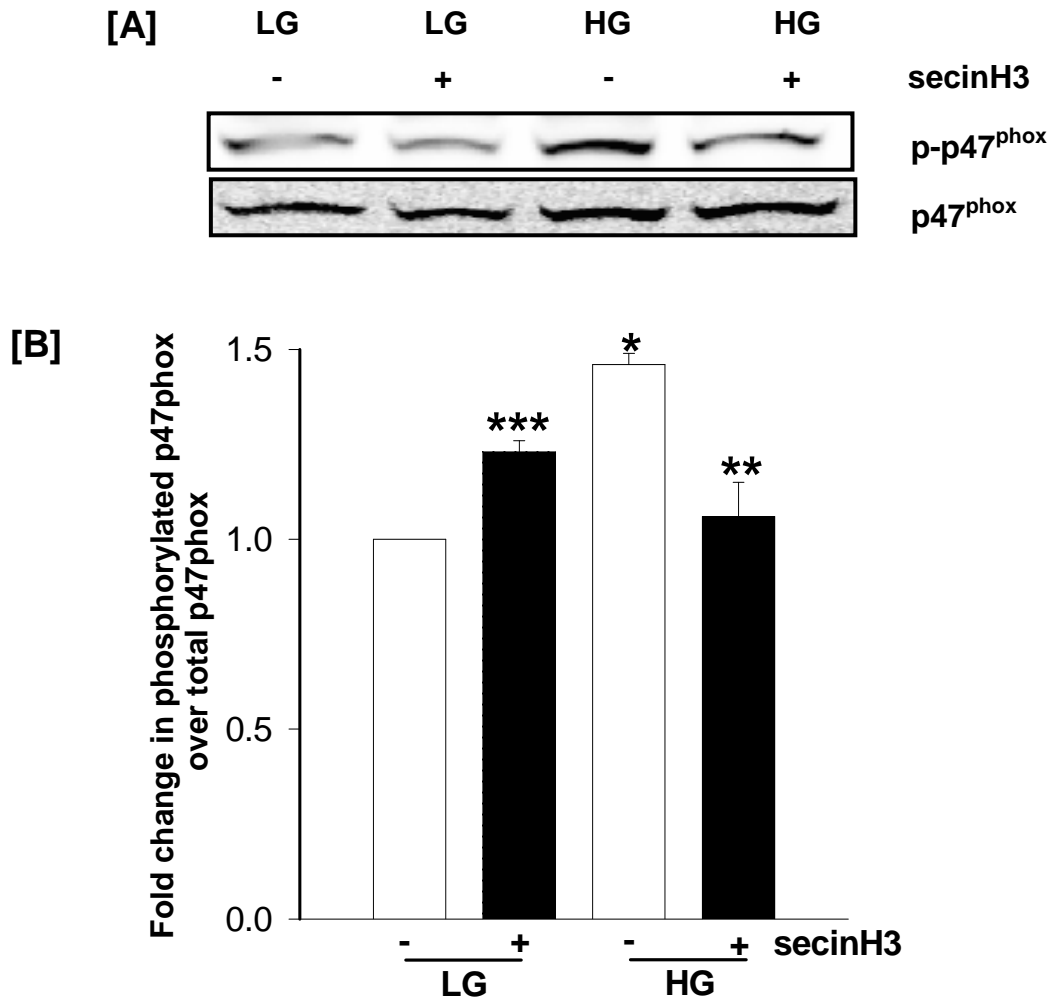


Figure 4-7: SecinH3 inhibits glucose-induced phosphorylation of p47^{phox}

Panel A: INS 832/13 cells were starved in low serum-low glucose overnight \pm secinH3 [50 μ M] and stimulated with either low glucose [LG, 2.5 mM] or high glucose [HG, 20 mM] \pm secinH3 [50 μ M] for 60 min. Lysates were prepared in RIPA lysis buffer substituted with appropriate phosphatase and protease inhibitors. Quantitated amounts of proteins were loaded and probed for p-p47^{phox}. The blot was reprobbed for total p47^{phox}. A representative blot from three independent experiments is shown here.

Panel B: Data shown above are expressed as fold change over basal and mean \pm SEM from three independent experiments. In Panel A, * represents $p < 0.05$ vs. low glucose without secinH3; high glucose with secinH3 and low glucose with secinH3. Similar symbols do not differ significantly.

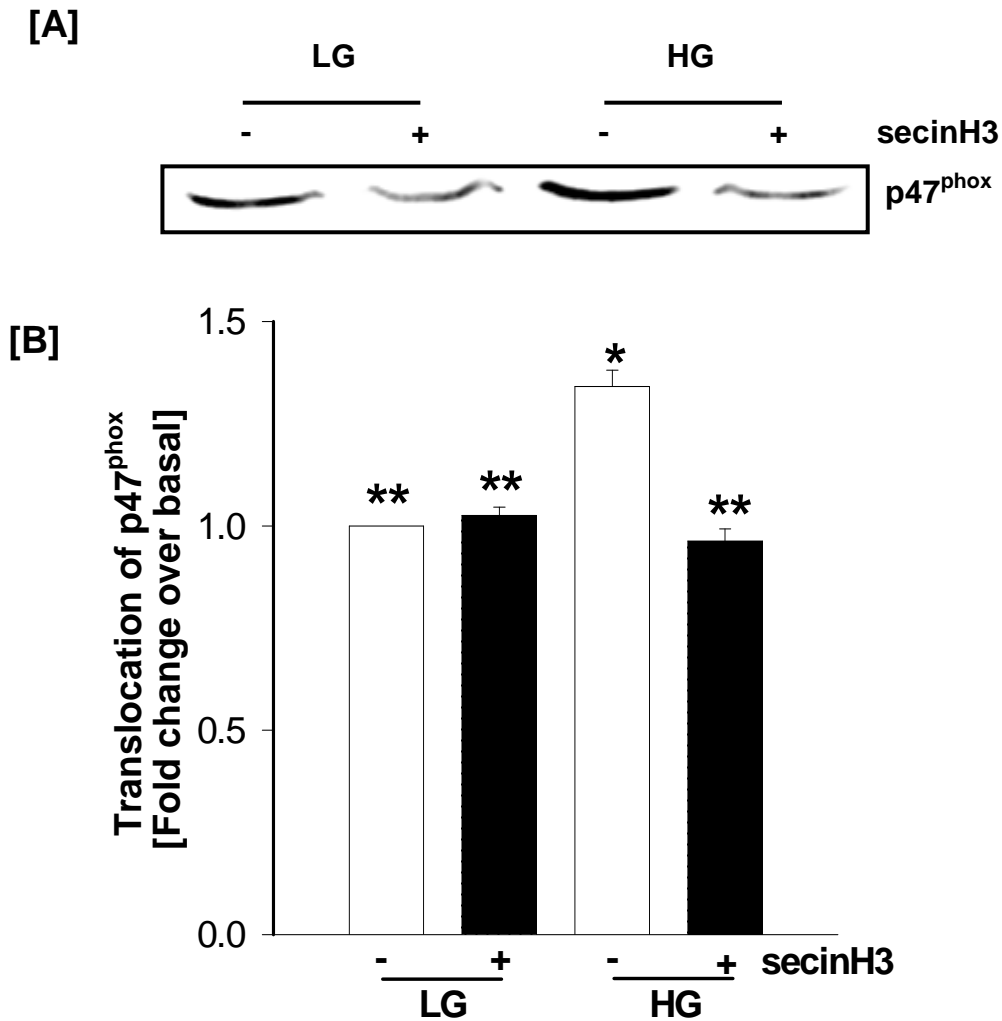


Figure 4-8: SecinH3 inhibits glucose-induced translocation of p47^{phox} to membrane

Panel A: INS 832/13 cells were starved in low serum-low glucose overnight \pm secinH3 [50 μ M] and stimulated with either low glucose [LG, 2.5 mM] or high glucose [HG, 20 mM] \pm secinH3 [50 μ M] for 60 min. At the end of stimulation, cells were lysed in homogenization buffer. Lysates were then differentially centrifuged [methods] to separate cytosol and membranous fractions. Total amount of proteins were quantitated in membrane fractions and separated onto SDS-PAGE. Proteins transferred onto nitrocellulose membrane were probed for p47^{phox}. A representative blot from three independent experiments is shown here.

Panel B: Data shown above are expressed as fold change over basal and mean \pm SEM from three independent experiments. In Panel A, * represents $p < 0.05$ vs. low glucose without secinH3; high glucose with secinH3 and low glucose with secinH3. Similar symbols do not differ significantly.

Actin remodeling

Arf6/ARNO has demonstrated many roles in regulation of actin cytoskeleton [163-166]. Particularly in neuroendocrine cells, cortical actin is shown to act as a barrier to movement of preformed dense core granules [167]. Upon stimulation, the actin network is dissolved thus allowing the granules to traverse the cell in order to access the plasma membrane. In addition to prominent roles of ARNO/Arf6 in actin cytoskeletal remodeling, cofilin is considered as a prototype of a family of actin-binding proteins that modulate cytoskeleton organization [168]. Cofilin is a well-studied protein that can bind and sever actin filaments and is involved in actin remodeling. Inactive cofilin is defined by its phosphorylated state. Activation of a phosphatase in response to stimulus, dephosphorylates cofilin and thus enabling it to bind actin [169, 170]. In order to study modulation of cytoskeleton organization, I investigated the effect of ARNO/Arf6 on the activation of cofilin. First, I determined the time-kinetics for the dephosphorylation or phosphorylation cycle of cofilin upon glucose stimulation. When INS 832/13 cells were stimulated with glucose for 0, 15, 30 and 60 min, cofilin was dephosphorylated significantly after 30 min time-point. This is in line with second-phase of insulin secretion where the reserve pool of granules needs to access the plasma membrane. To imply a role for active Arf6 in actin remodeling we tested the effect of secinH3 on glucose-induced dephosphorylation of cofilin. When activation of Arf6 is blocked using secinH3, cofilin is no longer dephosphorylated [Figure 4-7 A and B]. This above data correlated Arf6/ARNO to be an essential upstream regulator actin remodeling via the actin-severing agent, cofilin.

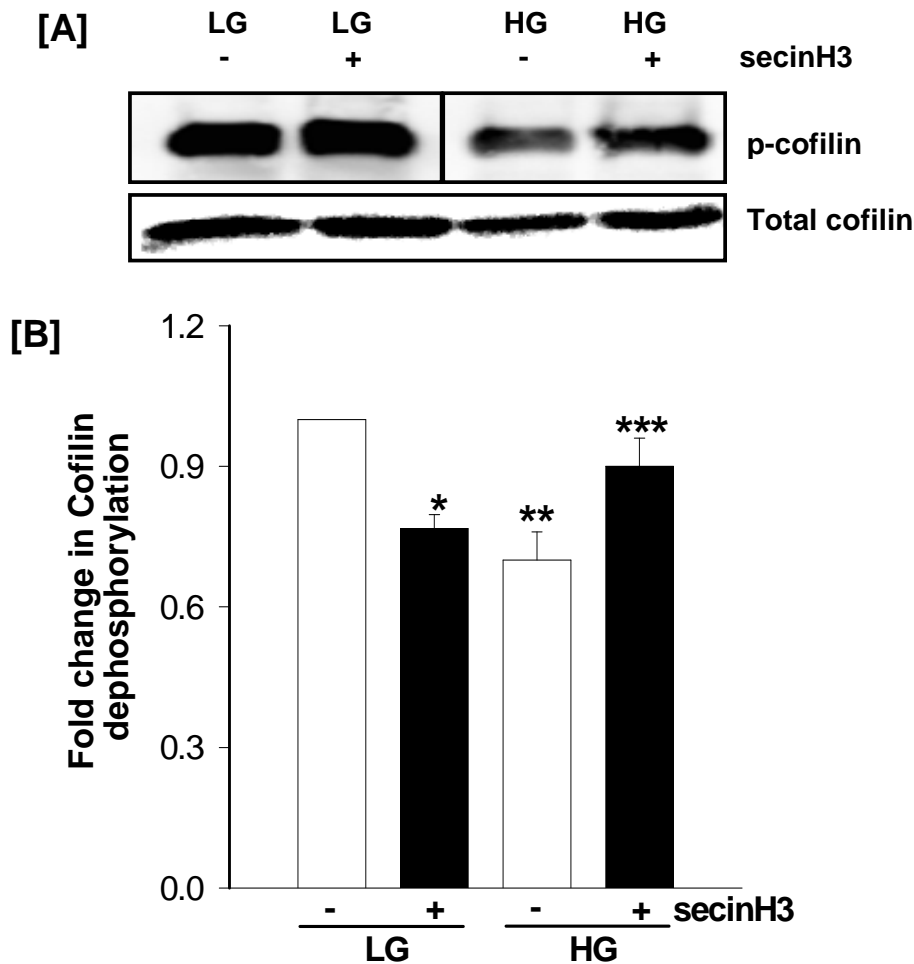


Figure 4-9: Pharmacological inhibition of ARNO function attenuates glucose-induced cofilin activation in INS 832/13 cells

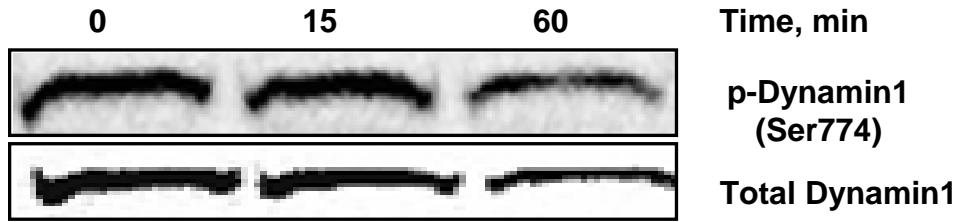
Panel A: INS 832/13 cells were starved overnight in the presence or absence of secinH3 [50 μ M] and were stimulated with low glucose [LG, 2.5 mM] and high glucose [HG, 20 mM] for 30 min in the continuous presence or absence of secinH3. Lysates were prepared in RIPA lysis buffer substituted with appropriate phosphatase and protease inhibitors. Quantitated amounts of proteins were loaded and probed for p-cofilin [Ser 3]. The blot was reprobated for total cofilin.

Panel B: Data were densitometrically analyzed and is expressed as fold change in ratio of p-cofilin over total cofilin and are mean \pm SEM of three independent experiments yielding similar results. * represents $p < 0.05$ vs. low glucose without secinH3; ** $p < 0.05$ vs. low glucose without secinH3 and *** $p < 0.05$ vs. high glucose without secinH3.

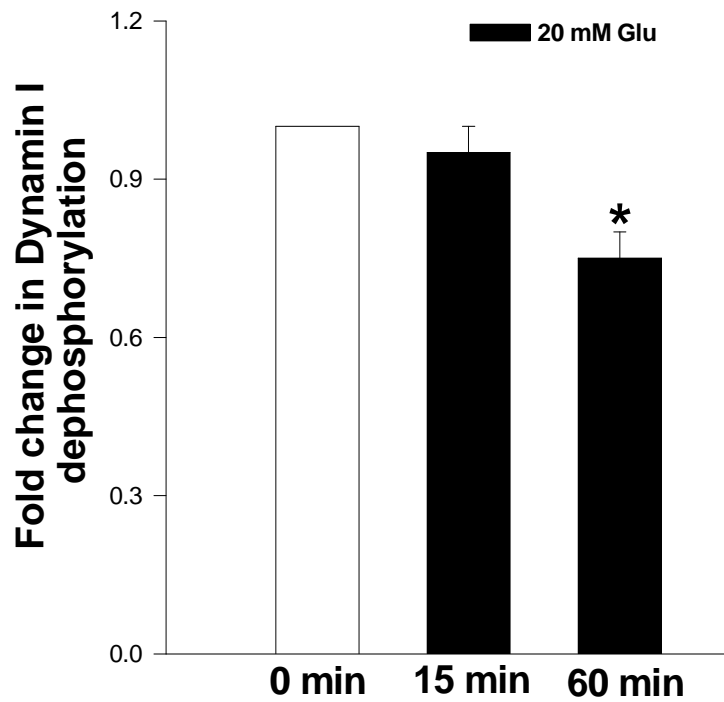
Active Arf6 regulates endocytosis

Dynamin-1, found in abundance in neuroendocrine cells, specifically regulates recycling of secretory vesicles after exocytosis [171, 172]. Among the three domains, the PRD domain plays a crucial role in mediating interaction with other proteins. The phosphorylation cycle of dynamin regulates protein-protein interactions and protein-lipid interactions [173, 174]. In a resting nerve terminal dynamin remains phosphorylated, but on stimulation phosphatases like calcineurin is activated, which dephosphorylates dynamin [114]. Moreover, it is directly demonstrated that dynamin-1 is dephosphorylated at Ser 774 after stimulation in neurons *in vivo* [175]. Dynamin-1 dephosphorylation in response to glucose stimulation is not yet investigated. This is an essential determinant of stimulus-induced activity. I challenged pancreatic beta-cell cultures with stimulatory concentrations of glucose at increasing time-point [0, 15 and 60 min]. Glucose challenge dephosphorylated dynamin 1 at Ser-774 with increasing time. In INS 832/13 cells, we found dynamin-1 to be dephosphorylated [active] to the maximum at 60 min upon glucose stimulation coinciding with endocytotic process [Figure 4-9]. Arf6 is a potential regulator of membrane traffic. It seems to efficiently control both exocytosis and endocytosis of vesicles as evident in many cell types. There has been direct implication of active Arf6 recruiting dynamin to membrane for endocytosis [137]. With this in regard, we tested whether active Arf6 functioned as an upstream regulator in the activation of dynamin-1. Upon treatment with secinH3, stimulation with glucose could not dephosphorylate dynamin-1 anymore [Fig 4-10]. This is clear evidence for active Arf6 to regulate the endocytic limb of the secretory process.

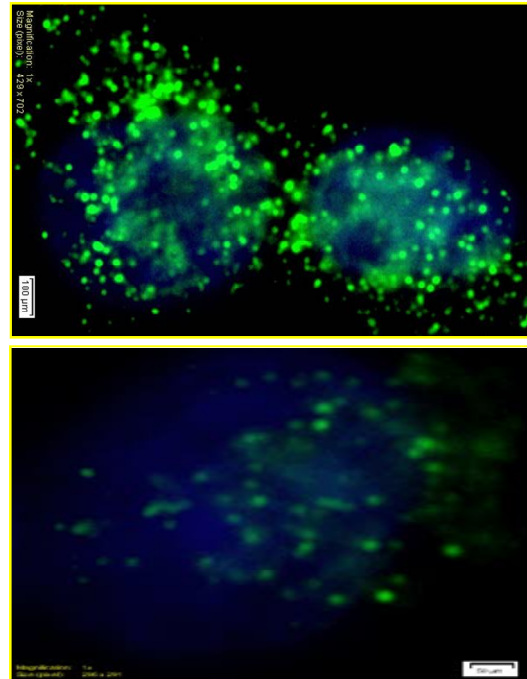
[A]



[B]



[C]



Green- p-Dynamin 1

Blue - Nucleus

Figure 4-10: Glucose induces activation of dynamin-1 over time in INS 832/13 cells

Panel A: INS 832/13 cells were starved overnight and further stimulated with low glucose [LG, 2.5 mM] and high glucose [HG, 20 mM] for indicated time-points. Lysates were prepared in RIPA lysis buffer substituted with appropriate phosphatase and protease inhibitors. Quantitated amounts of proteins were loaded and probed for p-p-dynamin-1. The blot was reprobed for total dynamin-1.

Panel B: Data were densitometrically analyzed and is expressed as fold change in ratio of p-dynamin-1 over total dynamin-1 and are mean \pm SD of two independent experiments yielding similar results. * represents $p < 0.05$ vs. p-dynamin-1 at 0 and 15 min time point.

Panel C: INS 832/13 cells were starved overnight and further stimulated with low glucose [LG, 2.5 mM] and high glucose [HG, 20 mM] for indicated time-points. Cells were fixed and permeabilized and stained with p-Dynamin-1 antibody and Hoechst nuclear stain for immunofluorescence [Methods]. The signal intensity of p-dynamin-1 is reduced in cells stimulated with glucose for 60 min as compared to 0 min. A representative image from multiple cells on two sets of coverslips is shown here.

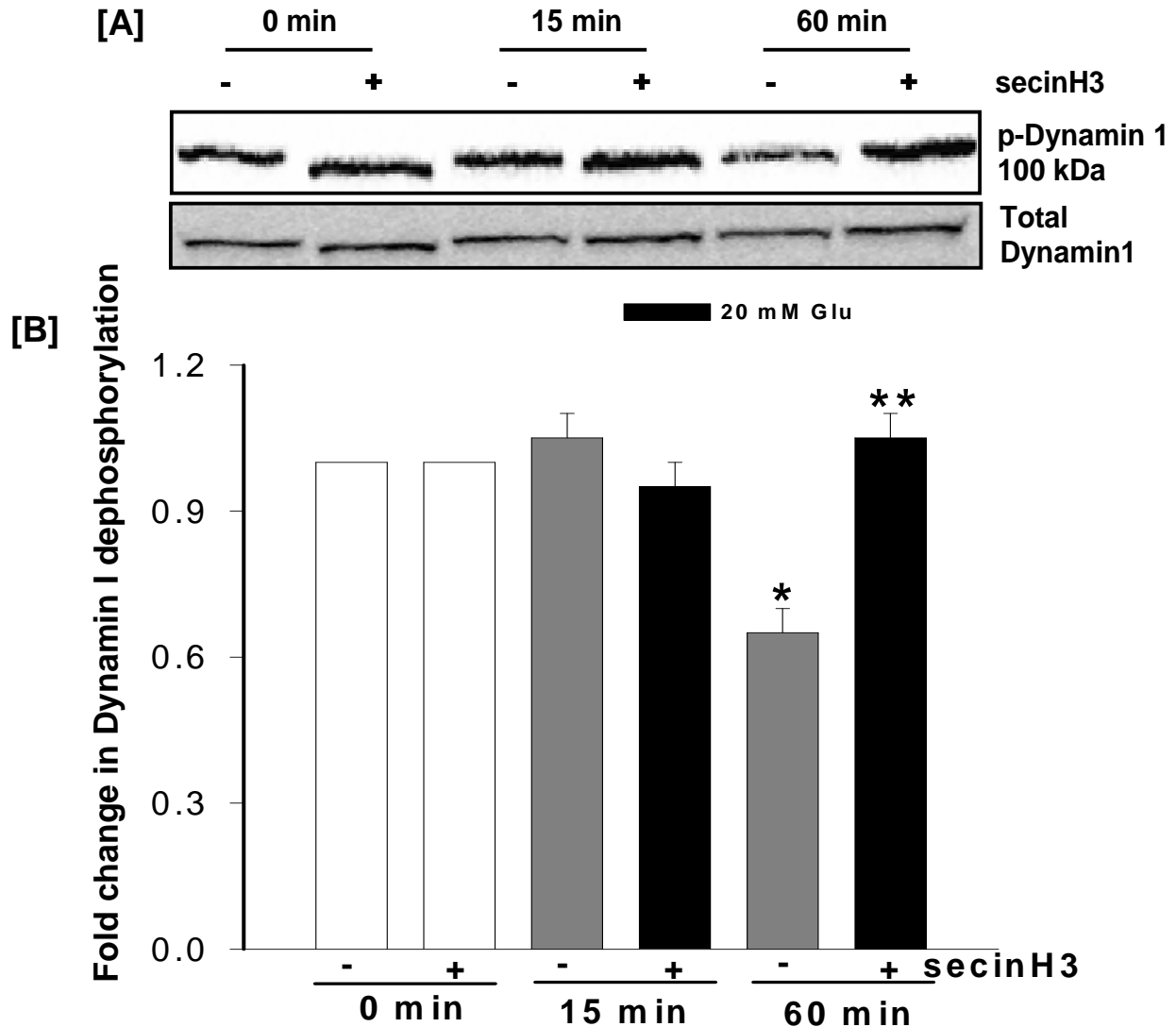


Figure 4-11: SecinH3 attenuates glucose-induced dynamin-1 activation in INS 832/13 cells

Panel A: INS 832/13 cells were starved overnight in the presence or absence of secinH3 [50 μ M] and were stimulated with low glucose [LG, 2.5 mM] and high glucose [HG, 20 mM] for 0, 15 and 60 min in the continuous presence or absence of secinH3. Lysates were prepared in RIPA lysis buffer substituted with appropriate phosphatase and protease inhibitors. Quantitated amounts of proteins were loaded and probed for p-dynamin-1. The blot was reprobed for total dynamin-1.

Panel B: Data were densitometrically analyzed and is expressed as fold change in ratio of p-dynamin-1 over total dynamin-1 and are mean \pm SD of two independent experiments yielding similar results. * represents $p < 0.05$ vs. p-dynamin-1 at 0 and 15

min time point with or without secinH3 and ** represents $p < 0.05$ vs. p-dynamin-1 at 60 min stimulation without secinH3.

Role(s) of Posttranslational Modification of Small G-Proteins in Glucose-Stimulated Insulin Secretion

For decades it is well known that small G-proteins undergo certain other chemical modifications apart from the addition of GTP to confer functional activity [69, 176]. A growing body of evidence indicates that G-proteins undergo isoprenylation and methylation. These chemical modifications are termed as post-translational modifications which enable small G-proteins to attach to plasma membrane. Recent years have seen advances in elucidating these modifications that confer biological activity upon small G-proteins. Ras family of G-proteins consist a CAAX motif [C,Cys; A, Aliphatic amino acid and X, any amino acid] at the C-terminal end [120]. The processing of CAAX-containing proteins undergoes prenylation [farnesylation or geranylgeranylation] [177] followed by a prenyl-cysteine carboxylmethylation. The enzymes that catalyze the above processes are of utmost interest for their therapeutic potential as anti-cancer agents and have also been a valuable tool in evaluating the importance of such modifications in stimulated insulin secretion. Prenylation process involves the addition of prenyl intermediates that are generated from acetyl CoA and acetoacetyl CoA in the cholesterol biosynthetic pathway at the CAAX motif. The candidate protein is incorporated with either a farnesyl group [15-carbon derivative of mevalonic acid] by farnesyltransferases [FTase] or a geranylgeranyl group [20 carbon derivative of mevalonic acid] by geranylgeranyltransferases [GGTase] [178]. The major farnesylated proteins include Ras, nuclear lamins and also gamma-subunits of trimeric

G-proteins whereas the geranylgeranylated proteins include Cdc42, Rac1 and Rho [140].

Extensive evidence from our laboratory has shown the localization and involvement of prenylation signaling event to be an essential step in GSIS from pancreatic beta cells [150, 158, 179]. Earlier studies have also demonstrated a potential regulatory role for prenylation in physiological insulin secretion with the use of pharmacological inhibitors [177].



From Kowluru A, Endocr Rev. 2010

Figure 4-12. Image depicting a biosynthesis of farnesyl and geranyl pyrophosphates. [140].

Followed by the addition of a prenyl group to the C-terminal cysteine, the three amino acids after the prenylated cysteine are cleaved by Ras-converting enzyme 1 [Rce 1], thus exposing the carboxylate anion. A secondary step involves the methylation of carboxylate site by isoprenylcysteine-O-carboxyl methyltransferase [ICMT] [180, 181]. It is widely accepted that the two steps increase the hydrophobicity of the candidate proteins for optimal targeting to their relevant membranous sites for the regulation of effector proteins [181]. While a significant number of recent studies have focused on putative roles of G-protein prenylation in glucose-stimulated insulin secretion (GSIS), very little is known with regard to potential roles of carboxymethylation in islet function [181]. Original studies from our laboratory have attempted to address the roles of carboxymethylation in islet function, including insulin secretion [75, 77]. Therein, using selective inhibitors of ICMT such as acetyl farnesyl cysteine (AFC) we have been able to demonstrate that Cdc42 and G γ subunits undergo carboxymethylation in response to glucose in clonal β -cells, normal rat islets and human islets [75, 77]. Follow-up studies by Li and coworkers characterized ICMT in insulin-secreting cells for its subcellular localization and regulation by known second messengers of insulin secretion [182]. In the current study, I have revisited this area of islet biology to precisely determine the role of carboxymethylation and the identity of methylated proteins to further evaluate their roles in the signaling events leading to insulin secretion.

Along these lines, emerging evidence implicates novel regulatory roles for phagocyte-like NADPH oxidases (Nox) in physiological insulin secretion. For example, using selective inhibitors (e.g., DPI or apocynin) and molecular biological tools (e.g., antisense and siRNAs for Nox subunits), several recent studies have demonstrated

“second messenger” roles for Nox-derived reactive oxygen species in glucose-stimulated insulin secretion [183-185]. Some of these aspects, including downstream targets for reactive oxygen species signals have been reviewed by Pi and Collins recently [153]. Furthermore, recent studies from our laboratory have also demonstrated a novel regulatory role for Rac1 in Nox-derived generation of reactive oxygen species, thus suggesting that glucose-induced Rac1 activation step might be necessary for Nox-mediated generation of reactive oxygen species and insulin secretion. For example, using selective inhibitors of prenylation (e.g., GGTI-2147), we have demonstrated that post-translational prenylation of Rac1 is important for its regulation of generation of reactive oxygen species [158]. Therefore, based on the above evidence and as a logical extension to studies to suggest obligatory roles of ICMT-mediated carboxymethylation of Rac1 function for its subcellular localization and function [186, 187], we undertook the current investigation to determine the regulatory roles of ICMT in glucose-induced Rac1 activation, generation of reactive oxygen species and insulin secretion in INS 832/13 cells. We have accomplished this goal by two distinct approaches to compromise the β -cell endogenous ICMT function, *via* siRNA-mediated knockdown of ICMT expression and pharmacological inhibition of ICMT by AFC. Indeed, data accrued from the current studies underscores the importance of carboxymethylation of Rac1 in glucose-induced Nox activation and associated generation of reactive oxygen species and insulin secretion.

ICMT is expressed in INS 832/13 cells

At the outset, I determined the immunological localization and subcellular distribution of ICMT in INS 832/13 cells. For this, total particulate and soluble fractions were isolated

from INS 832/13 cells by a single step centrifugation method and relative abundance of ICMT was determined in these fractions by Western blotting. Data in **Figure 4-13** suggested a predominant membrane association of ICMT in these cells. It should be also noted that we consistently observed a doublet for ICMT on Western blots, which might represent a post-translationally modified form of this protein.

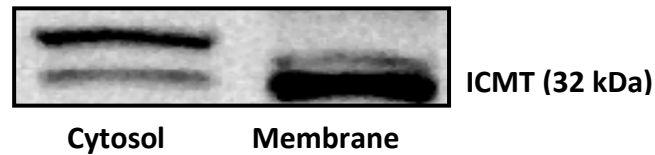


Figure 4-13: Expression and subcellular distribution of ICMT in INS 832/13 cells

Total particulate and soluble fractions were isolated from INS 832/13 cells by a single step centrifugation method described under Methods. ICMT expression was determined in these fractions by Western blotting. A representative of three blots is shown here.

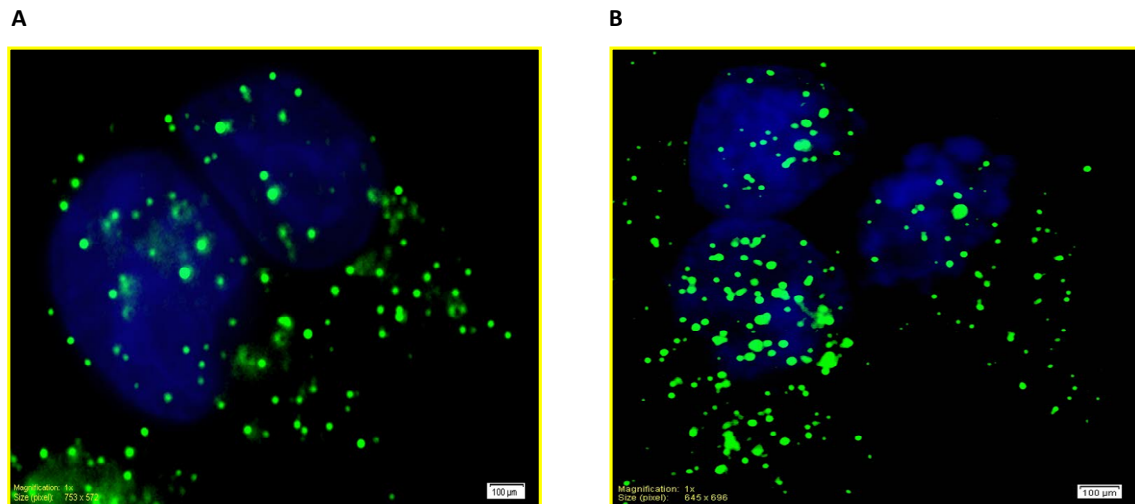


Figure 4-14: Localization of ICMT in INS 832/13 cells by immunofluorescence under basal and glucose-stimulated conditions

INS 832/13 cells were plated on coverslips and cultured overnight in low serum low glucose media prior to the incubation with either 2.5 mM (**Panel A**) or 20 mM glucose (**Panel B**) for 45 min at 37°C. The cells were fixed in 4% paraformaldehyde solution in PBS for 15 min and permeabilized using 0.2% triton X-100 for 15 min. Fixed cells were

examined for ICMT (stained in green) and nuclei (stained in blue) as described under Methods.

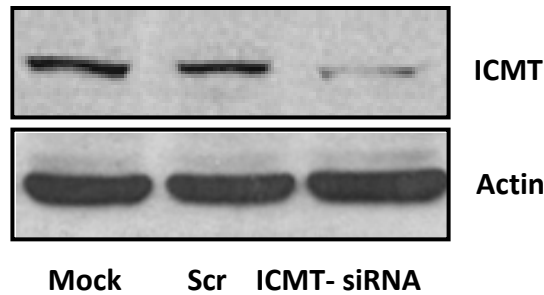
In the next series of studies we determined the distribution of ICMT in INS 832/13 cells by immunofluorescence method. Data in **Figure 4-14** suggested that ICMT [green] remain diffused throughout the cell under basal (Panel A; LG; 2.5 mM glucose) conditions. Further, we observed no clear effects of stimulatory glucose (Panel B; HG; 20 mM glucose) on ICMT distribution in these cells.

siRNA-mediated knockdown of ICMT attenuates glucose-, but not KCl-induced insulin secretion in INS 832/13 cells

We next investigated potential regulatory roles of ICMT in glucose-induced insulin secretion in these cells. To address this, we knocked down the endogenous expression of ICMT by siRNA methodology. Data in **Figure 4-15 A and B** indicated more than ~70 % inhibition in the expression of ICMT following siRNA-ICMT transfection. Data in **Figure 4-15 C** suggested no significant effects of scrambled siRNA transfection either on basal or glucose-induced insulin secretion (bars 1 vs. 3 and 2 vs 4). However, transfection of siRNA-ICMT in these cells led to a modest increase in basal secretion (bars 1 or 2 vs. bar 5), but insulin secretion elicited by stimulatory glucose was significantly reduced in ICMT knocked down cells (bars 2 or 4 vs. 6). These data suggested that activation of ICMT is necessary for glucose-stimulated insulin secretion to occur. We then determined potential requirement for ICMT in insulin secretion elicited by a membrane depolarizing concentration of KCl. Data shown in **Figure 4-15 D** suggested no significant effects of ICMT knockdown on KCl-induced insulin secretion.

Together, data in **Figure 4-15 C and D** suggest that glucose, but not KCl-evoked insulin secretion is mediated *via* activation of ICMT.

[A]



[B]

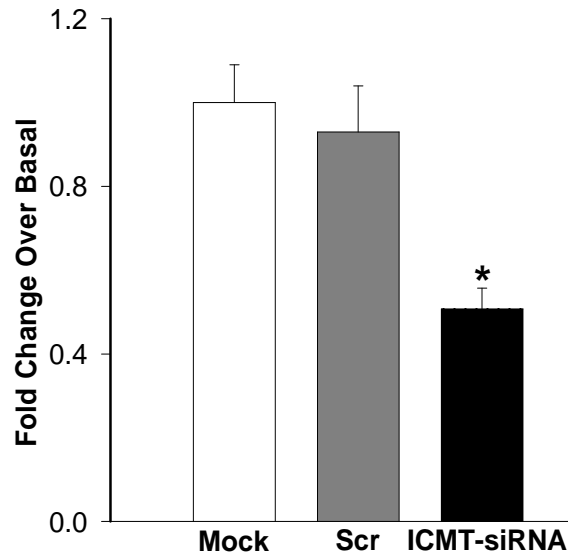
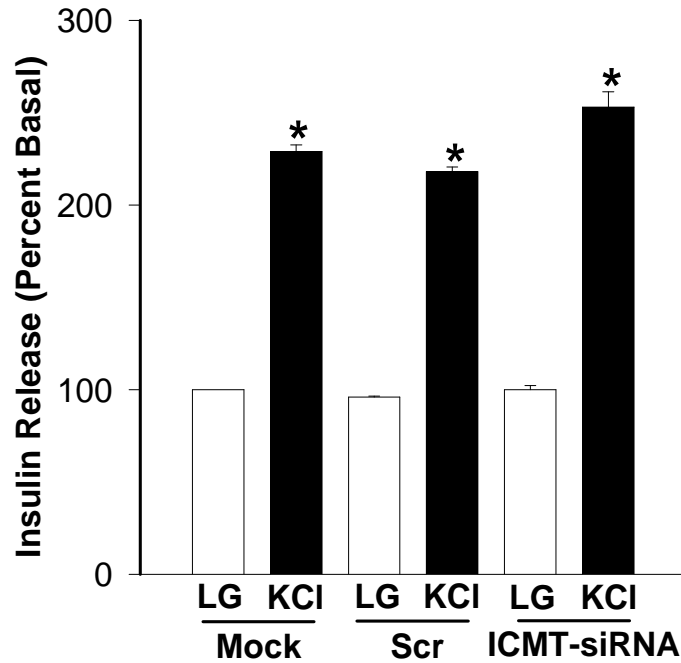


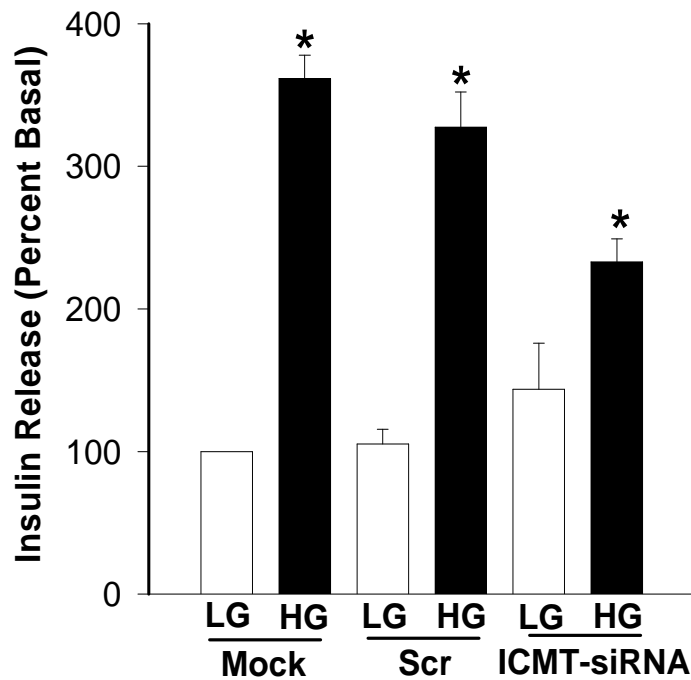
Figure 4-15: Glucose-, but not KCl-stimulated insulin secretion, is attenuated in INS 832/13 cells following siRNA-mediated knockdown of ICMT

INS 832/13 cells were either mock transfected or transfected with scrambled siRNA or siRNA-ICMT at a final concentration of 100 nM and cultured for 24 h. Transfection efficiency was determined by separating equal amounts of proteins on SDS-PAGE and probing with ICMT antibody (**Panel A**; representative of three transfections is shown here). Data in **Panel A** was densitometrically analyzed and expressed as fold change over basal (**Panel B**). * represent $p < 0.05$ compared with mock or scrambled siRNA transfected cells.

[C]



[D]



Further, transfected cells were incubated either with low glucose (LG; 2.5 mM) or high glucose (HG; 20 mM: **Panel C**) or a membrane depolarizing concentration of KCl (60 mM; osmolality adjusted by lowering NaCl; **Panel D**) for 45 min at 37°C. Insulin released

into the medium was quantitated by ELISA. Data are expressed as percentage of basal and are mean \pm SEM from three independent determinations. * $p < 0.05$ vs. respective low glucose controls; ** $p < 0.05$ vs. mock transfected cells.

siRNA-mediated knockdown of ICMT attenuates glucose-induced Rac1 activation in INS 832/13 cells

Published evidence from several laboratories, including our own have suggested that activation of Rac1, a small G-protein, is a requisite step in the signaling events leading to glucose-insulin secretion [139, 140, 188]. Furthermore, using inhibitors of post-translational geranylgeranylation [e.g., GGTI-2147] or a dominant negative mutant of the α -subunit of geranylgeranyl transferase, we have demonstrated a requirement for post-translational geranylgeranylation in glucose-induced Rac1 activation and insulin secretion [179]. Since Rac1 undergoes carboxymethylation, we investigated if silencing of ICMT affects glucose-induced Rac1 activation. Data shown in **Figure 4-16** demonstrated a significant increase in glucose-induced Rac1 activation (lane 1 vs. 3). siRNA-mediated knockdown of ICMT failed to exert any clear effects on basal Rac1 activation (lane 1 vs. 2), but significantly attenuated glucose-induced Rac1 activation (lane 3 vs. 4). Pooled data from multiple experiments are provided in **Figure 4-16 B**. Together, these findings suggested a requirement for carboxymethylation for glucose-induced activation of Rac1.

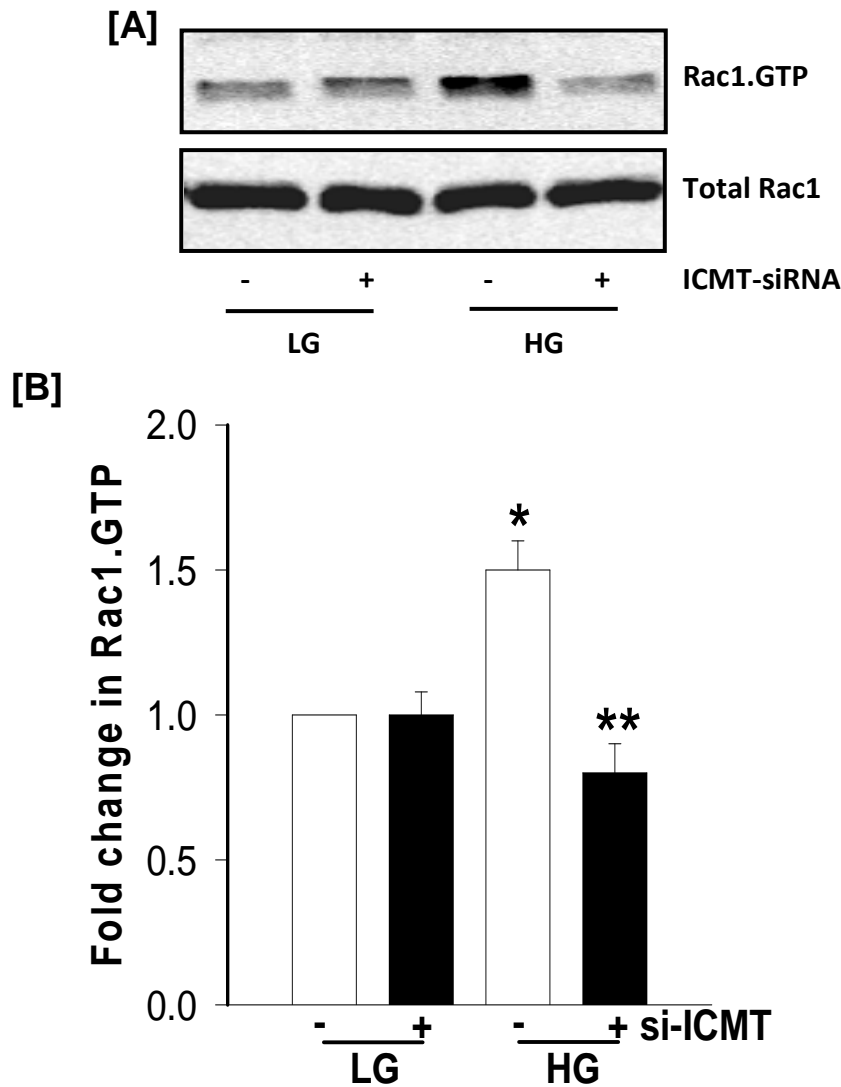


Figure 4-16: Depletion of endogenous ICMT markedly attenuates glucose-induced activation of Rac1 in INS 832/13 cells.

INS 832/13 cells were transfected with ICMT-siRNA or mock transfected and cultured for 24 h. At confluence, cells were starved overnight and stimulated with either low (2.5 mM) or high (20 mM) glucose for 30 min. The extent of Rac1 activation in these cells was quantitated by PAK-PBD pull-down assay. Total and activated (Rac1.GTP) were determined by Western blotting (**Panel A**) and quantitated by densitometry (**Panel B**).

Data are expressed as fold change in Rac1 activation and are mean \pm SEM from three independent determinations. * $p < 0.05$ vs. mock transfected low glucose; ** $p < 0.05$ vs. mock transfected high glucose.

siRNA-mediated knockdown of ICMT markedly inhibits glucose-induced reactive oxygen species generation in INS 832/13 cells

Emerging evidence from multiple laboratories appears to suggest novel second messenger roles for reactive oxygen species in GSIS [153]. It has also been shown that ROS generated *via* the activation of phagocyte-like NADPH oxidase (Nox) plays such regulatory roles in GSIS since pharmacological (e.g., apocynin or DPI) or molecular biological (e.g., siRNA or antisense for p47^{phox}) inhibition of Nox led to inhibition of GSIS [184, 185]. Since Rac1 represents one of the members of Nox holoenzyme [140, 159], we investigated if siRNA-mediated knockdown of ICMT exerts any regulatory effects on glucose-induced generation of reactive oxygen species in INS 832/13 cells. Data in **Figure 4-17** suggested no significant effects of ICMT knockdown on basal levels of reactive oxygen species in these cells (bar 1 vs. 2). However, glucose-induced generation of ROS was markedly attenuated in cells in which expression of ICMT was knocked down (bar 3 vs. 4). Taken together, these data demonstrated that glucose-induced Rac1 activation (Figure 4-16), generation of ROS (Figure 4-17) and insulin secretion (Figure 4-15) are regulated by ICMT in INS 832/13 β -cells.

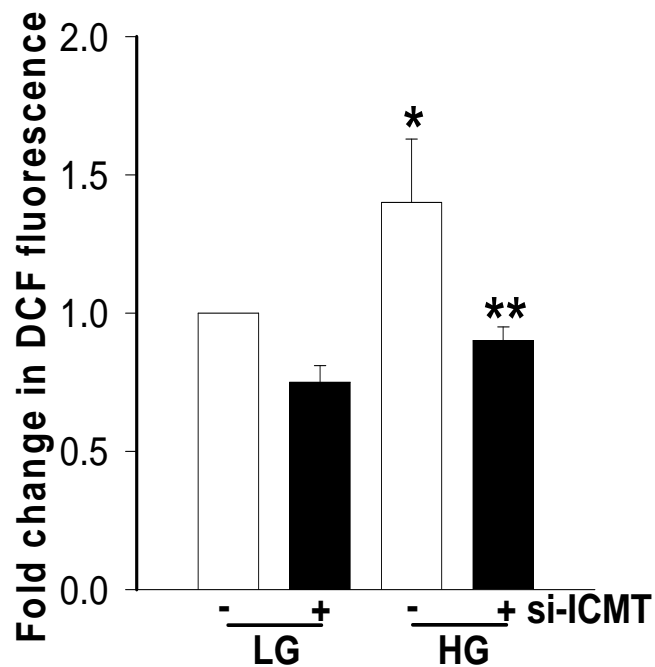


Figure 4-17: Glucose-induced ROS generation was attenuated in INS 832/13 cells following siRNA-mediated knockdown of ICMT

INS 832/13 cells transfected with ICMT-siRNA (or mock transfected) following which cells were stimulated with low glucose (2.5 mM) or high glucose (20 mM) for 1 h and were incubated with DCHFDA (10 μ M; 30 min) and harvested for quantitation of DCF fluorescence. Data expressed as DCF fluorescence and are mean \pm SEM from three independent determinations. * $p < 0.05$ vs. respective low glucose; ** $p < 0.05$ vs. high glucose in mock transfected cells.

Acetyl farnesyl cysteine (AFC), a selective inhibitor of ICMT, attenuates glucose-induced generation of reactive oxygen species and insulin secretion in INS 832/13 cells

We next confirmed the above data accrued through the use of siRNA-ICMT by a pharmacological approach. In the following studies, we determined the effects of acetyl farnesyl cysteine (AFC), a selective inhibitor of ICMT [74, 77], on glucose-induced generation of reactive oxygen species and insulin secretion. Data shown in **Figure 4-18 A** indicated a modest, but significant inhibition in basal level of reactive oxygen species in these cells following exposure to AFC (Figure 4-18; bar 1 vs. 2). However, increase in the level of reactive oxygen species seen in the presence of stimulatory glucose was significantly inhibited by AFC (Figure 4-18; bar 3 vs. 4). Furthermore, insulin secretion elicited by stimulatory (Figure 4-18; Panel B; bar 3 vs. 4), but not basal glucose (Figure 4-18; Panel B; bar 1 vs. 2), was markedly attenuated by AFC. In addition, in a manner akin to siRNA-ICMT effects, we observed no significant effects of AFC on KCl-induced insulin secretion (Figure 4-18; Panel C). Together, our above described findings confirm that glucose-, but not KCl-mediated effects on insulin secretion require activation of ICMT. Furthermore, along these lines, we also noticed a significant inhibition of glucose-induced activation of Rac1 by AFC under the conditions it inhibited glucose-induced generation of reactive oxygen species ($\sim 41 \pm 10\%$ inhibition by AFC; mean \pm SEM from three pull down assays; $p < 0.05$ vs. diluent) and insulin secretion (additional data not shown). Together, these data further confirm our siRNA-ICMT findings and support our hypothesis that ICMT-mediated carboxymethylation of specific proteins (e.g., Rac1)

plays a positive modulatory role in the cascade of events leading to glucose-induced generation of reactive oxygen species and insulin secretion in INS 832/13 cells.

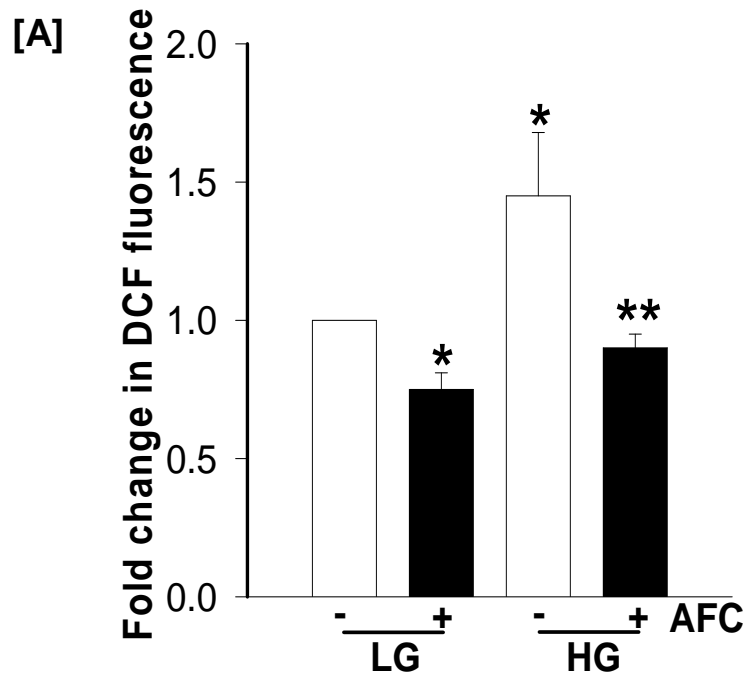
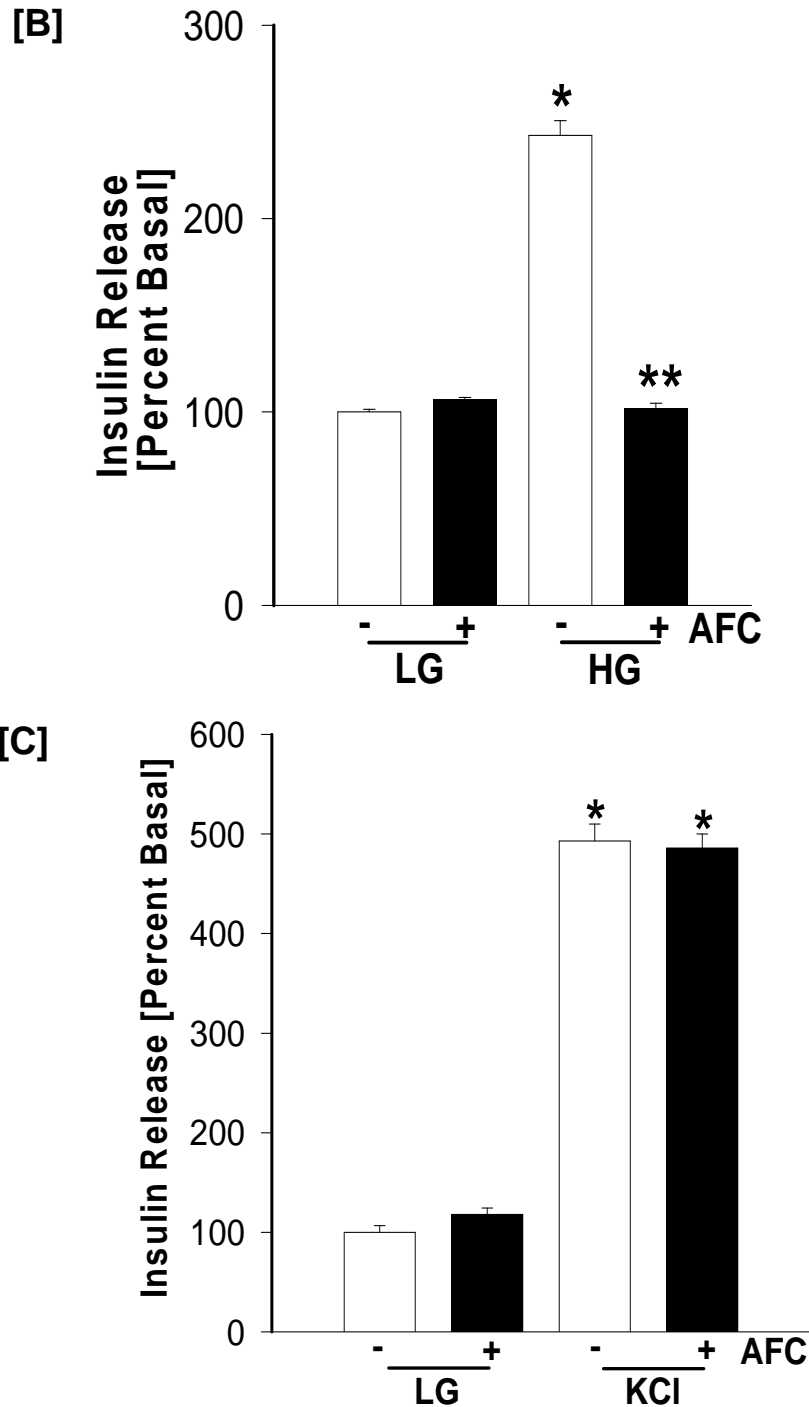


Figure 4-18: AFC, a competitive inhibitor of ICMT, attenuates glucose-induced ROS generation and insulin secretion in INS 832/13 cells

INS 832/13 cells were cultured overnight with low-glucose and low-serum medium and then incubated in KRB in the presence of diluent or AFC (100 μ M; 1 h) as indicated in the figure. Cells were further stimulated with either low glucose (LG; 2.5 mM) or high glucose (HG; 20 mM) for 1 h in continuous presence or absence of diluent or inhibitor. At the end of stimulation, ROS generation was determined by quantitating DCF fluorescence (**Panel A**) as described in Figure 4-16.



In a separate set of studies glucose- and KCl-stimulated insulin secretion was quantitated (**Panel B and C**) under conditions described in Methods section. Data in Panel B are mean \pm SEM from three independent determinations. * $p < 0.05$ vs. low glucose without AFC; ** $p < 0.05$ vs. high glucose without AFC whereas data in **Panel C** are mean \pm SEM from 12 determinations in each case. * $p < 0.05$ vs. low glucose without AFC and low glucose with AFC.

Inhibition of ICMT does not affect cell viability

We next investigated potential cytotoxic effects of ICMT knockdown (*via* siRNA-ICMT) or inhibition of ICMT activity (by AFC) on INS 832/13 cells. We asked this question to be sure that either inhibition in Rac1 activation, reactive oxygen species generation or insulin secretion seen under these conditions are not due to potential loss in cell viability or cell demise following inhibition of ICMT expression and/or activity. We addressed this by two independent experimental approaches. In the first, we quantitated activation of caspase-3, a hallmark of cellular apoptosis, in both siRNA-ICMT transfected cells and AFC-treated cells. In the second approach, we quantitated the metabolic viability of siRNA-ICMT transfected or AFC-treated cells using the MTT assay. Data shown in **Figure 4-19 A and B** indicated no caspase 3 activation following siRNA-ICMT transfection or AFC treatment. However, a significant activation of caspase 3 was seen in INS 832/13 cells treated with etoposide, which causes apoptosis in cells *via* caspase 3 activation. Together, these data in **Figure 4-19 A and B** suggest no cell death in INS 832/13 cells following inhibition of expression and activity of ICMT. In addition, we observed only a modest inhibition in cell viability as assessed by the MTT in cells following ICMT knockdown *via* siRNA-ICMT (**Figure 4-19 C**) or AFC treatment [**Figure 4-19 D**]. Together, these findings suggest that the observed inhibition of glucose-induced Rac1 activation, generation of reactive oxygen species and insulin secretion following inactivation of ICMT are specific and do not involve cytotoxic mechanisms.

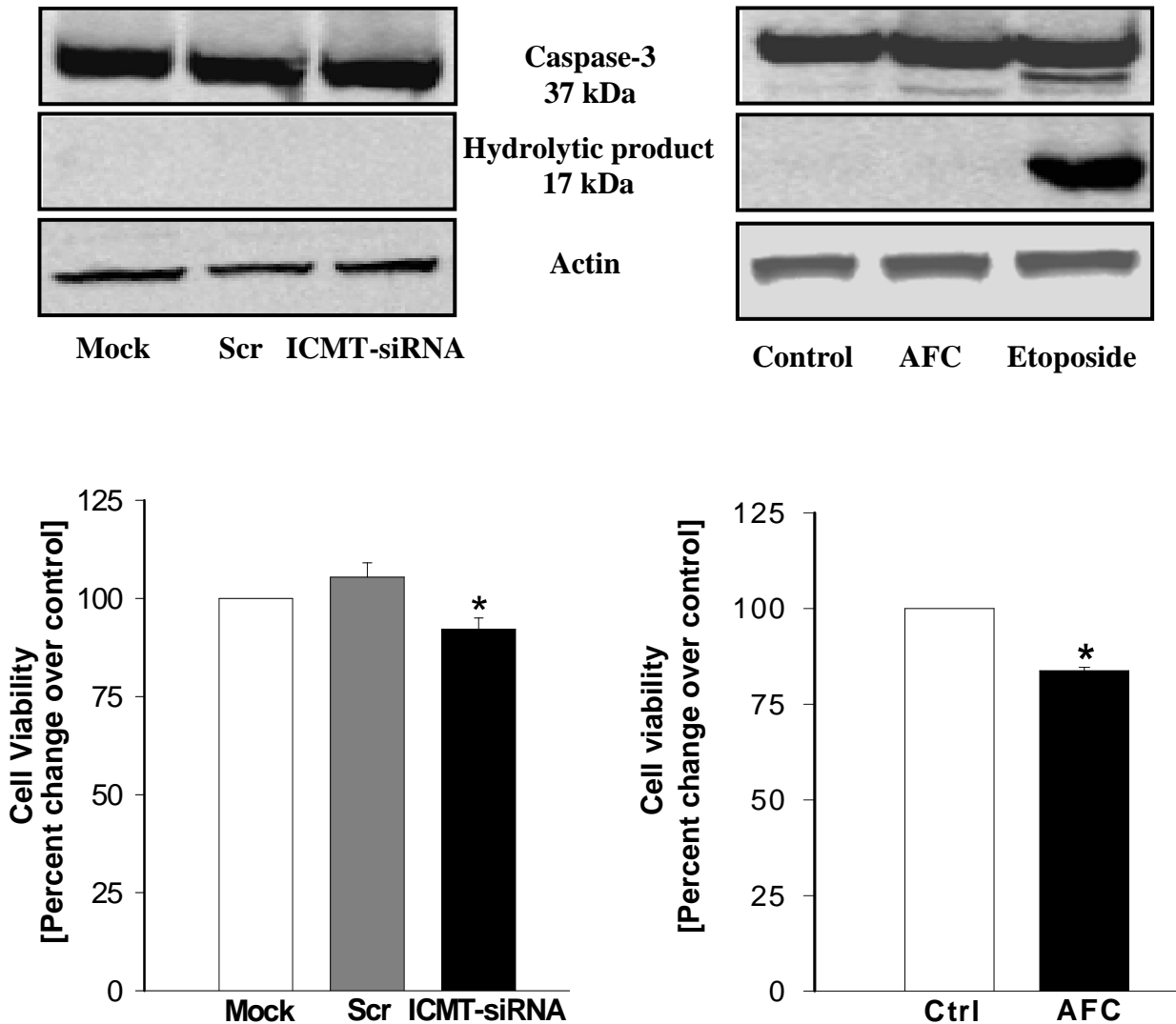


Figure 4-19: ICMT inhibition does not affect cell viability.

Panel A INS 832/13 cells treated with AFC [100 μ M, 1h] were incubated with MTT [5 mg/mL, 4h] as described in Materials and Methods. Cell viability was determined by quantitating reduction of MTT by metabolically active cells at 570 nm. Data are means \pm SEM from two independent experiments yielding identical results with n: >12 in each group and expressed as percent change over control. * represents $p < 0.05$ compared with control. In **Panel B**, INS 832/13 cells were mock transfected or transfected either with ICMT siRNA or scrambled siRNA (100 nmol, 24h). Cell viability in transfected cells was determined by MTT reduction method as described above. Data are means \pm SEM from two independent experiments yielding identical results with n: >12 in each group and expressed as percent change over control. * represents $p < 0.05$ compared with mock or scrambled siRNA transfected cells.

Alterations in ICMT expression in *in vitro* models of gluco-, lipo-, glucolipotoxicity and endoplasmic reticulum stress

A growing body of evidence implicates that long-term exposure of β -cells to saturated fatty acids [i.e., lipotoxicity], glucose [i.e., glucotoxicity] or both [i.e., glucolipotoxicity] leads to severe metabolic dysfunction and eventual demise of the β -cell [189]. Furthermore, exposure of these cells to thapsigargin, leads to endoplasmic reticular stress *via* depletion of calcium pools culminating in cellular dysfunction [190, 191]. Therefore, in the last series of these studies we investigated potential alterations in the expression of ICMT in INS 832/13 cells following exposure to palmitate, glucose or thapsigargin. Data shown in **Figure 4-20** indicated a significant increase in the expression of ICMT in cells exposed to gluco-, lipo- or glucolipotoxic conditions. However, no detectable changes were seen in the expression of ICMT protein in thapsigargin-treated cells.

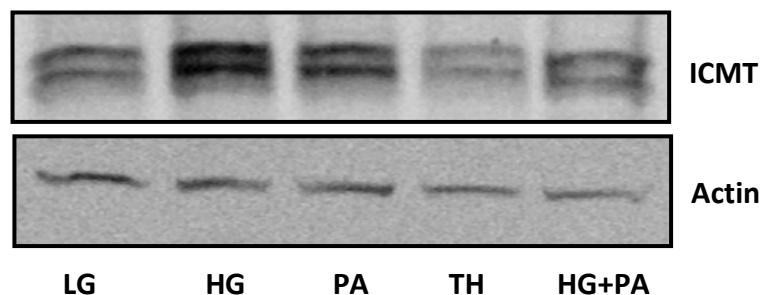


Figure 4-20: Expression of ICMT in lysates of INS 832/13 cells under the duress of gluco-, lipo-, glucolipotoxicity and endoplasmic stress

INS 832/13 cells were plated in six-well plates, grown to 70 % confluence and treated with low glucose (LG, 2.5 mM, 48 h), high glucose (HG, 50 mM, 48 h), palmitic acid (PA, 300 μ M; 48 h), HG plus PA (48 h) and thapsigargin (TH, 0.5 μ M, 9 h). ICMT expression was determined by Western blotting. A representative of two blots is shown here. Actin was used as a loading control.

Chapter 5

Functional status of the ARNO/Arf6 signaling cascade and its down-stream metabolic steps in models of impaired insulin secretion and type 2 diabetes.

Type 2 diabetes [T2D] is characterized by a progressive loss of beta-cell function throughout the course of the disease. Investigators have established the course of the disease starting with an initial loss in early or first phase insulin secretion, which is followed by a decreasing maximal capacity of glucose to potentiate all non-glucose signals. Apart from these, disproportionate hyperinsulinemia and impaired basal or steady-state insulin-secretion [192] add to the symptoms. The disease in clinical settings is defined at the end stage of this process and demonstrates all the above symptoms. But the remarkable finding is that impaired glucose potentiation and second-phase defect are compensated by hyperglycemia, at the intermediate stages of final beta-cell failure such that even non-glucose secretagogues stimulate release of insulin [193-195]. T2D has been shown to develop mainly in subjects that are unable to sustain the beta cell compensatory response. Longitudinal studies in T2D, which involved a continued observation of variables in a set of subjects over time, indicated a rise in levels of insulin in normoglycemic and prediabetic phases that maintain normal blood glucose levels inspite of insulin resistance. It was later followed by a decline in insulin levels when fasting glycemia surpassed the upper limit of basal [5.5 mM] due to beta-cell failure.

Hyperglycemia is one of the major determinants in the development of diabetic complications. Many biochemical and molecular events of high concentration of blood glucose underlie the pathogenesis of complications but the exact mechanism is still

unclear. With significant advances in tight glucose control, by blood-glucose monitoring and therapeutics, debilitating complications still remain the same. It is found that as early as the '80s, the concept of a "metabolic memory" has been prevalent, that is diabetic complications like vascular stress and retinopathy persist even after glucose normalization. This memory phenomenon has been supported by experimental evidence seen in both diabetic animals and isolated cells exposed to high glucose, followed by normalization to physiological concentrations of glucose and in early 2000, in results from clinical trials [196]. Furthermore, researchers have implicated nonenzymatic glycation of cellular proteins along with excessive ROS/RNS maintain the stress signaling even after tight glucose control thus contributing to metabolic memory.

Various evidences implicate a role for epigenetic modulation as a factor contributing to maintenance of stress levels even after normalization of blood glucose levels [197-199]. The idea that early glycemic environment is remembered in target organs of diabetic complications [i.e. eye, heart, kidney and extremities] [200-202], I wanted to study whether islet beta cells maintained the same metabolic memory. It is clearly evident that both Arf6 and Rac1 are essential modulatory factors mediating GSIS from pancreatic beta cells. Evidences also indicate a disruption in insulin secretion in T2DM. Thus, I was curious to investigate the above phenomenon with respect to signaling events associated with insulin secretion, we incubated INS 832/13 cells with either 5 or 30 mM glucose for 24 hr followed by normalization and stimulating with 20 mM glucose for time periods appropriate to activation of Arf6 and Rac1. When compared to control incubated in 11.1 mM, the activation of Arf6 and Rac1 was reduced in cells exposed to glucotoxic conditions [Figure 5-1 & 5-2]. Under physiological

glucose concentrations [20 mM], both Arf6 and Rac1 demonstrated an increase in fold activation which was lost under glucotoxic conditions. But there was no apparent change in total protein expression of Arf6 or Rac1. The probable dysfunction of the proteins might be due to a possibility of some chemical modification of the proteins like glycation which has not been reported yet. This is a clear indication that the beta cell is also conditioned to memory process which seems to be irreversible.

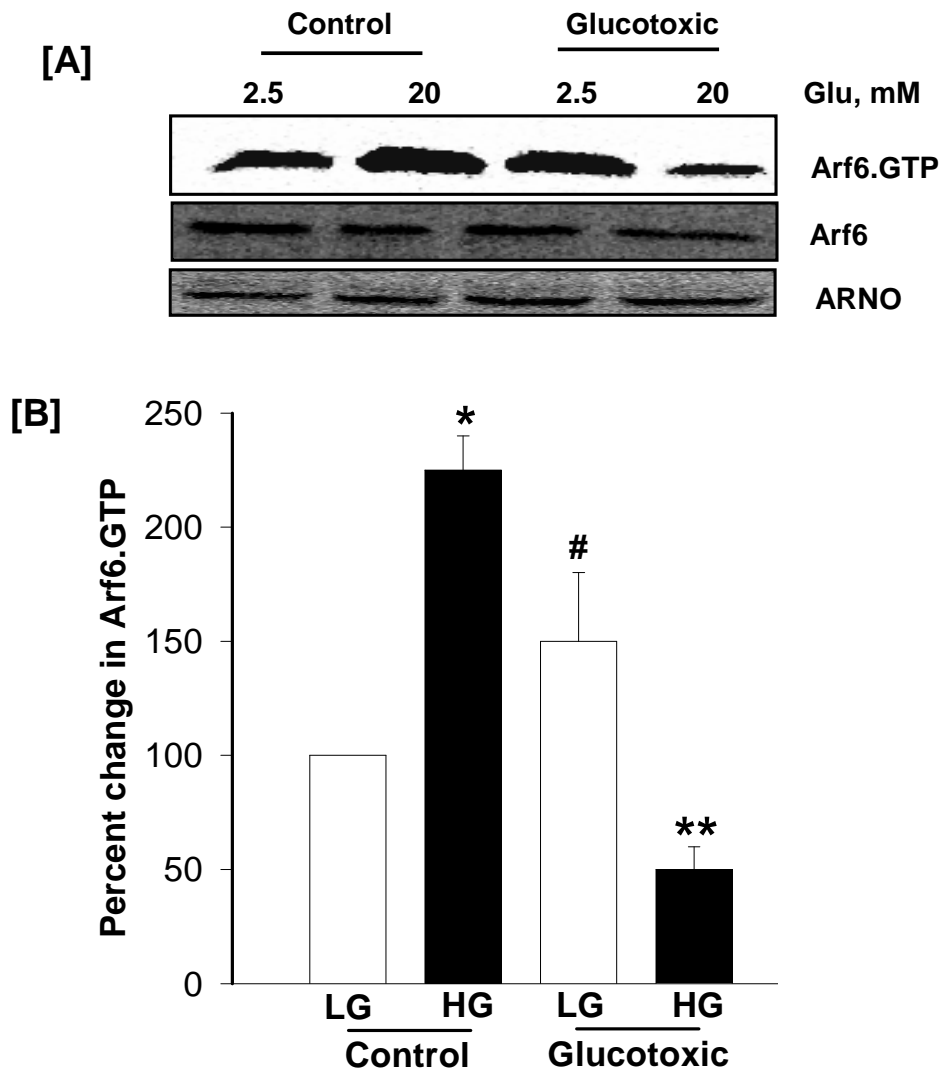


Figure 5-1: Effect of glucotoxicity on Arf6 activation to stimulatory concentrations of glucose

Panel A: INS 832/13 cells were exposed to either 11.1 (control) or 5 or 30 mM glucose for 24 hr. Thereafter, both control and glucotoxic cells were starved overnight in low serum-low glucose. Cells were then stimulated with either 2.5 or 20 mM glucose in KRB for 3 min. Lysates were collected and used for the detection of Arf6.GTP. Total Arf6 was used a loading control. Expression of ARNO under these conditions was also analyzed.

Panel B: Amount of activated Arf6 was quantitated densitometrically and expressed as percent change over control. The values obtained are mean \pm SEM three independent experiments. * and *** indicates $p < 0.05$ vs. control 2.5 mM glucose, ** vs. control 20 mM glucose.

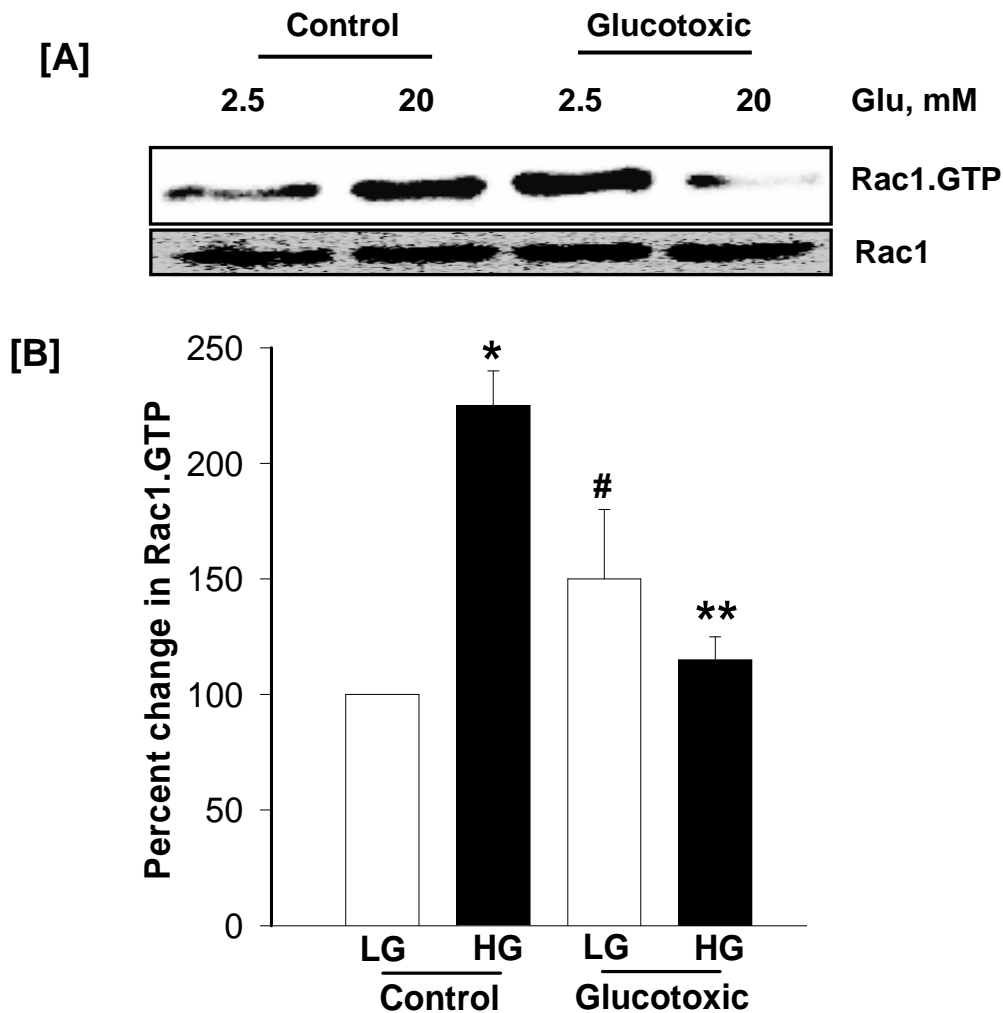


Figure 5-2: Effect of glucotoxicity on Rac1 activation to stimulatory concentrations of glucose

Panel A: INS 832/13 cells were exposed to either 11.1 (control) or 5 or 30 mM glucose for 24 hr. Thereafter, both control and glucotoxic cells were starved overnight in low serum-low glucose. Cells were then stimulated with either 2.5 or 20 mM glucose in KRB for 20 min. Lysates were collected and used for the detection of Rac1.GTP. Total Rac1 was used as a loading control.

Panel B: Amount of activated Rac1 was quantitated densitometrically and expressed as percent change over control. The values obtained are mean \pm SEM four independent experiments and three out of four experiments followed a similar trend as shown. * and *** indicates $p < 0.05$ vs. control 2.5 mM glucose, ** vs. control 20 mM glucose.

Table 5-1: Characteristics and islet histopathology of various rodent models of TTDM [203].

Model (14+ weeks)	Plasma glucose		Typical fasting plasma insulin (pM)	Typical fasting plasma triglyceride (mM)	Islet pathology
	12+ hours fasting, 0 min into OGTT (mM)	60 min into OGTT (mM)			
Normoglycemic Wistar rats	4 ¹	5 ¹	84 ¹	0.38 ²	Well delineated islets with central β cells surrounded by α - and δ -cell mantle
ZF rats	5 ³	13 ³	1850 ³	55.9 ⁴	Hypertrophied islets with β -cell proliferation. Reduced insulin granulation. Some degenerate islets.
ZDF rats	11 ⁵	22 ⁵	241 ⁴	17.3 ⁴	Most islets are poorly delineated, showing extensive fibrosis and a severe reduction in β cells. Remaining β cells show reduced insulin granulation.
Goto-Kakizaki rats	7 ¹	16 ¹	124 ¹	59 ²	"Starfish shaped" islets where previously hypertrophied islets have atrophied and fibrotic projections remain.
<i>P. obesus</i>	25.1 ⁶ (nonfasted)		5300 ⁶ (nonfasted)	26.9 ⁵ (nonfasted)	β -Cell vacuolation and reduced insulin granulation.
HIP rats	17 ^z	—	100 ^z	—	Progressive amyloidosis with a high frequency of β -cell apoptosis

Model (14+ weeks)	Plasma glucose		Typical fasting plasma insulin (pM)	Typical fasting plasma triglyceride (mM)	Islet pathology
	12+ hours fasting, 0 min into OGTT (mM)	60 min into OGTT (mM)			
STZ with high-fat diet diabetes in Rats	6.9 ^g	22 ^g	186 ^g	7.5 ^g	Irregular islets, β -cell vacuolation and apoptosis. Reduced insulin granulation.
Partially Pancreatectomized Rats	5.5 ^g	22 ^g	84 ^g	—	Islet hypertrophy with some fibrosis and occasional inflammatory infiltration of islets.

- Note: TTDM, type 2 diabetes mellitus; OGTT, oral glucose tolerance test; ZF, Zucker fatty; ZDF, Zucker diabetic fatty.

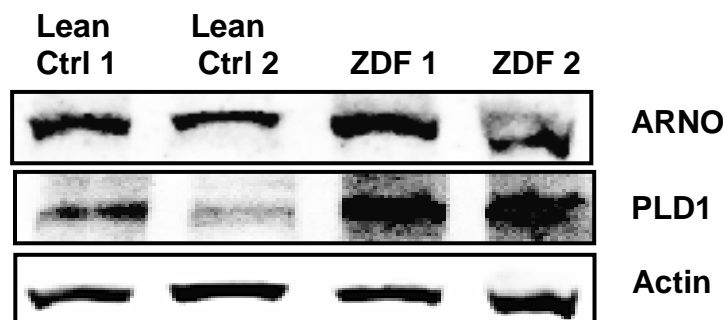
Table 1 elucidates the various rodent models available in market that were generated by spontaneously mutated genes and induced to develop T2DM. The ZDF rat is a rodent model of NIDDM which shows a predictable progression of the disease from prediabetic to diabetic state. The ZDF rat carries a mutation in the gene coding leptin receptor (*fa/fa*) in addition to a mutation that leads to spontaneous hyperglycemia at 7 to 10 weeks of age in males [204]. Female rats tend to become hyperglycemic only when fed a diabetogenic diet, with this additional mutation still unidentified [205]. Rats expressing *fa/-* genotype are lean and do not develop hyperglycemia. Leptin is a hormone that controls feeding behavior and a mutation in the leptin receptor results in obesity.

I used the male ZDF model to assess some of the key signaling events in the regulation of insulin secretion and thus may provide an understanding in the pathogenesis of diabetes characterized by defective insulin secretion. Changes in protein expression were also evident in islets from diabetic ZDF rats compared with age-matched lean control animals. Following the onset of diabetes, insulin secretion is severely reduced. The islets appear hypertrophic and show degenerative changes. By

14 weeks, the islet faces severe degenerative changes leading to defective insulin secretion, as the islet has to meet an increased secretory demand added to insulin resistance, in response to sustained, raised glucose levels. And there is also a loss in reserve pool due to inadequate repletion [206, 207].

Expression profile of membrane trafficking proteins under diabetic conditions

In another study, we quantitated protein expression of Arf6, ARNO, PLD1 and actin in islets of both ZDF and diabetic human [Figure 5-3]. The expression levels of the indicated proteins were significantly increased compared to the normal phenotype. A study from Rutter's laboratory published evidence for a functional and gene profiling analysis of proteins that might be responsible for defective GSIS from ZDF rat islets [208]. Microarray analyses of several "glucose-sensing" and exocytotic genes were significantly altered in ZDF islets. They also saw marked changes in expression of proteins involved in regulation of vesicle traffic and exocytosis. Another set of key players, notably the actin remodelers like gelsolin, actin and scinderin were upregulated in ZDF islets [208].



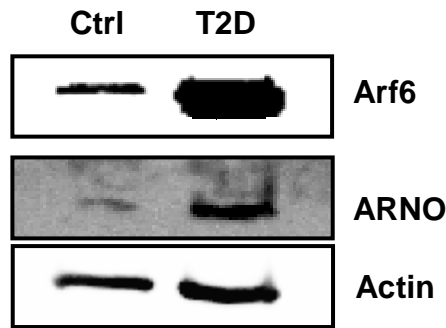
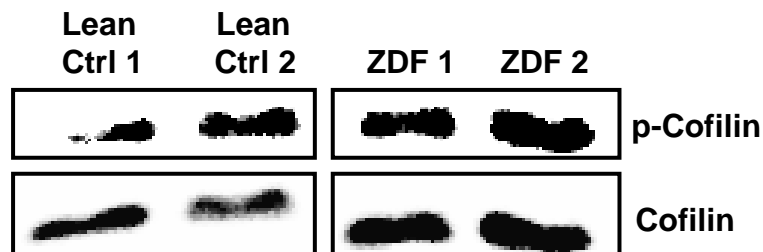


Figure 5-3: Increased expression of membrane trafficking proteins in ZDF rat and T2D islets compared to respective control

Islets from ZLC or ZDF rats were lysed using RIPA buffer. Equal amount of lysate proteins were resolved by SDS-PAGE. Expression of phosphorylated and total cofilin was determined by Western blotting. A representative blot is provided in Figure 5-3 A and B for ZDF and TDM islets.

In line with these studies and our own findings on cofilin, a marker used for actin remodeling, we investigated the activation of cofilin in ZDF and diabetic human islets. Apart from a significant increase in expression levels of both actin and cofilin under diabetic conditions, the phosphorylation of cofilin was upregulated [Figure 5-4]. This indicated that actin remodeling might be impaired under beta-cell dysfunction contributing to impaired insulin secretion.



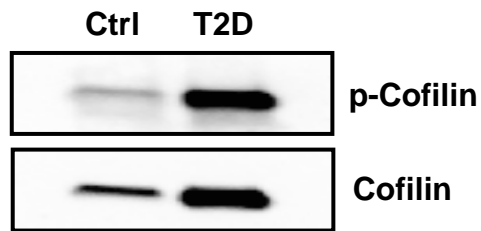


Figure 5-4: Increased expression and phosphorylation of cofilin in ZDF rat and T2D islets compared to respective control

Islets from ZLC or ZDF rats were lysed using RIPA buffer. Equal amount of lysate proteins were resolved by SDS-PAGE. Expression of phosphorylated and total cofilin was determined by Western blotting. A representative blot is provided in Figure 5-4 A and B for ZDF and TDM islets.

Thus, emerging evidence that hyperglycemia leaves an imprint to the development of future complications has potential therapeutic applications. All these evidences suggest that 'glucotoxic memory' persists even after good glycemc control is achieved. This evidently calls for early therapeutic treatment of hyperglycemia from diabetes, a strategy that may benefit T1DM with concerns of early insulin use in T2DM subjects.

Chapter 6

DISCUSSION

Metabolic coupling factors generated by glucose and hormones play a regulatory role in insulin exocytosis. A rise in intracellular calcium followed by an increase in ATP/ADP ratio is an important trigger for exocytosis. Ca^{2+} mediated action is mediated via calcium sensors like synaptotagmins and exocytosis takes place at proximity to voltage-dependent Ca^{2+} channels. SNARE complex formation aided by its chaperones NSF and CSP is essential for exocytosis. Insulin exocytosis is also sensitive to GTP and major exploratory work has been done to understand the organization of its reputed targets, small monomeric and heterotrimeric G-proteins. In this study, I have described a role for Arf6/ARNO in stimulated secretion of insulin from pancreatic beta cells. As stated in the Introduction, small G-protein Arf6 and its exchange factor ARNO play a key regulatory in stimulated-insulin secretion. This cascade involves Rho family proteins, lipid-modifying enzymes and actin-severing protein.

One of the main objectives of my doctoral work was to elucidate a role for Arf6/ARNO in GSIS and identify putative effectors for the pair in the cascade leading to insulin secretion. These studies were based on the overall hypothesis that ARNO-mediated activation of Arf6 is necessary to activate downstream effectors in the signaling cascade that primes insulin granules and remodels actin cytoskeleton. To address this, I have used clonal pancreatic beta cells [INS 832/13 cells] and rat islets. I have quantitated several indices both biochemical and molecular biological aspects to

validate the above hypothesis. The use of pharmacological tools aided in validating the hypothesis.

In over the years, Arf family of G-proteins has been traditionally portrayed to play key regulatory roles in membrane trafficking [95]. From among a family of six, only Arf1 and Arf6 are well-explored and have been implicated to regulate several cellular events including cell motility, vesicular transport, and cortical actin rearrangements [97, 127, 143]. Arf1, is best characterized as a recruiter from cytosol to Golgi complex. At the Golgi it mediates binding of coat proteins and adaptins to Golgi membranes. But recent work has shown Arf6 to play distinct functions in eukaryotes. In contrast to Arf1 which is restricted to Golgi, Arf6 is majorly associated with secretory granules and plasma membrane. Apart from playing a role in granule translocation, Arf6 also serves to modulate actin cytoskeleton. In view of early investigations to test functions of Arf family in regulated-exocytosis, Arf6 is the most-famed participant. Our localization studies remain on par with findings described in chromaffin cell, a model neuroendocrine cell for regulated-secretion [120].

On examining localization data of Arf6, it was predominantly associated with membrane fraction and on further separation into various cellular compartments, Arf6 was majorly associated with secretory granules. In chromaffin cells, secretory granule-bound Arf6 was found to associate with plasma membrane on stimulation. And this substantiates the involvement of the protein in intracellular vesicle trafficking.

Key to our understanding of Arf6 function in insulin exocytosis will be regulatory factors that activate the protein. One important regulatory factor for Arf6 is the GEF,

ARNO. In our study we report the presence of ARNO, predominantly localized in the cytosol. To show that they are hydrophilic proteins we performed phase partitioning experiment with Triton-X 114 as integral membrane proteins separate out in the hydrophobic detergent phase. In the present study we demonstrated that ARNO might subserve the function of a GEF for Arf6 in the islet β -cell. There are enough reports for the involvement of this small G-protein in regulated secretion in chromaffin cells as well as in MIN6, another clonal pancreatic beta cell.

To confirm their involvement in GSIS, dominant negative mutants [inactive conformations] of these proteins were overexpressed and its effect on GSIS was quantitated. To add more weightage to the above data, endogenous expression of both Arf6/ARNO was silenced and GSIS was quantitated. Data accrued from insulin secretion studies, indicated a positive modulatory for Arf6/ARNO in GSIS. We further wanted to test if Arf6/ARNO modulated K^+ -stimulated insulin secretion. We transfected cells with siRNA-ARNO and found that K^+ -stimulated insulin secretion was reduced by ~60%. Though glucose is a major insulin secretagogue, there are other nutrients like amino acids which potentiate GSIS. A detailed study on the dose- and glucose-dependent effects of amino acids on insulin secretion made us to question if Arf6/ARNO played a role in amino-acid potentiated GSIS. We tested this hypothesis using siRNAs of both Arf6 and ARNO and found that they attenuated L-arginine potentiated GSIS. All the above evidence corroborated the involvement of Arf6/ARNO in regulated-insulin secretion.

The next step in the project was only apt to determine the time-course dependence of glucose-stimulated activation of Arf6. With recent advances in molecular

biology, we were able to study the time kinetics of Arf6 activation in pancreatic beta cells. We employed GST-GGA3 beads in a pull-down assay, which essentially recognizes only active form of Arf6 [Arf6-GTP]. Glucose stimulation at multiple time-points starting from 0-30 min indicated a constitutive activation of Arf6. Surprisingly, K^+ stimulation also activated the protein unlike other G-proteins.

We also demonstrated that ARNO is one of the regulatory factors for glucose-mediated activation of Arf6 using pharmacological and gene-silencing tools.

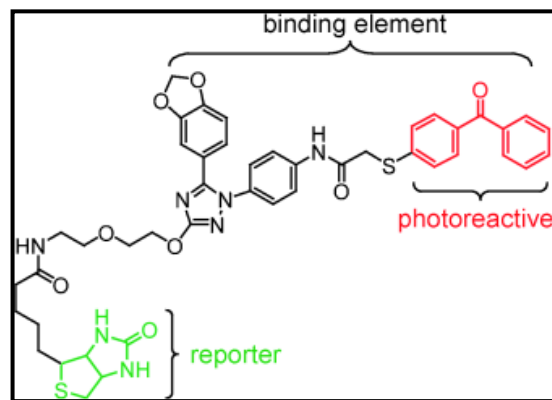


Figure 6-1. Structural representation of secinH3 with binding element that binds to cytohesin sec-7 domain [134].

Until very recently, only one small-molecule inhibitor, brefeldin A, an inhibitor of Arf GDP/GTP exchange, was available. But it was found brefeldin A was not sensitive towards cytohesins. The availability of secinH3, a novel small-molecule inhibitor of cytohesins, made it easy to dissect the role of ARNO. Above is the chemical structure of secinH3 designed by Famulok group [134]. SecinH3 gets its name for its high affinity to the functional domain of cytohesins, sec7. When used in mice, flies and human liver cells, it impaired Arf6/ARNO signaling pathway. In their paper describing synthesis and application for secinH3, they tested the selectivity of the inhibitor and found it to be

highly selective for cytohesin family with minimal or no effects against other Arf family of GEFs or other GTPases [134]. Hafner utilized the compound to study the role of Arf6/ARNO in insulin signaling pathway. It further substantiated the potential of secinH3 to inhibit ARNO-mediated activation of Arf6 [107].

We found that GTP-bound Arf6 upon glucose stimulation was significantly reduced when functional role of ARNO was compromised.

Salient features of our study are:

- I. Arf6/ARNO is expressed in clonal β -cells, rodent islets and human islets
- II. Overexpression of inactive mutants of ARNO or Arf6 or siRNAs of Arf6 or ARNO reduces insulin secretion elicited by glucose, arginine and KCl in insulin-secreting cells
- III. SecinH3, a selective inhibitor of ARNO/Arf6 signaling pathway, also inhibits GSIS in INS 832/13 cells and rodent islets
- IV. Insulinotropic concentration of glucose/KCl stimulates Arf6 activation
- V. Glucose-induced Arf6 activation is inhibited by secinH3 or siRNA-ARNO, suggesting a critical involvement of ARNO/Arf6 in insulin secretion and
- VI. Glucose promotes association between ARNO and Arf6 as evidenced by co-immunoprecipitation and confocal microscopic studies.

These findings provide the first evidence to implicate novel roles for Arf6/ARNO in insulin secretion.

2. What are the potential effectors of Arf6/ARNO signaling pathway?

Over decades, research has progressed to understand signal transduction involved with insulin secretion. And over the course, new proteins have been discovered and many pathways have been built. So far, we had worked on establishing a role for Arf6/ARNO in regulating stimulated-insulin secretion. Now we were interested in identifying downstream effectors in this signaling cascade. We studied the effects of compromised Arf6/ARNO on the activity of putative targets. Our first target was nm23H1, a tumor suppressor, whose function is to provide a localized supply of GTP. But its role as a regulator of heterotrimeric GTP-binding proteins gained much importance [209]. In 1979, Kimura and Nagata showed that NDP kinase transphosphatidylated GDP to GTP thus aiding in hormonal activation of adenylyl cyclase [210]. The GTP-supply of nm23H1 operated by NDP kinase serves to maintain maximal activation of G-protein coupled signaling. The idea that nm23H1 could act as a localized GEF for Arf6 was interesting. In support of this, Randazzo PA examined potential substrates for NDP kinase activity. They examined the nucleotide exchange activity of NDP kinase on purified Arf [211]. So I investigated if knockdown of Nm23H1 affected the status of GTP-bound Arf6. Data obtained from this study indicated a positive role for Nm23H1 in maintaining active form of Arf6.

Next in my line of targets were the famed Rho class of G-proteins, Cdc42 and Rac1. Several laboratories have contributed the current understanding of Rho GTPases in insulin secretion. In particular, Cdc42 and Rac1 have been characterized in insulin-secreting cells and their functional importances have been established. Time kinetics for glucose-induced activation of both proteins has been demonstrated. It is a well-established fact that both Cdc42/Rac1 work together to cause insulin secretion upon

glucose stimulation. A study from Thurmond's laboratory serves as evidence to suggest involvement of active Cdc42 in cytoskeletal rearrangement [139]. Using gene-silencing methodology, they showed glucose-mediated sequential activation of Cdc42 to PAK1 to Rac1 in insulin secretion. Using *Clostridium difficile* toxins, Kowluru et al., demonstrated a potential role for Rac1 in GSIS [212]. Further on, several studies were documented to define essential roles for Rac1. Both Cdc42 and Rac1 may be essential for regulating actin dynamics in phase of secretion. There are many regulatory factors like GEFs and GAPs. But what was the upstream signal that was required to activate these proteins? Studies done in primary human fibroblast cells clearly indicated an enhancing role of Arf6 on Rac1 in actin remodeling [213]. Soon, several other studies followed suit to indicate a regulatory role for Arf6 on activation of Rac1. In HeLa and CHO cells, Arf6 influenced the redistribution of Rac1 from endosomes to cell periphery [213, 214]. An Arf6-dependent was also shown to be essential for polarized recruitment and activation of Cdc42 in astrocytes [215]. Another group demonstrated a role for active Arf1 to bind GAP of Cdc42 thus enabling it be activated by its GEF [216]. All the above evidences urged us to investigate if Arf6 had a regulatory hold on Cdc42/Rac1. Cells treated with secinH3 or transfected with siRNA-ARNO showed a reduction in GTP-bound forms of Cdc42/Rac1. These data indicated ARNO-activated Arf6 is an important upstream signal for the activation of Cdc42/Rac1.

Arf6/ARNO has been shown to activate several lipid-modifying enzymes like PI4P-5K and PLD to generate fusogenic lipids. The generated lipids aid in activating downstream effectors or in preparing the plasma membrane for fusion process [217, 218].

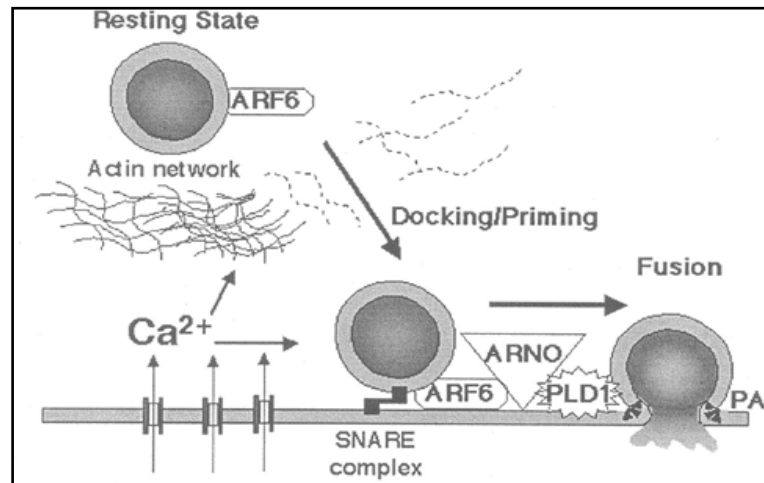


Figure 6-2: An illustration to represent Arf6/ARNO-regulated membrane trafficking and actin rearrangement for secretion of vesicles in chromaffin cells [219].

Earlier observations also demonstrated potential involvement of PLD activation in the signaling mechanisms leading to GSIS [220, 221]. More recent findings by Ma and coworkers further implicated Arf6 in glucose-induced PLD activation in MIN6N8 cells, which suggested binding of Arf6 to PLD. Furthermore, brefeldin A, a known inhibitor of Arf6, decreased glucose-induced PLD activity and insulin secretion [147]. So when I investigated the effect of secinH3 on PLD activity, we found its activity to be reduced.

A growing body of evidence pointed towards glucose-induced activation of ERK1/2 mediated via G-protein activation. Evidence from my own lab established a farnesylation-dependent signaling that leads to ERK1/2 activation in insulin secreting cells. And also several reports suggesting an Arf6-regulated activation of ERK1/2 [144, 222] led us to investigate effect of secinH3 on activation of ERK1/2. SecinH3 was found

to inhibit the phosphorylation of ERK1/2 via glucose stimulation. This placed ERK1/2 as a downstream effector of ARNO/Arf6 in the signaling cascade leading to insulin secretion. Whether Arf6/ARNO is a direct mediator of ERK1/2 activation needs to be investigated.

Investigation of prenylation roles in Nox activation suggested a role for Rac1-GTP in generation of ROS. And to test if Arf6/ARNO played a role in Rac1-mediated activation of Nox and generation of ROS, we used secinH3 and quantitated ROS generation. My findings provided in this document suggest a clear role for Arf6/ARNO mediated induction of ROS generation in both clonal pancreatic beta cells and rat islets. Several published reports indicated a role for small G-proteins, lipid-modifying enzymes, certain kinases and also ROS to bring about cytoskeletal remodeling in a stimulated cell.

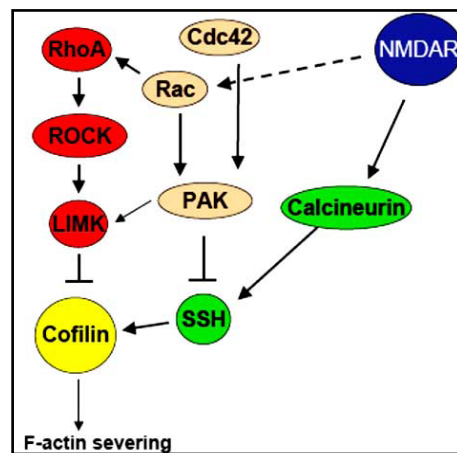


Figure 6-3: An illustration to represent regulatory factors that govern the activity of cofilin [223].

Arf6/ARNO by itself has been reported to act as actin remodeling agents. It is without doubt that breakdown of actin barrier is essential for insulin release. One among

the several actin-binding proteins, cofilin, was investigated in this aspect. Cofilin severs actin filaments by binding to F-actin and stabilizes the twisted filament [224].

Cofilin activity is regulated by dephosphorylation/phosphorylation at Ser3 [225]. Phosphorylation of cofilin does not confer any changes in protein conformation rather generates a charge repulsion that prohibits it from binding actin [226].

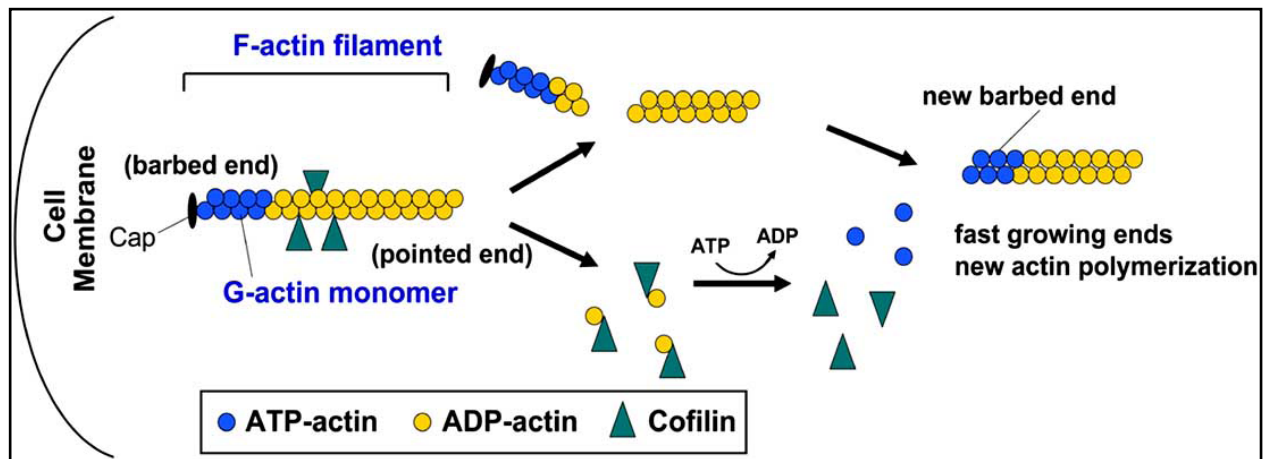


Figure 6-3: An illustration to represent activity of cofilin as an actin-severing agent, promoting F-actin remodeling [223].

Phosphorylation of cofilin is regulated by LIMK, which lie downstream to Rho-GTPases as seen in the illustration. Dephosphorylation is controlled by a phosphatase, specific to cofilin, slingshot. Glucose-induced dephosphorylation of cofilin activated the protein to sever actin filaments. Treatment of INS 832/13 cells with secinH3 attenuated dephosphorylation of cofilin via glucose.

Having examined the exocytotic partners in insulin release, we tested if Arf6/ARNO played any role in endocytotic pathway as implicated in other cell types. In several cases, literature has demonstrated a large GTPase, dynamin, to be a major mediator of endocytosis. Recurring evidence suggested active dynamin to aid in

recycling of secretory vesicles after insulin release. A report published in Nature depicted an Arf6-regulated role of dynamin in endocytosis. It is likely that active Arf6 regulated phosphorylation/dephosphorylation cycle of dynamin. This was confirmed with the use of secinH3 which inhibited dephosphorylation of dynamin.

Several earlier studies have implicated activation of small G-proteins (e.g., Arf6, Cdc42 and Rac1) in physiological insulin secretion. Such conclusions were drawn from studies involving the use of Clostridial toxins, dominant negative mutants, siRNAs and inhibitors of post-translational modifications, including prenylation, carboxymethylation and palmitoylation [140]. To the best of our knowledge, the current study provides the first evidence to implicate carboxymethylation of Rac1 in the signaling cascade leading to glucose-induced ROS generation and insulin secretion. We have presented supporting evidence via two distinct approaches, namely siRNA-mediated knockdown or selective pharmacological inhibition of ICMT, which mediates the carboxymethylation of these signaling proteins.

At least two distinct carboxymethyl transferases have been identified in insulin secreting cells. The first one is involved in methylating the carboxy terminal leucine (Leu-309) of the catalytic subunit of protein phosphatase 2A; such a signaling step has been implicated in subunit interaction and catalytic activation of the enzyme [227]. The second enzyme, which is the focus of the current study, is the ICMT. In a previous study, Li and associates characterized the ICMT in insulin-secreting cells and normal rat islets [182]. Such an activity was monitored by quantitating the degree of methylation of AFC by the islet ICMT in the presence of [3H] S-adenosylmethionine as the methyl donor. Subcellular fraction assays revealed that this enzyme activity is enriched in the

endoplasmic reticulum [182] Along these lines, using the pharmacological approaches, we have demonstrated that glucose promotes the carboxymethylation of Cdc42, another small G-protein involved in cytoskeletal remodeling and glucose-stimulated insulin secretion [75]. It was also demonstrated that the G γ -subunits also undergo carboxymethylation in a glucose-sensitive manner in clonal β -cells, normal rat islets and human islets [77]. Not much has been reported since then with regard to potential functional consequences of carboxymethylation in islet function primarily due to lack of experimental tools (e.g., siRNA) to selectively deplete the expression of ICMT in isolated β -cells. Indeed, data from the current investigation further reinforce our original hypothesis that in addition to prenylation, carboxymethylation of specific G-proteins (e.g., Rac1) plays regulatory roles in physiological insulin secretion. Such regulatory effects may, in part, be due to the ability of methylated Rac1 to increase the activation of Nox and associated generation of reactive oxygen species.

Data accrued in the current studies implicate carboxymethylation as one of the requisite signaling steps for glucose-induced activation by Rac1 in a stimulated β -cell. Moreover, the carboxymethylation of Rac1 appears to be necessary for glucose-induced Nox activation and generation of reactive oxygen species. In this context, using reconstituted systems and the C-terminal Rac1 peptides, Kreck and coworkers have provided experimental support to implicate participatory roles for Rac1 in cell-free activation and assembly of NADPH-oxidase [228]. Compatible with these findings are our recent data to implicate inhibition of glucose- or mitochondrial-fuel-induced Nox activation and generation of reactive oxygen species in INS 832/13 cells and normal rat islets by inhibitors of protein prenylation. These studies thus provided evidence for

requisite roles for prenylation in the functional regulation of Nox in the islet β -cell [158]. Data from the current investigation indicate that in addition to prenylation, the carboxymethylation of specific G-proteins may be necessary for optimal regulation of Nox by glucose. More importantly, my current findings also suggest that carboxymethylation is necessary for glucose-induced activation of Rac1, since pretreatment of isolated β -cells with AFC or selective depletion of ICMT by siRNA markedly attenuated glucose-induced Rac1 activation. These findings are in agreement with recent findings of Cushman and Casey demonstrating inhibition of EGF-induced Rho A and Rac1 activation by cysmethynil, a selective inhibitor of ICMT, in MDA-MD-231 cells [229]. Together, based on the above discussion it is concluded that both prenylation and carboxymethylation of Rac1 are necessary for glucose-induced Nox-mediated ROS generation and insulin secretion. It is important to note that palmitoylation of cysteine residues upstream to prenylated and carboxylmethylated Rac1 may not be involved in this signaling cascade at least based on recent studies from Roberts and associates who reported no known consensus palmitoylation motifs for Rac1 [230] although this remains to be verified experimentally in the islet β -cell.

Emerging evidence appears to implicate a significant contributory role for Nox in the generation of oxidative stress and the onset of mitochondrial dysfunction in multiple cell types, including the islet β -cell. For example, it has been shown that chronic exposure of isolated β -cells to high concentrations of saturated fatty acids (e.g., palmitate; lipotoxicity), glucose (i.e., glucotoxicity) or both (i.e., glucolipotoxicity) or a mixture of cytokines (e.g., IL-1 β , TNF α and IFN γ) culminates in increased oxidative stress, mitochondrial dysfunction and apoptosis in these cells [231-233]. Inhibition of

protein prenylation of Rac1 by pharmacological approaches (e.g., GGTI-2147) or Rac1 activation by Tiam1, a known guanine nucleotide exchange factor for Rac1 (using NSC23766) markedly attenuated metabolic dysfunction of the β -cell [232, 233]. Along these lines, data described herein suggest a significant increase in the expression of ICMT under glucolipotoxic conditions. It remains to be verified if such an increase in the expression translates into increased ICMT activity. Nonetheless, it may be likely that use of selective inhibitors of carboxymethylation might prove to be valuable in preventing oxidative stress induced under the duress of glucolipotoxicity and/or cytokines. These are being studied in our laboratory currently. Based on the data accrued in the current studies we conclude that ICMT regulates glucose-induced Rac1 activation, generation of reactive oxygen species and insulin secretion in pancreatic β -cells.

Synchronized with obesity epidemic, the incidence of T2DM is also increasing at an alarming rate. T2DM results from a failure of endocrine pancreas to secrete sufficient insulin to meet the metabolic demands due to acquired dysfunction of beta-cells along with decreasing beta-cell mass. Whether or not insulin secretory dysfunction is a cause for the dysfunction, beta-cell loss seems to play an important role in the pathogenesis of T2DM. As seen in obese individuals who do not develop diabetes exhibit an increase in beta-cell mass and a compensatory mechanism for the metabolic load. This very same beta-cell adaptation seems to have failed in obese individuals who developed T2DM. Several defects in insulin secretion are well documented in human T2DM; however the role in the pathogenesis and the possible clinical relevance of high frequency rapid pulsatile insulin secretion is still unclear. Chronic elevation of FFA and glucose has

been implicated to cause beta-cell dysfunction. Whereas acute exposure of beta-cells to FFA has been shown to stimulate insulin secretion, chronic exposure to FFA inhibits insulin secretion. Chronic elevation of blood glucose concentration impairs beta-cell function largely due to activation of oxidative stress and increased generation of ROS. Both conditions of gluco- and lipotoxicity attenuate GSIS in addition to downregulation of diverse groups of genes that are responsible for insulin biosynthesis and processing.

In addition to the above factors that have been established in the progression to T2DM, several observations have suggested significant abnormalities in various intracellular signals that contribute to defective insulin secretion in G-protein knockout animal models. One example of this is the Rac1-null mice [β Rac1^{-/-} mice] where an impaired glucose tolerance and hypoinsulinemia was noticed [234]. This observation was in concordance with the in vitro studies in cultured beta-cells where GSIS was attenuated when function of Rac1 was compromised. In addition to its positive modulatory roles, Rac1 has been observed to negatively modulate metabolic dysfunction of beta-cells by participating in the generation of ROS. Numerous observations from our laboratory have demonstrated a role for Tiam1/Rac1/Nox signaling in beta-cell dysregulation under the duress of glucolipotoxicity. Both the activity and expression of Rac1 were increased in ZDF islets and in human islets exposed to glucotoxicity.

Using the animal model Goto-Kakizaki [GK] rat, Metz et al reported that insulin secretory defects were due to a defect late in the signal transduction leading to exocytosis and not due to levels of insulin found in the cell. And the secretory defect was corrected by the use of Mastoparan [Mas] proved that a G-protein involved in

exocytosis was responsible for the abnormality [235]. Another group used galparan to stimulate insulin secretion from islets derived from GK rat. They found galparan to stimulate secretion of insulin at a distal in stimulus-secretion [236]. Systematic approach to identify more G-proteins that are compromised during diabetic conditions need to be identified.

Pancreatic beta-cell failure, starts early and progresses to T2DM, is a phenomenon that determines the progression from impaired glucose tolerance to overt diabetes. It has been demonstrated that a dysfunction in secretory process in ZDF islets is a result of altered expression of wide variety of genes. It includes both key factors involved in glucose-sensing and vesicle trafficking events. Data from G-protein knockout animal models definitely are promising and encourage the idea that G-protein signaling play an important role in insulin secretion and maintain optimal beta-cell mass. Additionally, evaluation of post-translational modifications and their enzymes need to be studied on the same importance as their substrate, small G-proteins. Posttranslational modifications have been an indispensable feature to for translocation or for membrane targeting. Roberts et al. provided sufficient evidence in favor of posttranslational modifications such as CAAX motif and methyltransferase [237]. Future studies, involving a systematic assessment of the above targets under diabetic conditions will be necessary to delineate their contribution to the secretory phenotype.

Chapter 7

Conclusions and Future directions

On a concluding note, the vast expanse of research to understand the regulatory roles of small G-proteins has seen tremendous progress since early '90s. A review of the presented data and published evidence from multiple laboratories indicates a significant role for Arf6 in regulating GSIS from the pancreatic beta-cell. This is confirmed in a wide variety of insulin-secreting cells including clonal beta-cells, rat, mouse and human islets. As an added step, the regulatory factor GEF-ARNO for Arf6, has been implicated in modulating its activity upon stimulation. Herein, based on all the above evidences, I built a working model **[Figure 7-1]**, for ARNO/Arf6 signaling cascade in the events leading insulin secretion with the probable key molecules [PLD, ERK1/2, Rac1, Nox, dynamin-1 and cofilin]. Apart from the regulatory factors for the small G-proteins, I have determined posttranslational modification i.e., carboxymethylation, on the activity of Rac1 and its role in insulin release and generation of ROS.

Future studies in light of the presented data will involve:

1. Identifying whether PLD is upstream/downstream to ERK1/2
2. What are the possible posttranslational modifications Arf6 undergoes in the event of insulin secretion.
3. Does PLD regulate activity of dynamin-1?
4. Determine the molecular events after exocytosis and how nm23H1/Arf6/dynamin-1 regulate the recycling of vesicles after secretion.

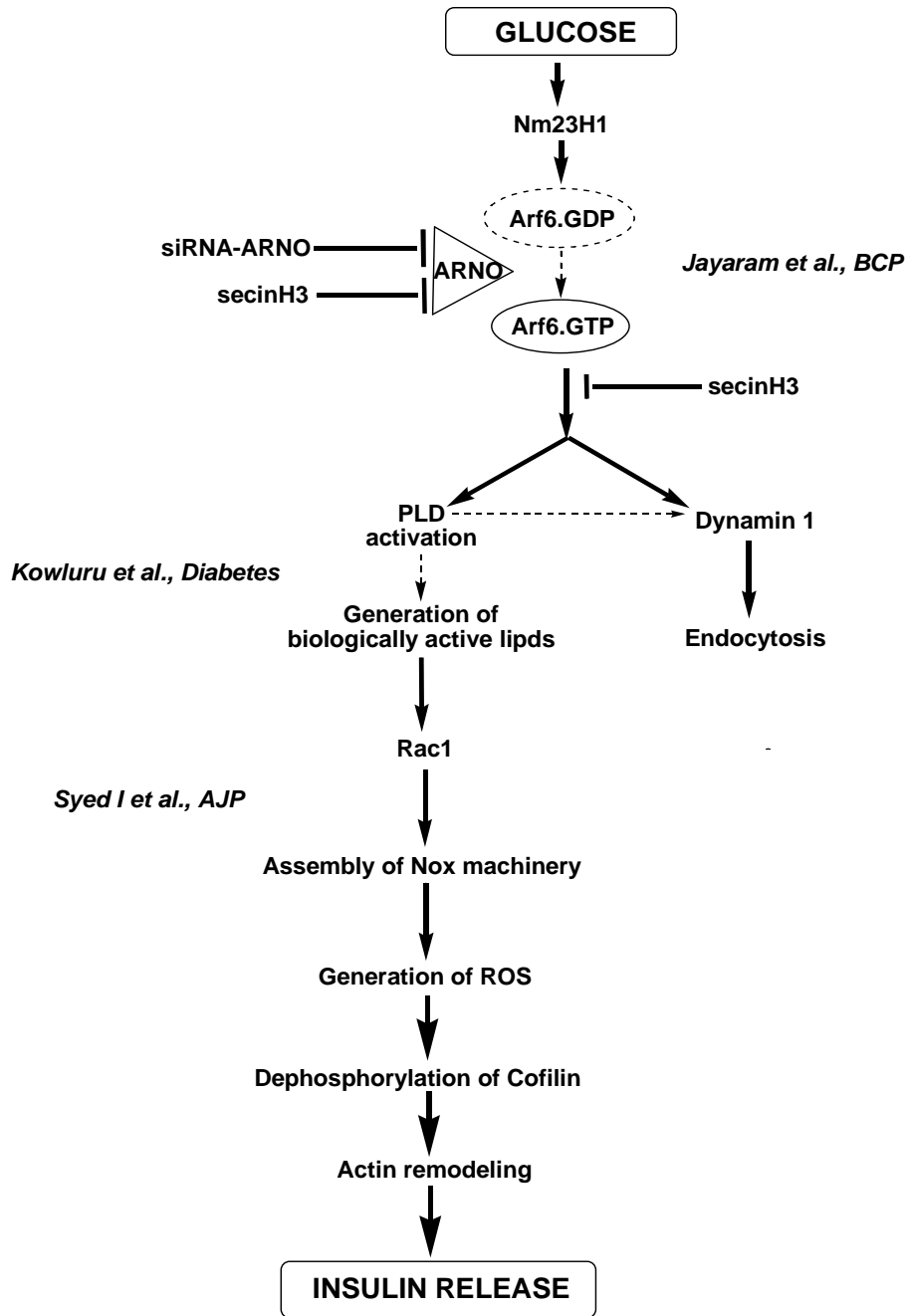


Figure 7-1: Working model placing all the key players in the event leading to exo-endocytosis of insulin-laden secretory granules.



Arf nucleotide binding site opener [ARNO] promotes sequential activation of Arf6, Cdc42 and Rac1 and insulin secretion in INS 832/13 β -cells and rat islets

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ARTICLE INFO

Article history:

Received 8 November 2010
Accepted 12 January 2011
Available online 26 January 2011

Keywords:

Insulin secretion
Pancreatic islet
ARNO
Arf6
Rac1
SecinH3

ABSTRACT

Glucose-stimulated insulin secretion [GSIS] involves interplay between small G-proteins and their regulatory factors. Herein, we tested the hypothesis that Arf nucleotide binding site opener [ARNO], a guanine nucleotide-exchange factor [GEF] for the small G-protein Arf6, mediates the functional activation of Arf6, and that ARNO/Arf6 signaling axis, in turn, controls the activation of Cdc42 and Rac1, which have been implicated in GSIS. Molecular biological [i.e., expression of inactive mutants or siRNA] and pharmacological approaches were employed to assess the roles for ARNO/Arf6 signaling pathway in insulin secretion in normal rat islets and INS 832/13 cells. Degrees of activation of Arf6 and Cdc42/Rac1 were quantitated by GST-GGA3 and PAK-1 kinase pull-down assays, respectively. ARNO is expressed in INS 832/13 cells, rat islets and human islets. Expression of inactive mutants of Arf6 [Arf6-T27N] or ARNO [ARNO-E156K] or siRNA-ARNO markedly reduced GSIS in isolated β -cells. SecinH3, a selective inhibitor of ARNO/Arf6 signaling axis, also inhibited GSIS in INS 832/13 cells and rat islets. Stimulatory concentrations of glucose promoted Arf6 activation, which was inhibited by secinH3 or siRNA-ARNO, suggesting that ARNO/Arf6 signaling cascade is necessary for GSIS. SecinH3 or siRNA-ARNO also inhibited glucose-induced activation of Cdc42 and Rac1 suggesting that ARNO/Arf6 might be upstream to Cdc42 and Rac1 activation steps, which are necessary for GSIS. Lastly, co-immunoprecipitation and confocal microscopic studies suggested increased association between Arf6 and ARNO in glucose-stimulated β -cells. These findings provide the first evidence to implicate ARNO in the sequential activation of Arf6, Cdc42 and Rac1 culminating in GSIS.

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1. Introduction

It is widely accepted that small G-proteins regulate various cellular functions including proliferation, survival and demise. At least four major classes of small G-proteins have been identified in the pancreatic β -cell. These include the Ras, Rho, Rab and ADP-ribosylation factor [Arf] family of G-proteins [1], of which Arf family of G-proteins is less studied in the islet. Though originally identified as an ADP-ribosylator for cholera toxin, Arf has gained much importance as a critical modulator of membrane traffic in eukaryotic cells. Among the six members of the Arf family, Arf6 is a well-documented protein for its positive modulatory roles in

multiple cell types including regulation of various effector proteins [e.g., phospholipase-D; PLD] and trafficking of secretory granules to the plasma membrane for exocytosis [2]. Regazzi and coworkers first described localization and regulation of Arfs in insulin-secreting RINm5f cells [3,4]. More recently, Lawrence and Birnbaum have demonstrated regulatory roles for Arf6 in insulin secretion mediated by glucose, GTP γ S and membrane depolarization. They further demonstrated that Arf6 regulates insulin secretion by maintaining plasma membrane phosphatidylinositol-4,5-bisphosphate (PIP₂; [5]). Existing evidence also supports a potential role for PLD in physiological insulin secretion [6–8].

Arf6 cycles between the GDP-bound [inactive] and GTP-bound [active] configurations; which are tightly regulated by two distinct classes of regulatory factors namely the GTPase activating proteins [GAPs] and the GTP/GDP exchange factors (GEFs; [9,10]). GAPs inactivate Arf6 by promoting its conversion to the inactive GDP-bound form, while GEFs facilitate its activation. The GEF activity is a rate-defining step and involves coordination of multiple intracellular signals. In this context, many GEFs with distinct size and structure have been identified for Arf6 [11–16]. However, in

Abbreviations: Arf6, ADP-ribosylation factor 6; ARNO, Arf nucleotide binding site opener; GAP, GTPase activating protein; GDI, GDP-dissociation inhibitor; GEF, guanine nucleotide-exchange factor; GGA3, Golgi-localized c-ear homology domain Arf-binding protein-3; GSIS, glucose-stimulated insulin secretion; PA, phosphatidic acid; PIP₂, phosphatidylinositol-4,5-bisphosphate; PLD, phospholipase-D.

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the majority of the signaling events, only one member belonging to the cytohesin family has been closely linked to activate Arf6 [13,14,17,18]. Recently, Hafner et al. reported a small molecule inhibitor, secinH3, which selectively blocks ARNO-mediated activation of Arf6 [19]. Previous studies have utilized secinH3 to determine the regulatory roles for ARNO/Arf6 signaling in cellular signal transduction [20,21].

In an attempt to identify precise regulatory mechanisms involved in glucose-mediated activation of Rac1 and insulin secretion, we first proposed [22] and subsequently confirmed experimentally [23] that certain biologically active lipid second messengers [e.g., PA, PIP₂] promote dissociation of Rac1 from Rac1/GDI complex to facilitate activation of Rac1 in rodent islets and clonal β -cells. Therein, we also proposed that Arf6 could represent one of the upstream regulators of Rac1 activation by generating relevant lipid second messengers via phospholipase activation to dissociate the Rac1/GDI complex [22,23]. Studies from Thurmond's laboratory have demonstrated the requirement of Cdc42, a Rho family GTPase in the Rac1 activation process for actin remodeling and insulin exocytosis [24,25]. The current study is undertaken to test the hypothesis that ARNO mediates sequential activation of Arf6, Cdc42 and Rac1 leading to GSIS. Using molecular biological and pharmacological approaches we provide below the first evidence to in support of this hypothesis in normal rodent islets and insulin-secreting INS 832/13 cells.

2. Materials and methods

2.1. Materials

SecinH3 was from Tocris Biosciences [Ellisville, MO]. siRNA-Arf6 consisting of pools of three to five target-specific 19–25 nt siRNAs were from Santa Cruz Biotechnology [Santa Cruz, CA]. siRNA-ARNO was from Dharmacon [Lafayette, IL]. The rat insulin ELISA kit was from American Laboratory Products [Windham, NH]. Antisera directed against Arf6, ARNO and Dbl were from Santa Cruz Biotechnology [Santa Cruz, CA]. Cdc42 and Rac1 antisera were from BD Biosciences [San Jose, CA]. Cdc42 and Rac1 activation kits were from Cytoskeleton Inc. [Denver, CO]. Arf6 activation assay kit and the Classic Co-IP kit were from PIERCE [Rockford, IL]. Alexa-fluor secondary antibody was from Invitrogen Molecular Probes [Carlsbad, CA]. All other reagents used in these studies were from Sigma Aldrich Co. [St. Louis, MO] unless stated otherwise.

2.2. Insulin-secreting INS 832/13 cells, rat islets and human islets

INS 832/13 cells were kindly provided by Dr. Chris Newgard (Duke University Medical Center, Durham, NC). The cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum supplemented with 100 IU/ml penicillin and 100 IU/ml streptomycin, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol, 11 mM glucose, and 10 mM HEPES (pH 7.4). Islets were isolated from pancreas of male Sprague–Dawley rats (Harlan Laboratories, Oxford, MI), using collagenase digestion and a ficoll gradient as we described previously [22]. All experiments were reviewed and approved by the Wayne State University Institutional Animal Care and Use Committee. Human pancreatic islet lysates were kindly provided by Dr. Karl Olson [Michigan State University, Lansing, MI].

2.3. Hydrophilic and hydrophobic phase partitioning method using Triton X-114

Total hydrophobic and hydrophilic phases of lysates derived from INS 832/13 cells and pancreatic islets were separated using Triton X-114 according to method described earlier by us [22].

Briefly, about 400 μ g of cell [INS 832/13 cell or islet] homogenate protein, prepared in 400 μ l of buffer (20 mM Tris–HCl, pH 7.5, 0.5 mM EGTA, 2 mM MgCl₂, 10 μ g/ml leupeptin, and 2 μ g/ml aprotinin), supplemented with 1% (w/v) Triton X-114 was overlaid on 400 μ l sucrose cushion 6% (w/v) prepared in 20 mM Tris–HCl buffer (pH 7.4) containing 0.06% (w/v) Triton X-114. Following brief incubation at 30 °C, samples were centrifuged at 300 \times g for 3 min and the aqueous phase was mixed with 0.5% (w/v) fresh Triton X-114 at 4 °C. Following dissolution, the mixture was again overlaid on the same sucrose cushion, incubated for 3 min at 30 °C and centrifuged at 300 \times g for 3 min. The lower hydrophobic phase was diluted to a final volume of 400 μ l with homogenization buffer, while the aqueous phase was transferred into a separate tube supplemented with 2% fresh Triton X-114, incubated for 3 min at 30 °C, and centrifuged at 300 \times g without sucrose cushion. The supernatant obtained thereof served as total hydrophilic phase. The relative abundance of ARNO in hydrophilic and hydrophobic phases was determined by Western blotting.

2.4. Transfection of Arf6 or ARNO mutants and siRNAs

INS 832/13 cells were subcultured at 50–60% confluency and transfected using Effectene [Qiagen, Valencia, CA], with 0.2 μ g of plasmid DNA constructs against either dominant-negative of Arf6 [T27N] or ARNO [E156K] per well of a 24-well plate. Endogenous Arf6 or ARNO expression was depleted by transfecting cells using small interfering RNA [siRNA; 100 nM] using HiPerfect transfection reagent [Qiagen, Valencia, CA]. Efficiency of mutant expression or protein knockdown was determined by Western blotting.

2.5. Insulin release studies

Arf6 or ARNO mutant or siRNA-transfected or secinH3 inhibitor-treated cells were cultured overnight in low serum and low glucose containing medium and then stimulated either with high glucose, KCl or arginine in Krebs–Ringer bicarbonate buffer [KRB, pH 7.4] for different time intervals as indicated in the text. In studies involving KCl-induced insulin secretion, we noticed that INS 832/13 cells were not responsive to 40 mM KCl in releasing insulin. However, higher KCl concentrations [60 mM] were found to elicit robust insulin release. Therefore, in KCl-stimulated insulin secretion studies, cells were incubated with 60 mM KCl in an osmolarity-balanced KRB medium [5]. For arginine [L-Arg]-stimulated insulin release, a stock solution was prepared in Tris–HCl [pH 7.4], and diluted to desired concentration with KRB. The insulin released into the medium was quantitated by ELISA [22].

2.6. Quantitation of Arf6-GTP in pancreatic β -cells

Active Arf6 was quantitated by a pull-down assay. Briefly, the incubation medium was aspirated and cells were washed with ice-cold PBS. Cells were lysed with 500 μ l lysis buffer and the lysate was clarified by centrifugation at 16,000 \times g at 4 °C for 15 min and incubated \sim 400 μ g protein with 100 μ l of glutathione resin and 100 μ g of GST-GGA3-PBD beads at 4 °C for 1 h with gentle rocking, following which the reaction mixture was spun at 6000 \times g for 30 s. The GST-tagged beads were washed [3 \times] and proteins were separated by SDS-PAGE and activated Arf6 was identified by Western blotting.

2.7. Quantitation of Cdc42 and Rac1 activation

Relative degree of activated Cdc42 and Rac1 [i.e., GTP-bound form] was determined by p21-activated kinase-p21-binding domain pull-down assay as described in [22]. Briefly, INS 832/

13 cells treated with either diluent or secinH3 [50 μ M] or cells were either mock-transfected or transfected with ARNO-siRNA were cultured overnight in low serum-low glucose media. Cells were stimulated with either low [2.5 mM] or high [20 mM] glucose for 3 or 30 min at 37 °C in the continued presence of either diluent or secinH3 with respect to inhibitor studies. The GTP-bound forms of Cdc42 and Rac1 in the pull-down samples were quantitated by Western blotting and densitometry.

2.8. Co-immunoprecipitation studies

Immunoprecipitation studies were performed using the Classic Co-IP kit as suggested by the manufacturer [26]. Briefly, β -cell lysates [500 μ g protein] were incubated with anti-ARNO for 2 h at 4 °C followed by incubation with agarose resin for an additional 1 h at 4 °C. Beads were washed; eluted using sample buffer and proteins were resolved by SDS page to quantify Arf6.

2.9. Immunofluorescence studies

INS 832/13 cells were plated onto coverslips and incubated with [2.5 or 20 mM] glucose for 30 min at 37 °C followed by washing in PBS and fixed with 4% paraformaldehyde solution for 15 min at room temperature. They were then permeabilized with 0.2% Triton X-100 for 15 min at room temperature. After blocking with 1% BSA for 1 h, the cells were further incubated with primary antibodies Arf6 [1:150] and ARNO [1:150] in 0.1% BSA solution for 1 h. After extensive washes, the cells were further incubated with secondary antibodies Alexa-fluor 488 anti-mouse [1:1000] and Alexa-fluor anti-goat 546 [1:1000] in 0.1% BSA solution for 1 h at 37 °C. The coverslips were then mounted on glass slides containing mounting media [DAKO Corporation, Carpinteria, CA] and visualized under a confocal LSM 510 microscope in the midplane using a 63 \times oil-immersion lens [26].

2.10. Statistical analyses

The statistical significance of the difference between the experimental conditions was determined by Student's *t*-test unless mentioned otherwise. *p* values <0.05 were considered significant.

3. Results

3.1. Distribution of ARNO in pancreatic β -cells

Data shown in Fig. 1A indicated that ARNO is expressed in normal rat islets, human islets and INS 832/13 cells. The relative abundance of ARNO in the total hydrophilic and hydrophobic compartments [isolated from cell lysates using Triton X-114 phase separation protocol; see Section 2] indicated that ARNO remains associated with the hydrophilic compartment [Fig. 1B].

3.2. Molecular biological inhibition of Arf6 or ARNO attenuates insulin release by various insulin secretagogues in INS 832/13 cells

We first verified potential consequences of overexpression of dominant negative mutants of Arf6 or ARNO on GSIS from INS 832/13 cells. To examine this, INS 832/13 cells were either mock-transfected or transfected either with Arf6-T27N or ARNO-E156K [see Section 2]. Transfection efficiency of mutants was verified by Western blotting [Fig. 2A]. Insulin secretion was quantitated in these cells in the presence of 2.5 or 20 mM glucose. Data in Fig. 2C showed a marked reduction in GSIS in cells expressing Arf6-T27N. Likewise, overexpression of ARNO-E156K, a mutant lacking the GEF function, significantly inhibited GSIS in these cells [Fig. 2D].

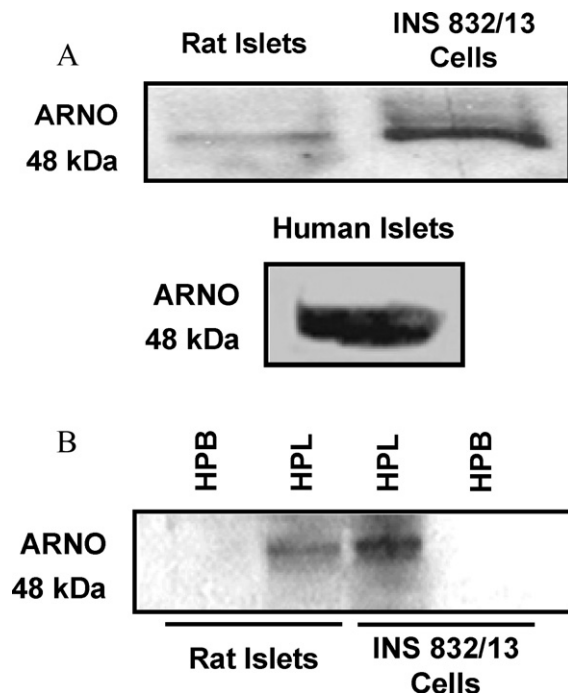


Fig. 1. Expression of ARNO in INS 832/13 cells, rat islets and human islets. *Panel A:* lysates from rat islets, human islets and INS 832/13 cells were separated by SDS-PAGE and probed for ARNO [48 kDa]. *Panel B:* hydrophilic [HPL] and hydrophobic [HPB] phases of the homogenates from rat islets and INS 832/13 cells were isolated using Triton X-114 partition technique [see Section 2 for additional details]. Proteins were separated by SDS-PAGE and probed for ARNO. A representative blot from three independent experiments is shown here.

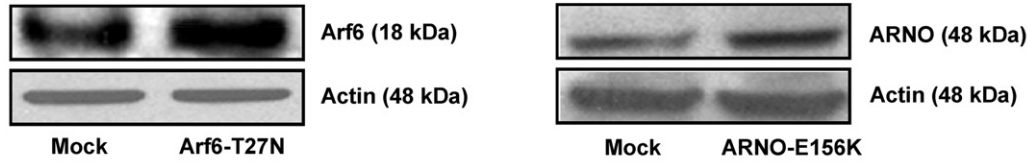
Together, these data suggested key roles for Arf6 and ARNO in signaling events leading to GSIS. Next, we verified if knockdown of endogenous Arf6 and ARNO by using siRNA affects insulin secretion elicited by high glucose. We observed 50–60% reduction in the expression of Arf6 or ARNO by Western blotting analysis [Fig. 2B]. Under these conditions, GSIS was significantly inhibited in Arf6- and ARNO-knocked-down cells [Fig. 2E and F]. Together, data in Fig. 2 [panels C–F] implicated Arf6/ARNO signaling axis in GSIS.

In the next series of studies, we determined potential roles of Arf6 or ARNO in insulin secretion elicited by a membrane-depolarizing concentration of KCl or arginine. To address this, INS 832/13 cells were transfected with either siRNA-Arf6 or siRNA-ARNO, and insulin secretion in the presence of KCl [60 mM; Fig. 2G] or arginine [20 mM; Fig. 2H and I] was quantitated. Data depicted in Fig. 2 [panels G–I] demonstrated that insulin secretion elicited by KCl or arginine was inhibited in cells in which the endogenous Arf6 or ARNO was knocked-down. Together, data described in Fig. 2 implicate regulatory roles for ARNO/Arf6 in insulin secretion elicited by a variety of secretagogues.

3.3. Glucose activates Arf6 in pancreatic β -cells

To further understand the role of Arf6 in stimulus-coupled secretion, we next examined whether insulinotropic concentrations of glucose activates Arf6 in the β -cell. This was accomplished by using a recently developed GST-GGA3 pull-down assay, which utilizes GGA proteins to capture activated forms of Arf6 by interacting with the Arf-binding domain [27]. Efficiency and specificity of the activation assay was confirmed by the ability of GTP γ S to stimulate Arf6 activation in broken cell preparations [additional data not shown]. A time-course study for Arf6 activation by glucose [Fig. 3A and B] suggested that glucose-induced activation was seen as early as 1 min and reached

A. Overexpression of dominant negative mutants of Arf6 or ARNO



B. Knockdown of endogenous expression of Arf6 or ARNO with siRNA

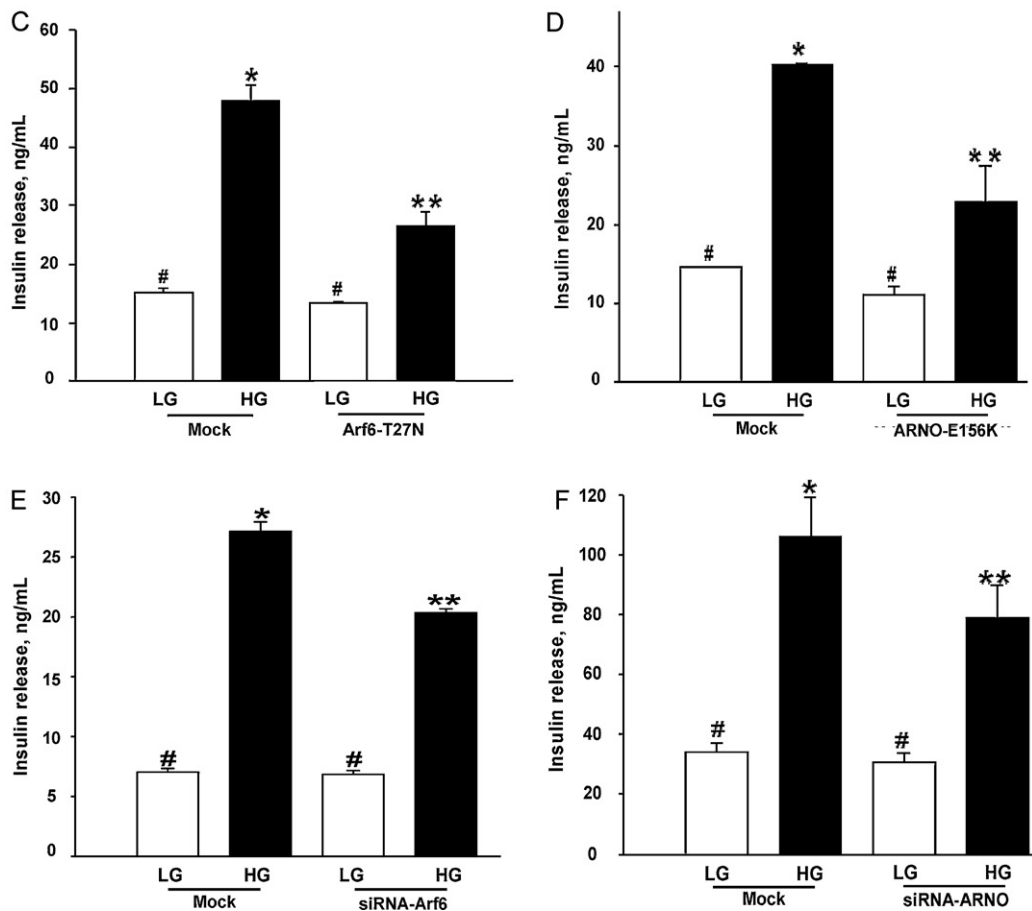
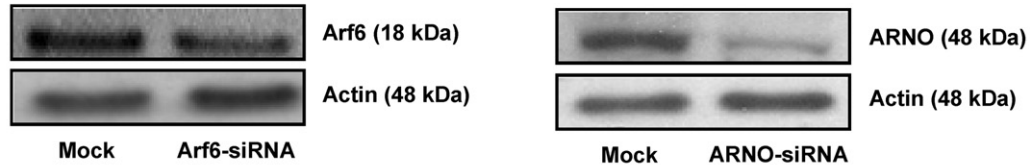


Fig. 2. Overexpression of inactive mutants or siRNA-Arf6 or siRNA-ARNO markedly inhibits glucose-induced insulin secretion in INS 832/13 cells. *Panel A:* INS 832/13 cells were either mock-transfected or transfected with dominant mutants of Arf6 [T27N] or ARNO (E156K; see Section 2). Relative degrees of expression of the mutants were verified by Western blotting. A representative blot from three independent studies yielding similar results is provided here. *Panel B:* INS 832/13 cells were either mock-transfected or transfected with siRNA-Arf6 or siRNA-ARNO as described in Section 2. Relative degrees of knockdown of these proteins were verified by Western blotting. A representative blot from three independent studies yielding similar results is shown here. *Panel C:* INS 832/13 cells were transfected with dominant negative Arf6 [T27N] at a final concentration of 0.2 μ g of DNA and cultured for 48 h. Following this, cells were stimulated with either low [2.5 mM] or high [20 mM] glucose in KRb for 30 min at 37 °C. Insulin released into the media was quantitated and expressed as ng/ml. Data are mean \pm SEM from three independent experiments. * represents $p < 0.05$ vs. mock low glucose; ** $p < 0.05$ vs. mock transfected cells treated with high glucose, and data points with similar symbol did not differ significantly. *Panel D:* INS 832/13 cells were transfected with dominant negative ARNO [E156K] at a final concentration of 0.2 μ g of DNA and cultured for 48 h following which cells were stimulated with either low [2.5 mM] or high [20 mM] glucose for 30 min at 37 °C. Insulin released into the medium was quantitated and expressed as ng/ml. Data are mean \pm SEM from three independent experiments. * represents $p < 0.05$ vs. mock transfected low glucose and ** $p < 0.05$ vs. mock transfected high glucose, and data points with similar symbol do not differ significantly. *Panel E:* INS 832/13 cells were either mock-transfected or transfected with Arf6-siRNA at a final concentration of 100 nM. After 48 h culture in regular medium, cells were stimulated with low [2.5 mM] or high [20 mM] glucose for 30 min. Insulin released into the medium was quantitated and expressed as ng/ml. Data are mean \pm SEM from three independent experiments. * represents $p < 0.05$ vs. mock low glucose; ** $p < 0.05$ vs. mock transfected cells treated with high glucose, and data points with similar symbol did not differ significantly. *Panel F:* INS 832/13 cells were either mock-transfected or transfected with ARNO-siRNA at a final concentration of 100 nM. After 48 h culture in regular medium, cells were stimulated with low

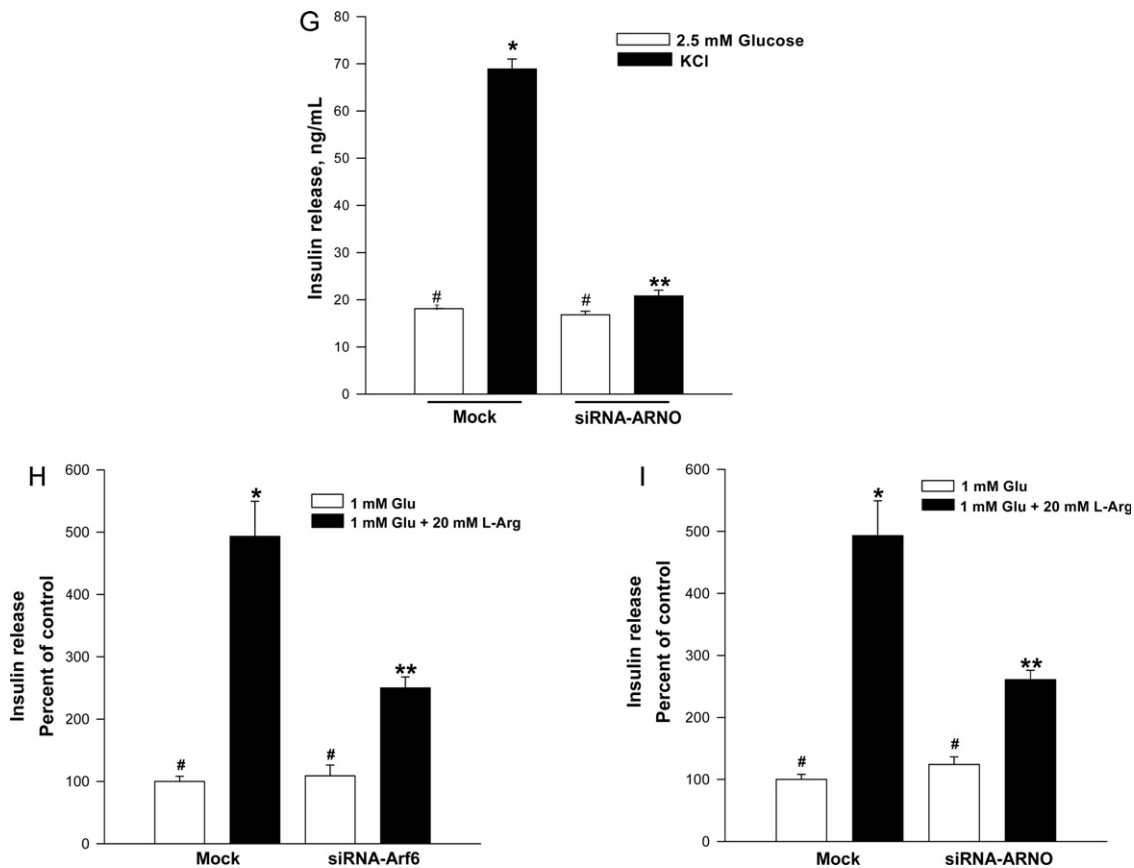


Fig. 2. (Continued). [2.5 mM] or high [20 mM] glucose for 30 min. Insulin released into the medium was quantitated and expressed as ng/ml. Data are mean \pm SEM from five independent experiments. * represents $p < 0.05$ vs. mock transfected low glucose; ** $p < 0.05$ vs. mock transfected high glucose. Insulin release values between mock low glucose and siRNA transfected low glucose did not differ significantly. **Panel G:** INS 832/13 cells were either mock-transfected or transfected with ARNO-siRNA at a final concentration of 100 nM. After 48 h culture in regular medium, cells were stimulated with low [2.5 mM] or KCl [60 mM; osmolarity adjusted] for 60 min. Insulin released into the medium was quantitated and expressed as ng/ml. Data are mean \pm SEM from three independent experiments. * represents $p < 0.05$ vs. mock-transfected low glucose; ** $p < 0.05$ vs. mock transfected K⁺. Insulin release values between mock low glucose and siRNA transfected low glucose did not differ significantly. **Panels H and I:** INS 832/13 cells were mock-transfected or transfected either with Arf6-siRNA/ARNO-siRNA at a final concentration of 100 nM. After 48 h culture in regular medium, cells were stimulated with 1 mM glucose [Glu] or 1 mM glucose + 20 mM arginine [L-Arg] for 15 min. Insulin released into the medium was quantitated and expressed as percent of control. Data are mean \pm SEM from replicates. * represents $p < 0.05$ vs. mock-transfected 1 mM glucose; ** $p < 0.05$ vs. mock-transfected 1 mM Glu + 20 mM L-Arg. Insulin release values between mock and siRNA transfected under low glucose [1 mM] conditions did not differ significantly.

optimum at 3 min time point. Even though, we noticed a reduction in activated Arf6 at 5 min time point, Arf6 remained active [Arf6-GTP] above basal till 30 min [additional data not shown]. Together, these data suggest that Arf6 activation might represent one of the early signaling steps leading to GSIS [see below].

3.4. siRNA-mediated knockdown of ARNO or pharmacological inhibition of ARNO/Arf6 signaling results in attenuated glucose-induced activation of Arf6

The conversion of the GDP-bound inactive forms of G-proteins to their GTP-bound active state is mediated by GEFs. The above data prompted us to investigate if ARNO represents one of the GEFs for glucose-mediated activation of Arf6. This was verified by two complementary methods. In the first, glucose-induced activation of Arf6 was examined in cells in which ARNO expression was reduced by siRNA-ARNO. Data in Fig. 4A and B indicated complete inhibition of glucose-induced activation of Arf6 under these conditions. This was further verified by a second approach involving pharmacological inhibition of ARNO/Arf6 signaling by secinH3 [19–21]. Data in Fig. 4C and D suggested a complete inhibition of glucose-induced activation of Arf6 by secinH3. Taken together, these data indicate that glucose-induced activation of Arf6 requires the intermediacy of ARNO.

3.5. SecinH3 markedly attenuates GSIS in INS 832/13 cells and rat islets

As a logical extension to the above studies, we next investigated potential impact of pharmacological inhibition of ARNO on GSIS in INS 832/13 cells and normal rat islets. Data in Fig. 5 demonstrated complete inhibition of GSIS by secinH3 in INS 832/13 cells [Fig. 5A] and normal rat islets [Fig. 5B]. Under these conditions secinH3 failed to increase basal secretion in either INS 832/13 cells or rat islets [Fig. 5A and B]. It should be noted that inhibitory effects of secinH3 on Arf6 activation [Fig. 4] or GSIS [Fig. 5] were not due to its cytotoxic effects since we noticed no significant effects of this inhibitor on the metabolic cell viability of β -cells under these conditions [not shown]. Together, these pharmacological data confirm the above molecular biological data to support our hypothesis that ARNO/Arf6 signaling cascade plays a positive modulatory role in GSIS.

3.6. siRNA-mediated knockdown of ARNO or pharmacological inhibition of ARNO leads to marked reduction in glucose-induced activation of Rac1 and Cdc42

Several recent studies, including our own, have implicated Rho subfamily of small G-proteins [e.g., Cdc42 and Rac1] in cytoskeletal remodeling leading to the translocation of insulin-laden secretory

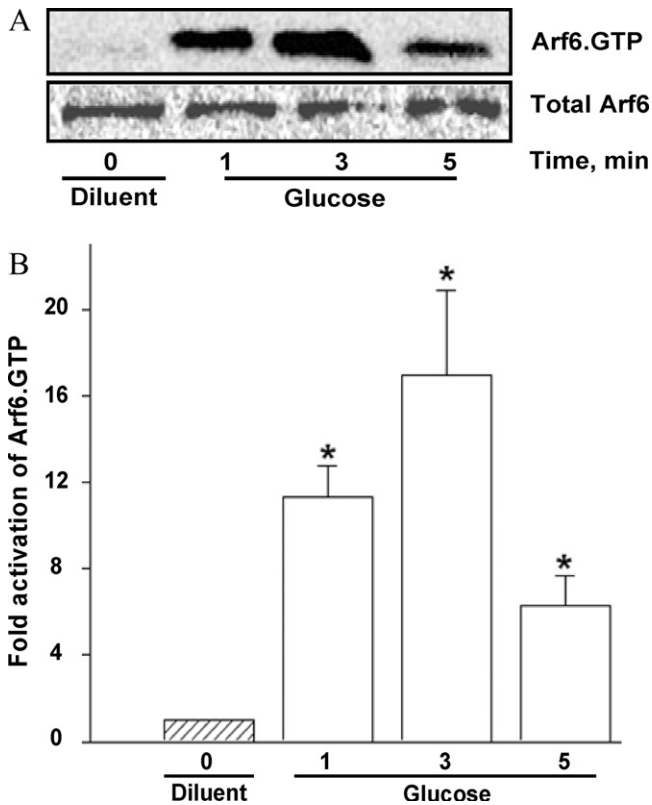


Fig. 3. Time-dependent activation of Arf6 by glucose in pancreatic β -cells. *Panel A:* INS 832/13 cells were incubated with KRB for 1 h and either left unstimulated [diluent] or stimulated with high glucose [20 mM] for different time points as indicated. Cell lysates were used for detecting activated Arf6 [Arf6-GTP] by GST-GGA3-PBD pull down assay [see Section 2]. Total Arf6 was used as the loading control and a representative blot from three independent experiments is shown. *Panel B:* densitometric quantitation of Arf6 activation depicted in panel A is shown here. * represents $p < 0.05$ vs. diluent. Statistical analysis was performed using one-way ANOVA, all pairwise multiple comparison method (Dunnett's).

granules to the plasma membrane for fusion and exocytotic secretion of insulin [28,29]. In this context, recent evidence appears to suggest a significant cross-talk between ARNO/Arf6 and Rac1 in the regulation of cell function in multiple cell types [30–35]. Therefore, we asked if ARNO/Arf6 signaling axis regulates glucose-induced Rac1 activation in the pancreatic β -cell. We addressed this question by quantitating glucose-induced Rac1 activation in INS 832/13 cells in which ARNO function is compromised via pharmacological [e.g., secinH3] or molecular biological [e.g., siRNA-ARNO] approaches. As expected, we noticed a significant Rac1 activation in control cells exposed to glucose [Fig. 6A]. Interestingly, siRNA-mediated knockdown of ARNO increased Rac1 activation under basal glucose conditions. However, glucose-induced activation of Rac1 was completely inhibited in ARNO-depleted β -cells [Fig. 6A]. Likewise, pharmacological inhibition of ARNO/Arf6 signaling axis with secinH3 abolished glucose-induced activation in these cells [Fig. 6B] suggesting that ARNO/Arf6 signaling step might be upstream to the Rac1 activation step in the cascade of events leading to GSIS. These data, which are compatible with our original proposal [22] also fit into the time-frame for glucose-induced activation of these proteins. We noticed in this study that Arf6 activation is seen as early as 1 min while glucose-induced activation of Rac1 is maximal at 15–20 min [28,29].

Along these lines, earlier studies from our laboratory have suggested that the carboxymethylation of Cdc42 is stimulated by glucose within 1 min of exposure [36]. More recent and comprehensive investigations by Thurmond's group [24,25] have

reported glucose-induced activation of Cdc42 within 3 min of exposure. They also demonstrated that Cdc42 activation is upstream to Rac1 activation in the cascade of events leading to GSIS [25]. Therefore, we examined if inhibition of ARNO/Arf6 signaling step affects Cdc42 activation. Our findings suggested ~50% inhibition of glucose-induced activation of Cdc42 in INS 832/13 cells following inhibition of ARNO/Arf6 by secinH3 [Fig. 6C]. Together, our findings are suggestive of sequential activation of Arf6, Cdc42 and Rac1 by ARNO in glucose-stimulated β -cell culminating in insulin secretion.

It should be noted that recent investigations from our laboratory have described the roles for Tiam1, a GEF for Rac1 in GSIS. For example, we have shown that siRNA-mediated knockdown of Tiam1 or pharmacological inhibition of Tiam1/Rac1 signaling axis [e.g., NSC23766] markedly attenuated GSIS in INS 832/13 cells [37]. Along these lines, Western blot data indicated expression of Dbl, a known GEF for Cdc42 [38], in INS 832/13 cells, normal rat islets and human islets [Fig. 6D]. Together, these data are suggestive expression of at least three GEFs [i.e., ARNO, Tiam1 and Dbl] in the islet β -cell. Potential cross talk between these GEFs, if any, in the context of GSIS, remains to be verified.

3.7. Glucose promotes association between Arf6 and ARNO in pancreatic β -cells

In the last series of studies we utilized co-immunoprecipitation and immunofluorescence approaches to further determine if exposure of isolated β -cells to an insulinotropic concentration of glucose promotes association between Arf6 and ARNO. Data shown in Fig. 7A indicate detectable levels of Arf6 in ARNO immunoprecipitates suggesting that these two proteins stay complexed under basal conditions. Moreover, incubation of these cells with stimulatory glucose resulted in a significant increase [~2-fold] in the amount of Arf6 in the ARNO immunoprecipitates [Fig. 7B]. Together, these data suggest that glucose promotes physical association between ARNO and Arf6 in insulin-secreting cells. We verified these findings via a complementary immunofluorescence approach. Data in Fig. 7C suggested that both Arf6 [green] and ARNO [red] remain diffused throughout the cell under basal conditions [2.5 mM glucose]. Merged images of subpanels a and b in Fig. 7 [i.e., subpanel c] further suggested that the two proteins remain localized in the cytosolic compartment. However, exposure of these cells to a stimulatory concentration of glucose [20 mM] led to a significant association of these proteins as evidenced in the merged images of subpanels d and e of Fig. 7 [i.e., subpanel f]. Together, these findings [Fig. 7] provide evidence for increased association of Arf6 and ARNO in the presence of glucose leading to the activation of ARNO/Arf6 signaling pathway followed by sequential activation of Cdc42/Rac1 culminating in insulin secretion.

4. Discussion

It is widely accepted that the Arf family of G-proteins play a key regulatory role in membrane trafficking [9]. Among these, Arf6 is well studied and has been shown to regulate several cellular events including cell motility, vesicular transport, and cortical actin rearrangements [17,32,39]. In addition, Arf6 has been shown to activate several G-proteins and enzymes of lipid metabolism including PLD to generate fusogenic lipids such as PA and PIP₂ [40,41]. Such experimental evidence led to the postulation that Arf6 might represent one of the G-proteins whose activation may be necessary for the trafficking of secretory vesicles to the plasma membrane and fusion to lease their contents into the circulation.

The functions of Arf6 are regulated by factors such as GEFs. Despite compelling evidence in many cell types, little is known

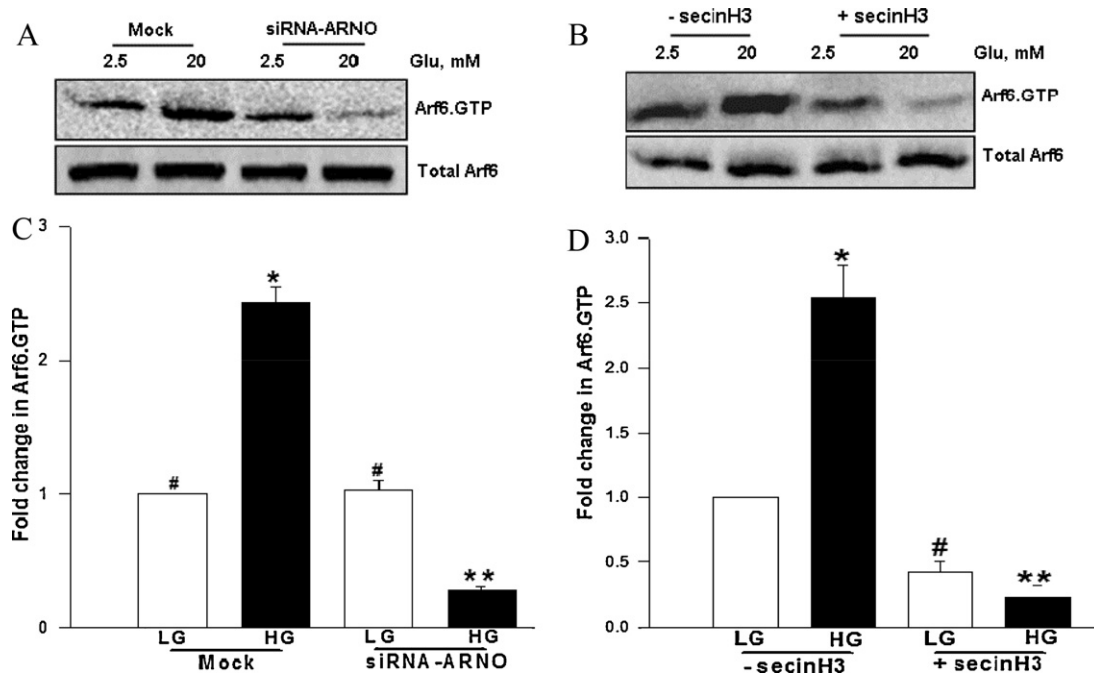


Fig. 4. Molecular biological or pharmacological inhibition of ARNO attenuates glucose-induced activation of Arf6 in INS 832/13 pancreatic β -cells. *Panel A:* INS 832/13 cells were either mock-transfected or transfected with siRNA-ARNO and cultured for 48 h following which cells were stimulated in the presence of either low glucose [LG, 2.5 mM] or high glucose [HG, 20 mM] for 30 min at 37 °C. The relative amounts of activated Arf6 [i.e., Arf6-GTP] were determined by pull down assay. Total Arf6 from cell lysates was used as the loading control and a representative blot from three independent experiments is shown. *Panel B:* data shown in the panel A were analyzed densitometrically and expressed as fold change in Arf6-GTP over basal and are mean \pm SEM of three independent experiments. * represents $p < 0.05$ vs. mock transfected low glucose; ** $p < 0.05$ vs. mock transfected high glucose, and data points with similar symbol do not differ statistically. *Panel C:* INS 832/13 cells were incubated overnight in the presence or absence of secinH3 [50 μ M] and stimulated with either low glucose [LG, 2.5 mM] or high glucose [HG, 20 mM] in the continuous presence or absence of secinH3 [50 μ M] for 30 min. Relative degrees of Arf6 activation were quantitated as described in panel A. Total Arf6 from cell lysates was used as the loading control and a representative blot from three independent experiments is shown. *Panel D:* data shown in the panel C are analyzed densitometrically and expressed as fold change in Arf6-GTP over basal. Data are mean \pm SEM from three independent experiments. * and # represent $p < 0.05$ vs. low glucose without secinH3; and ** $p < 0.05$ vs. high glucose without secinH3.

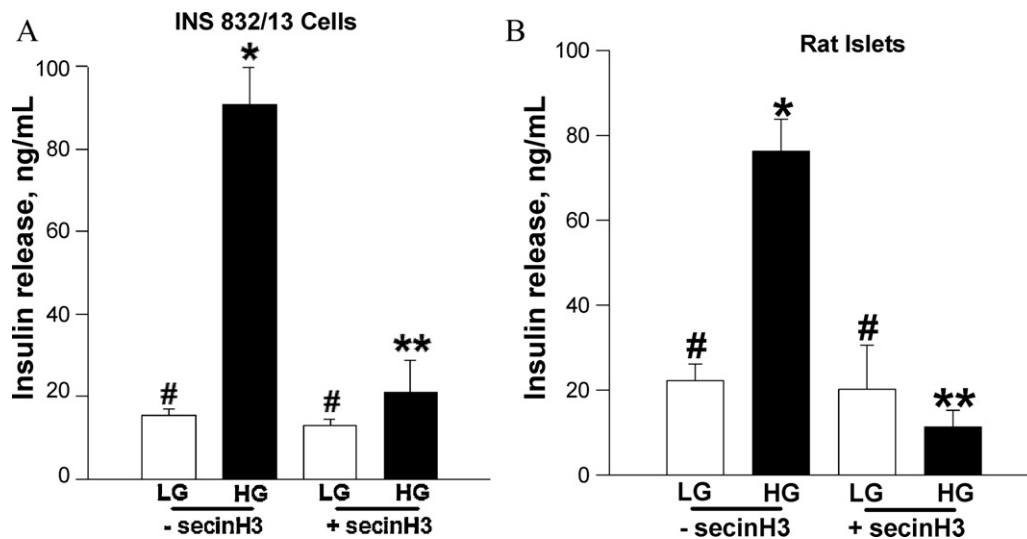


Fig. 5. SecinH3, a selective inhibitor of ARNO attenuates GSIS in INS 832/13 cells and normal rat islets. *Panel A:* INS 832/13 cells were incubated in low serum-low glucose overnight in the continuous presence of 50 μ M secinH3 or diluent and stimulated with either low [LG, 2.5 mM] or high glucose [HG, 20 mM] for 30 min in KRB. Insulin released into the medium was quantitated by ELISA and expressed as ng/ml. Data are mean \pm SEM from four independent experiments. * represents $p < 0.05$ vs. low glucose without secinH3; ** $p < 0.05$ vs. high glucose without secinH3 and data points with similar symbol do not differ statistically. *Panel B:* normal rat islets were incubated in low serum-low glucose overnight in the continuous presence of either 50 μ M secinH3 or diluent and stimulated with either low [LG, 2.5 mM] or high glucose [HG, 20 mM] for 30 min in KRB. Insulin released into the medium was quantitated by ELISA. Data are expressed as ng/ml insulin released and are mean \pm SEM from four independent experiments. * represents $p < 0.05$ vs. low glucose with secinH3 and without secinH3; and ** $p < 0.05$ vs. high glucose without secinH3. Data points with similar symbol do not differ significantly.

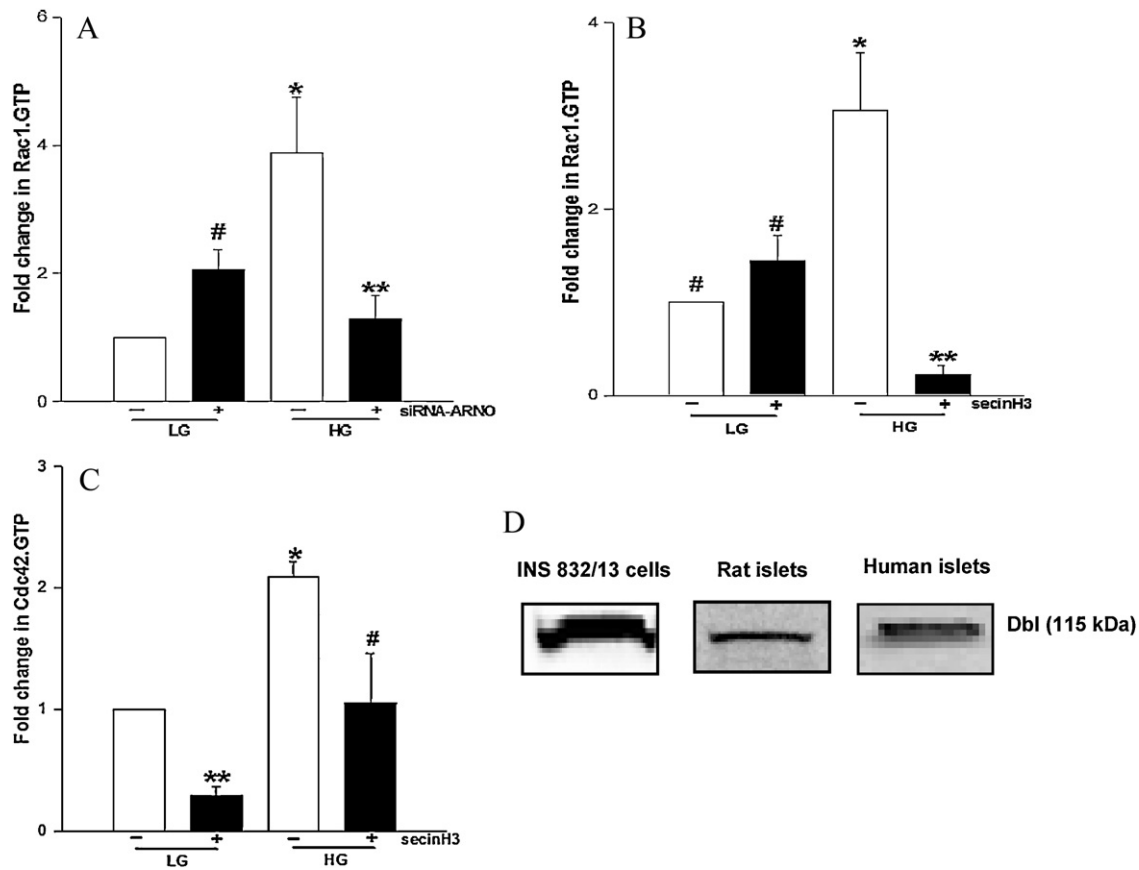


Fig. 6. Molecular biological or pharmacological inhibition of ARNO function attenuates glucose-induced Rac1 or Cdc42 activation in INS 832/13 cells. *Panel A:* INS 832/13 cells were either mock-transfected or transfected with siRNA-ARNO at a final concentration of 100 nM and after 48 h culture, cells were stimulated with either low glucose [LG, 2.5 mM] or high glucose [HG, 20 mM] for 30 min at 37 °C. The relative amounts of activated Rac1 [i.e., Rac1-GTP] were quantitated by PAK-PBD pull down [see Section 2 for additional details]. Total Rac1 from cell lysates was used as the loading control. Data were analyzed densitometrically and expressed as fold change in Rac1-GTP over basal. Data are mean \pm SEM of five independent experiments. * and # represent $p < 0.05$ vs. low glucose without siRNA-ARNO; and ** $p < 0.05$ vs. high glucose without siRNA-ARNO. *Panel B:* INS 832/13 cells were cultured overnight in the presence or absence of secinH3 [50 μ M] and further stimulated with low glucose [LG, 2.5 mM] and high glucose [HG, 20 mM] for 30 min in the continuous presence or absence of secinH3. The relative amounts of activated Rac1 [i.e., Rac1-GTP] were determined by PAK-PBD pull down assay as described in panel A. Total Rac1 from cell lysates was used as the loading control. Data were analyzed densitometrically and expressed as fold change in Rac1-GTP over basal and are mean \pm SEM of three independent experiments. * represents $p < 0.05$ vs. low glucose without secinH3; ** $p < 0.05$ vs. high glucose without secinH3, and data points with similar symbol do not differ statistically. *Panel C:* INS 832/13 cells were starved overnight in the presence or absence of secinH3 [50 μ M] and were stimulated with low glucose [LG, 2.5 mM] and high glucose [HG, 20 mM] for 3 min in the continuous presence or absence of secinH3. The relative amounts of activated Cdc42 [i.e., Cdc42-GTP] was determined by PAK-PBD pull down assay. Total Cdc42 from cell lysates was used as the loading control. Data were densitometrically analyzed and is expressed as fold change in Cdc42-GTP over basal and are mean \pm SEM of three independent experiments yielding similar results. * and ** represent $p < 0.05$ vs. low glucose without secinH3 and # $p < 0.05$ vs. high glucose without secinH3. *Panel D:* lysates from rat islets, human islets and INS 832/13 cells were separated by SDS-PAGE and probed for Dbl by Western blotting.

with regard to regulatory factors for Arf6 in β -cells. Therefore, in the current study, we tested the hypothesis that ARNO mediates the functional activation of Arf6, and that the ARNO/Arf6 signaling cascade, in turn, controls the activation of downstream regulatory proteins including Cdc42 and Rac1 leading to GSIS. In the present study we demonstrated that ARNO might subserve the function of a GEF for Arf6 in the islet β -cell. Salient features of our study are: [i] ARNO is expressed in clonal β -cells, rodent islets and human islets; [ii] overexpression of inactive mutants of ARNO or Arf6 or siRNAs of Arf6 or ARNO reduces insulin secretion elicited by glucose, arginine and KCl in insulin-secreting cells; [iii] secinH3, a selective inhibitor of ARNO/Arf6 signaling pathway, also inhibits GSIS in INS 832/13 cells and rodent islets; [iv] insulinotropic concentration of glucose stimulates Arf6 activation; [v] glucose-induced Arf6 activation is inhibited by secinH3 or siRNA-ARNO, suggesting a critical involvement of ARNO/Arf6 in insulin secretion; [vi] pharmacological or molecular biological inhibition of ARNO/Arf6 inhibits glucose-induced activation of Cdc42 and Rac1; and [vii] glucose promotes association between ARNO and Arf6 as evidenced by co-immunoprecipitation and confocal microscopic

studies. These findings provide the first evidence to implicate novel roles for ARNO in insulin secretion.

Regazzi et al. first reported [3,4] that incubation of permeabilized RINm5F cells with non-hydrolyzable analogs of GTP resulted in a significant redistribution of otherwise cytosolic Arfs to the Golgi and plasma membrane compartments. Based on these and insulin secretion data, they concluded that Arf is subjected to cycling between the membrane and soluble compartments and this cycling is governed by regulatory factors to mediate insulin secretion in clonal β -cells. And more recently Lawrence and Birnbaum demonstrated regulatory roles for Arf6 in insulin secretion from MIN6 cells [5]. Using adenoviral expression protocols involving the wildtype, dominant negative and constitutively active Arf6 mutants, these investigators provided compelling evidence to implicate regulatory roles for Arf6 in glucose-, KCl- and GTP γ S-induced insulin secretion. Along these lines, at least two other recent studies have verified roles for Arf6 in islet function and insulin secretion. Grodnitzky et al. have demonstrated a role for EFA6A, another GEF for Arf6 in somatostatin-mediated activation of PLD in clonal β [HIT-T15] cells. Interestingly,

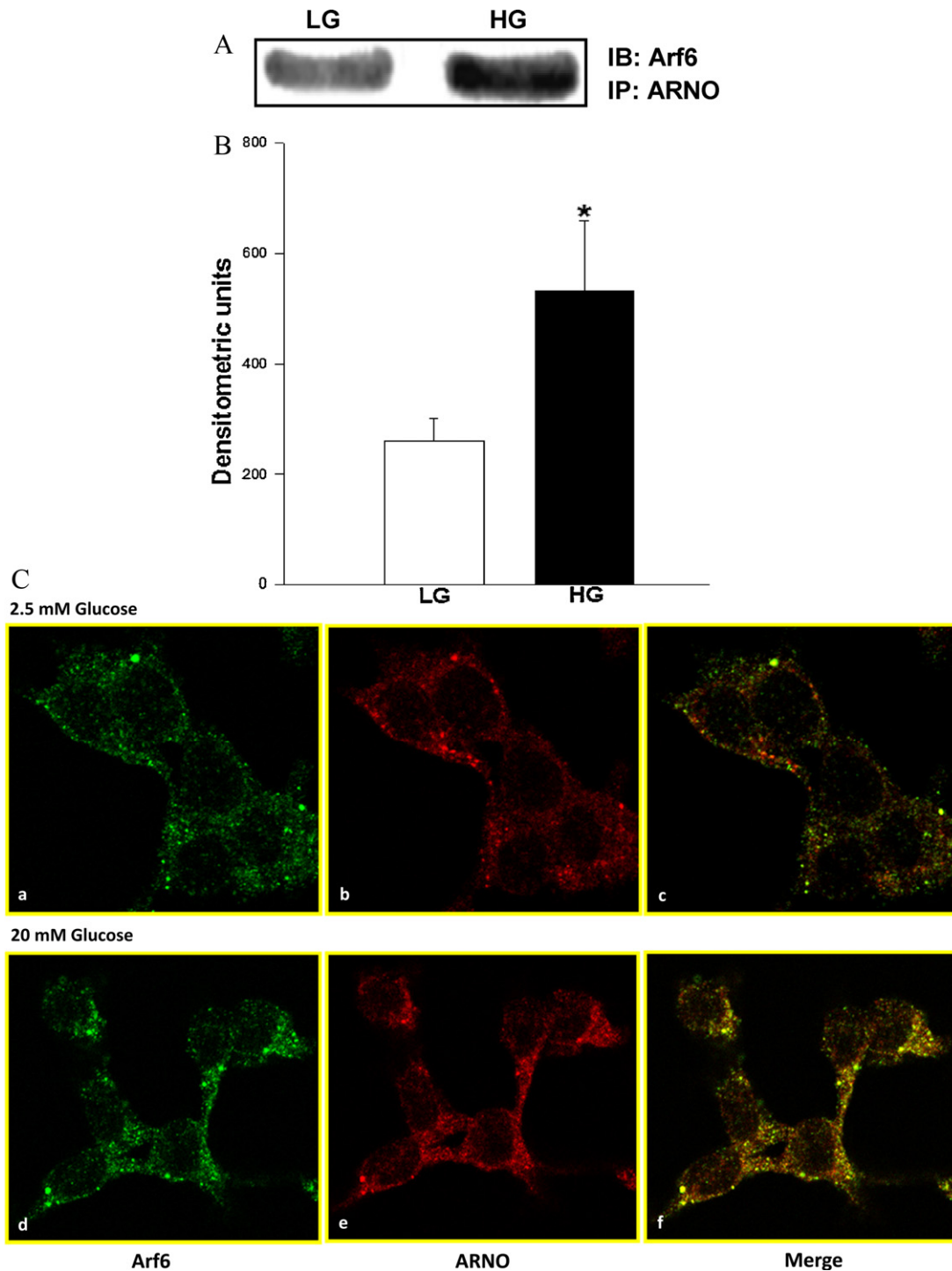


Fig. 7. Glucose promotes association between Arf6 and ARNO in INS 832/13 cells. *Panel A:* co-immunoprecipitation studies: herein, INS 832/13 cells were incubated in the presence of low glucose [LG, 2.5 mM] or high glucose [HG, 20 mM] for 30 min at 37 °C. ARNO was immunoprecipitated in the lysates using a specific antibody as described in Section 2. The immunoprecipitates were separated by SDS-PAGE and probed for Arf6. A representative blot from three studies is shown. *Panel B:* data from multiple studies shown in panel A are analyzed densitometrically and expressed as densitometric units and are mean ± SEM. * represents $p < 0.05$ vs. low glucose. *Panel C:* immunofluorescence studies using confocal microscopy: INS 832/13 cells were cultured on coverslips and cultured overnight prior to the incubation with either 2.5 mM or 20 mM glucose for 30 min at 37 °C. The cells were fixed in 4% paraformaldehyde solution in PBS for 15 min and permeabilized using 0.2% triton X-100 for 15 min. Fixed cells were examined for Arf6 [stained in green] and ARNO [stained in red] as described under Section 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

however, this pathway appears to be distinct from glucose-mediated effects in INS 832/13 cells and normal rat islets [current study] since overexpression of inactive ARNO mutant [E156K] failed to affect somatostatin-induced PLD activation [42]. More recent findings by Ma and coworkers further implicated Arf6 in glucose-induced PLD activation in MIN6N8 cells, which suggested

binding of Arf6 to PLD. Furthermore, brefeldin A, a known inhibitor of Arf6, decreased glucose-induced PLD activity and insulin secretion [43].

What then is the potential connection between glucose-induced ARNO/Arf6 signaling cascade and Rac1 activation that we have demonstrated in the current study? We proposed earlier

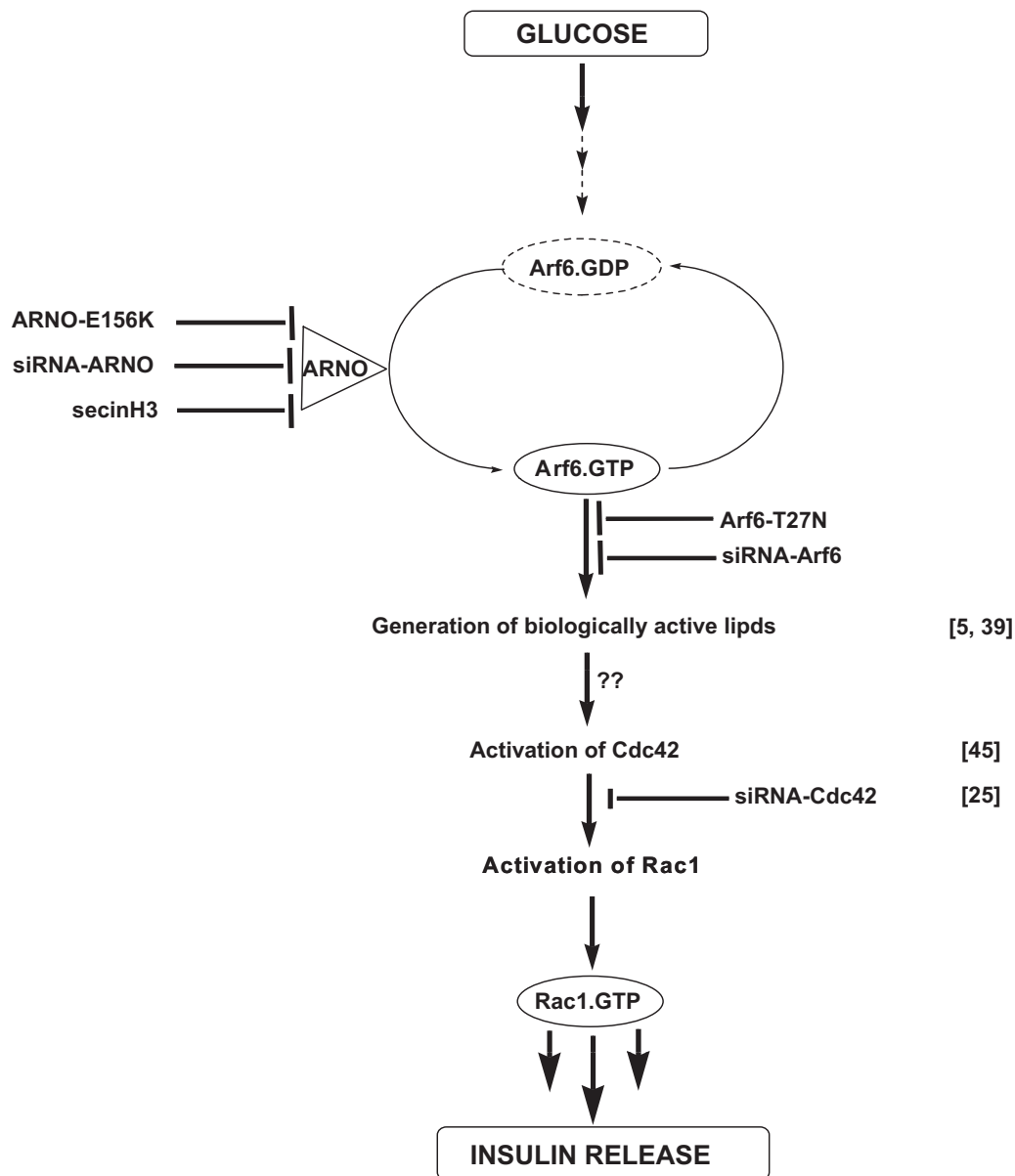


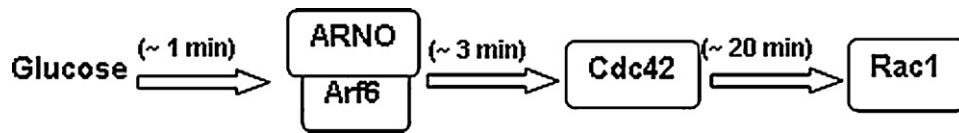
Fig. 8. A proposed mechanism for ARNO signaling axis in GSIS via sequential activation of Arf6, Cdc42 and Rac1 in pancreatic β -cells: based on the data described herein and our previously published data [22,23], we propose a model for potential involvement of ARNO as a GEF for Arf6 in promoting GSIS in pancreatic β -cells. We propose that glucose activates islet endogenous ARNO to facilitate the conversion of inactive GDP-bound Arf6 to its GTP-bound biologically active conformation. Essential nature for ARNO in this signaling cascade was demonstrated via the use of molecular biological [E156K mutant and siRNA-ARNO] and pharmacological approaches [e.g., secinH3]. A role for Arf6 in GSIS was confirmed via molecular biological [i.e., Arf6-T27N mutant and siRNA-Arf6] approaches. These data confirm the original observations of Lawrence and Birnbaum [5]. We propose that the activation of ARNO/Arf6 signaling pathway leads to the activation of PLD [43] leading to the generation of fusogenic lipids [e.g., PA], which in turn, promote dissociation of Rac1/GDI complexes leading to the activation and membrane association of Rac1 [22,23]. Our time course studies also suggest that ARNO-mediated activation of Cdc42 [within 1 min] is upstream to Rac1 activation [15–20 min] together suggesting that ARNO facilitates sequential activation of Arf6, Cdc42 and Rac1 to promote insulin secretion. Potential mechanisms underlying Arf6 mediated activation of Cdc42 remain to be determined. It might include dissociation of Cdc42/GDI complex by biologically active lipids as in the case of Rac1 [21,22] or inhibition of GAP activity specific for Cdc42 by ARNO/Arf6 signaling pathway [see Section 4]. These aspects are being investigated in our laboratory currently.

[22] that Rac1 activation might be downstream to Arf6 in the signaling events leading to GSIS. More recently we have provided further evidence to suggest that biologically active, fusogenic lipids regulate Rac1 signaling in the β -cell [23]. For example, using lysates derived from normal rat islets and INS 832/13 cells we have demonstrated that these lipids promote the dissociation of Rac1/GDI complex, which is necessary for the membrane translocation and activation of Rac1 [23]. Earlier observations also demonstrated potential involvement of PLD activation in the signaling mechanisms leading to GSIS [6,7,44–46]. Therefore, it is reasonable to

speculate that glucose-induced Arf6-mediated activation of PLD1 [43] results in biologically active, fusogenic lipids, which, in turn, facilitate the dissociation of Rac1/GDI complex and leading to the translocation, membrane association and activation of Rac1 culminating in the cytoskeletal remodeling and fusion of granules with the plasma membrane. This remains to be verified experimentally.

Our current findings from the time course studies suggested that Cdc42 activation [~ 3 min] step as an intermediate between Arf6 [~ 1 min] and Rac1 [~ 20 min] activation. In support of

sequential activation of Arf6, Cdc42 and Rac1, our current data demonstrated that inhibition of ARNO/Arf6 signaling leads to inhibition of Cdc42 and Rac1. Findings from Thurmond's laboratory have clearly demonstrated that Cdc42 plays an upstream regulatory role to Rac1 in GSIS [25]. Based on these findings, we propose that GSIS involves sequential activation of ARNO-mediated stimulation of Arf6, Cdc42 and Rac1 as shown below.



However, it remains to be verified if Cdc42 activation mechanism by ARNO/Arf6 also involves the dissociation of Cdc42/GDI complexes by biologically active lipids as in the case of Rac1 [23]. Further, it is likely that ARNO/Arf6-mediated Cdc42 activation in the β -cell might involve a mechanism similar to the one described in MCF7 cells by Dubois et al. [47]. These investigators have demonstrated that active Arf proteins bind to Cdc42. GTPase activating protein thereby preventing its GAP function and retain Cdc42 in the GTP-bound active conformation. Such a possibility remains to be verified for Arf6 in the islet.

Lastly, our current studies identify ARNO as one of the GEFs for glucose-induced activation of Arf6. The list of G-protein regulatory factors involved in GSIS continues to grow. Along these lines, we have identified Tiam1 as one of the GEFs for Rac1 [37]. Rho-GDI appears to subserve the roles of GDI for Cdc42 and Rac1 [29]. The above described Western blot data are suggestive of localization of Db1, a known GEF for Cdc42, in INS 832/13 cells, normal rat islets and human islets. Studies are underway to determine potential cross-talk between various GEFs and their corresponding G-proteins in the cascade of events leading to insulin secretion.

In conclusion, based on the existing experimental data including those described herein, we propose a mechanism [Fig. 8] involved in GSIS in pancreatic β -cells involving ARNO/Arf6 signaling steps. We propose that GSIS involves ARNO-mediated conversion of GDP-Arf6 [inactive] to GTP-Arf6 [active]. We verified this by overexpression of dominant negative mutants of ARNO and Arf6 as well as siRNA-mediated knockdown of these proteins in pancreatic β -cells. This postulation was further supported by our findings of pharmacological inhibition of glucose-induced ARNO-mediated activation of Arf6 by secinH3. Based on the data described herein and our previously published data [22,23], it is likely that glucose metabolic events lead to the activation of endogenous phospholipases, including PLD [44,45] and the subsequent generation of biologically active lipid second messengers [e.g., PA and PIP₂], which, in turn, facilitate the dissociation of Rac1/GDI complex for attaining the GTP-bound active conformation [23]. Potential involvement of ARNO/Arf6-mediated activation of Cdc42 and Rac1 via PLD remains to be verified. Finally, as we demonstrated previously [48], it is also likely that biologically active lipids could exert direct effects on the functional activation of specific GTPases by increasing their GTP binding function and decreasing their GTPase activities thereby retaining putative G-proteins in their GTP-bound active conformation [29].

Lastly, it may be germane to point out that data described above implicated regulatory roles for ARNO/Arf6 signaling axis in insulin secretion elicited by KCl. Our current observations are compatible with studies of Lawrence and Birnbaum implicating a role for Arf6 in KCl-induced insulin secretion [5]. However, it remains to be seen if ARNO/Arf6 signaling cascade regulates additional pathways which are independent of Cdc42/Rac1 activation in insulin-secreting cells. Such an examination is necessary since recent

studies have clearly implicated non-regulatory roles for Cdc42/Rac1 signaling pathways in KCl-induced insulin secretion ([28,29] and references therein). To the best of our knowledge, very little is known with regard to potential involvement of small G-proteins [e.g., Rac1] in insulin secretion facilitated by amino acids, such as arginine. These aspects, which are not included in the current model [Fig. 8], are being investigated in our laboratory currently.

Acknowledgements

This research was supported by a Merit Review Award from the Department of Veterans Affairs [to A.K.] and the National Institutes of Health [DK 74921 to A.K. and DK 55267 to C.J.R.]. A.K. is also the recipient of the Senior Research Career Scientist Award from the Department of Veterans Affairs. We thank Dr. Chris Newgard for INS 832/13 cells, Dr. Karl Olson for human islets, Dr. Michel Franco for Arf6 [T27N] and Dr. Nicolas Vitale for ARNO [E156K]. We also thank Carmel Harkins and Mary Olive [Wayne State University Confocal Facility] for their help with these studies.

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Isoprenylcysteine carboxyl methyltransferase facilitates glucose-induced Rac1 activation, ROS generation and insulin secretion in INS 832/13 β -cells

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Key words: Rac1, ROS, pancreatic islet, carboxymethylation and insulin secretion

Abbreviations: AFC, acetyl farnesyl cysteine; ICMT, isoprenylcysteine methyl transferase; DCHFDA, 2',7'-dichlorodihydrofluorescein diacetate; GSIS, glucose-stimulated insulin secretion; Nox, phagocyte-like NADPH oxidase

Isoprenylcysteine carboxyl methyltransferase (ICMT) catalyzes the post-translational methylation of C-terminal cysteines of isoprenylated proteins, including small G-proteins and the γ -subunits of heterotrimeric G-proteins. It is widely felt that carboxymethylation promotes efficient membrane association of the methylated proteins and specific protein-protein interactions. In the current study, we tested the hypothesis that ICMT-mediated carboxymethylation of specific proteins (e.g., Rac1) plays a regulatory role in glucose-stimulated insulin secretion (GSIS). Western-blot analysis indicated that ICMT is expressed and predominantly membrane associated in INS 832/13 β -cells. siRNA-mediated knockdown of endogenous expression of ICMT markedly attenuated glucose, but not KCl-induced insulin secretion. These findings were further supported by pharmacological observations, which suggested a marked reduction in glucose-, but not KCl-stimulated insulin secretion by acetyl farnesyl cysteine (AFC), a selective inhibitor of ICMT. In addition, glucose-induced Rac1 activation, a hallmark signaling step involved in glucose-stimulated insulin secretion, was markedly inhibited following pharmacological (AFC) or molecular biological (siRNA-ICMT) inhibition of ICMT. Lastly, we also noticed a marked reduction in glucose-induced acute increase in the generation of reactive oxygen species in INS 832/13 cells pre-treated with AFC or transfected with siRNA-ICMT. Together, these data suggest that ICMT regulates glucose-induced Rac1 activation, generation of reactive oxygen species and insulin secretion in pancreatic β -cells.

Introduction

It has been known for a long time that both monomeric G-proteins (e.g., Rac1 and Cdc42) and the γ -subunits of heterotrimeric G-proteins (G γ subunits) undergo post-translational modifications, such as isoprenylation and methylation at their C-terminal cysteine residues (often referred to as the CAAX motif).^{1,2} The first of the four step modification sequence includes incorporation of mevalonic acid-derived farnesyl or geranylgeranyl isoprenoid moiety onto the C-terminal cysteine. This is followed by the proteolytic cleavage of—AAX peptide by the Ras-converting enzyme1 (Rce1) endoprotease of microsomal origin, which leads to methylation of the prenylated cysteine by the carboxyl methyl transferase (ICMT) in the presence of S-adenosyl methionine serving as the methyl donor.³ It is widely accepted that the prenylation and carboxymethylation modification steps increase the

hydrophobicity of the candidate proteins for optimal targeting to their relevant membranous sites for the regulation of effector proteins.¹⁻³

While a significant number of recent studies have focused on putative roles of G-protein prenylation in glucose-stimulated insulin secretion (GSIS), very little is known with regard to the potential roles of carboxymethylation in islet function.³ Original studies from our laboratory have attempted to address the roles of carboxymethylation in islet function, including insulin secretion.^{4,5} Therein, using selective inhibitors of ICMT such as acetyl farnesyl cysteine (AFC), we have been able to demonstrate that Cdc42 and G γ subunits undergo carboxymethylation in response to glucose in clonal β -cells, normal rat and human islets.^{4,5} Follow-up studies by Li and coworkers characterized ICMT in insulin-secreting cells for its subcellular localization and regulation by known second messengers of insulin secretion.⁶

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Submitted: 12/07/10; Revised: 01/26/11; Accepted: 01/29/11

DOI:

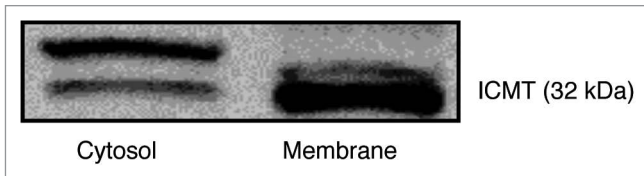


Figure 1. Expression and subcellular distribution of ICMT in INS 832/13 cells. Total particulate and soluble fractions were isolated from INS 832/13 cells by a single step centrifugation method described in Materials and Methods. ICMT expression was determined in these fractions by western blotting. A representative of three blots is shown here.

In the current study, we have revisited this area of islet biology to precisely determine the role of carboxymethylation and the identity of methylated proteins to further evaluate their roles in the signaling events leading to insulin secretion.

Along these lines, emerging evidence implicates novel regulatory roles for phagocyte-like NADPH oxidases (Nox) in physiological insulin secretion. For example, using selective inhibitors (e.g., DPI or apocynin) and molecular biological tools (e.g., antisense and siRNAs for Nox subunits), several recent studies have demonstrated “second messenger” roles for Nox-derived reactive oxygen species in glucose-stimulated insulin secretion.⁷⁻⁹ Some of these aspects, including downstream targets for reactive oxygen species signals, have been reviewed by Pi and Collins recently in reference 10. Furthermore, recent studies from our laboratory have also demonstrated a novel regulatory role for Rac1 in Nox-derived generation of reactive oxygen species, thus suggesting that glucose-induced Rac1 activation step might be necessary for Nox-mediated generation of reactive oxygen

species and insulin secretion. For example, using selective inhibitors of prenylation (e.g., GGTI-2147), we have demonstrated that post-translational prenylation of Rac1 is important for its regulation of generation of reactive oxygen species.¹¹ Therefore, based on the above evidence and as a logical extension to studies to suggest obligatory roles of ICMT-mediated carboxymethylation of Rac1 function for its subcellular localization and function,^{12,13} we undertook the current investigation to determine the regulatory roles of ICMT in glucose-induced Rac1 activation, generation of reactive oxygen species and insulin secretion in INS 832/13 cells. We have accomplished this goal by two distinct approaches to compromise the β -cell endogenous ICMT function, via siRNA-mediated knockdown of ICMT expression and pharmacological inhibition of ICMT by AFC. Indeed, data accrued from the current studies underscores the importance of carboxymethylation of Rac1 in glucose-induced Nox activation and associated generation of reactive oxygen species and insulin secretion.

Results

ICMT is expressed in INS 832/13 cells. At the outset we determined the immunological localization and subcellular distribution of ICMT in INS 832/13 cells. For this, total particulate and soluble fractions were isolated from INS 832/13 cells by a single step centrifugation method and relative abundance of ICMT was determined in these fractions by western blotting. Data in **Figure 1** suggested a predominant membrane association of ICMT in these cells. It should also be noted that we consistently observed a doublet for ICMT on western blots, which might

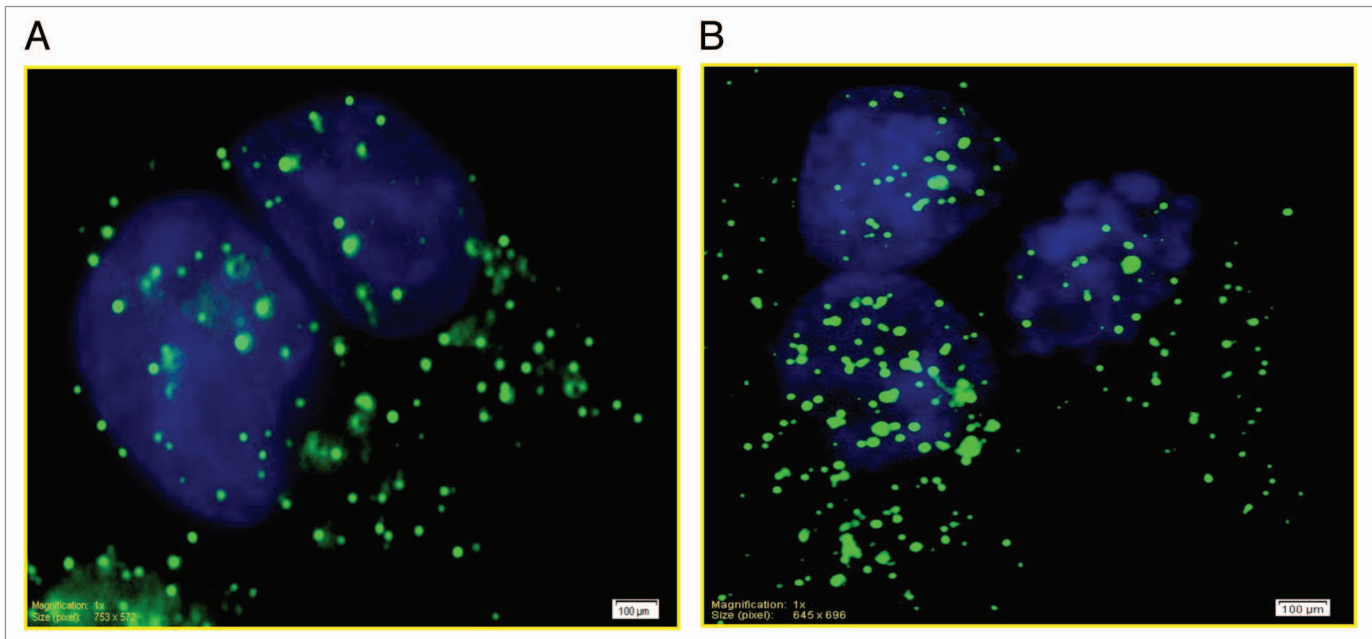


Figure 2. Localization of ICMT in INS 832/13 cells by immunofluorescence under basal and glucose-stimulated conditions. INS 832/13 cells were plated on coverslips and cultured overnight in low serum low glucose media prior to incubation with either 2.5 mM (A) or 20 mM glucose (B) for 45 min at 37°C. The cells were fixed in 4% paraformaldehyde solution in PBS for 15 min and permeabilized using 0.2% triton X-100 for 15 min. Fixed cells were examined for ICMT (stained in green) and nuclei (stained in blue) as described under Methods.

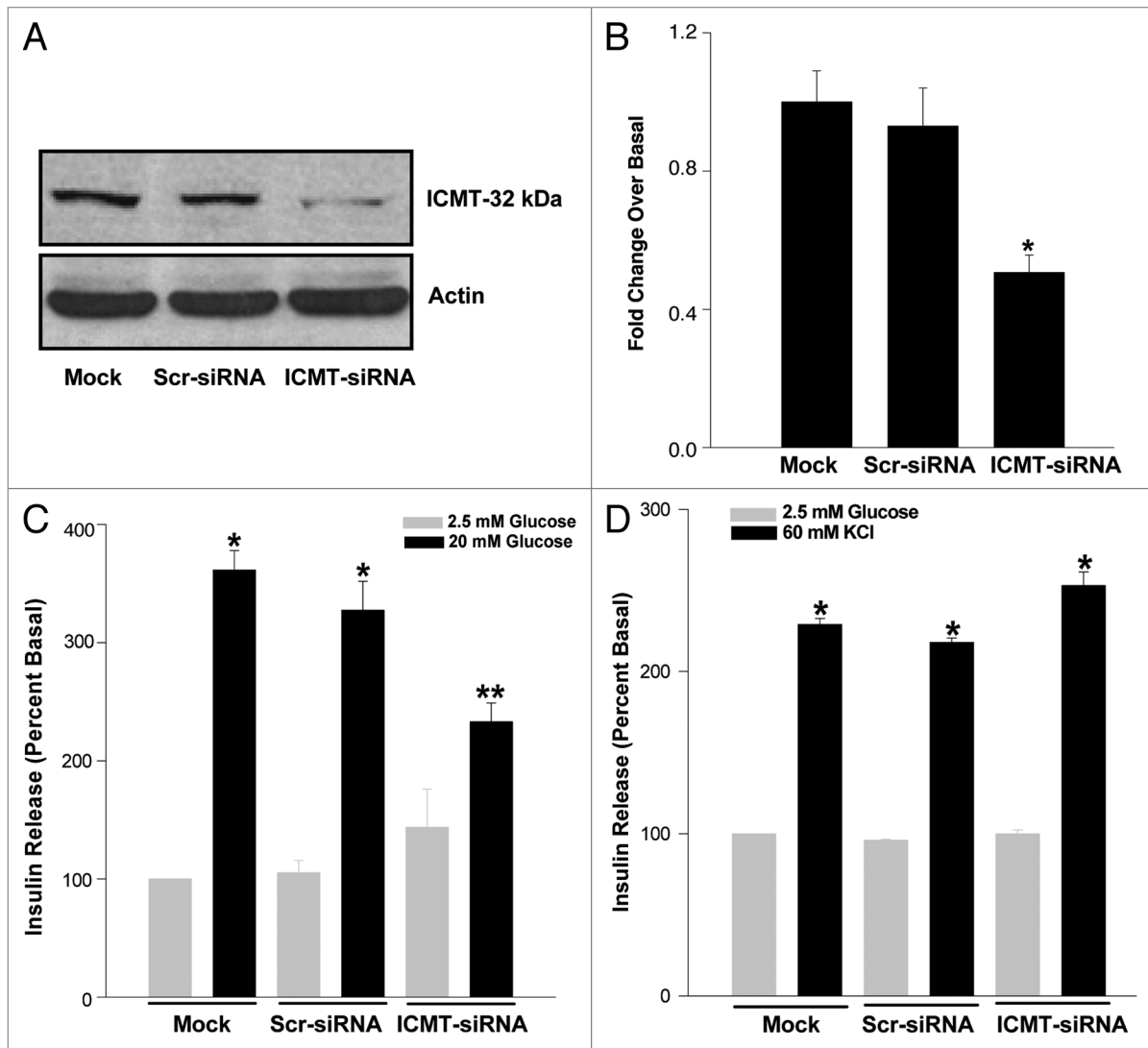


Figure 3. Glucose-, but not KCl-stimulated insulin secretion, is attenuated in INS 832/13 cells following siRNA-mediated knockdown of ICMT. INS 832/13 cells were either mock transfected or transfected with scrambled siRNA or siRNA-ICMT at a final concentration of 100 nM and cultured for 24 h. Transfection efficiency was determined by separating equal amounts of proteins on SDS-PAGE and probing with ICMT antibody [(A) representative of three transfections is shown here]. Data in (A) was densitometrically analyzed and expressed as fold change over basal (B). * $p < 0.05$ compared with mock or scrambled siRNA transfected cells. Further, transfected cells were incubated either with low glucose (LG; 2.5 mM) or high glucose [(C) HG; 20 mM] or a membrane depolarizing concentration of KCl [(D) 60 mM; osmolality adjusted by lowering NaCl] for 45 min at 37°C. Insulin released into the medium was quantitated by ELISA. Data are expressed as percentage of basal and are mean \pm SEM from three independent determinations. * $p < 0.05$ vs. respective low glucose controls; ** $p < 0.05$ vs. mock transfected cells.

represent a post-translationally modified form of this protein. In the next series of studies we determined the distribution of ICMT in INS 832/13 cells by immunofluorescence method. Data in Figure 2 suggested that ICMT (green) remain diffused throughout the cell under basal [(A) LG; 2.5 mM glucose] conditions. Further, we observed no clear effects of stimulatory glucose [(B) HG; 20 mM glucose] on ICMT distribution in these cells.

siRNA-mediated knockdown of ICMT attenuates glucose-, but not KCl-induced insulin secretion in INS 832/13 cells. We next investigated potential regulatory roles of ICMT in glucose-induced insulin secretion in these cells. To address this, we knocked down the endogenous expression of ICMT by siRNA methodology. Data in Figure 3A and B indicated more

than ~70 % inhibition in the expression of ICMT following siRNA-ICMT transfection. Data in Figure 3C suggested no significant effects of scrambled siRNA transfection either on basal or glucose-induced insulin secretion (bars 1 vs. 3 and 2 vs. 4). However, transfection of siRNA-ICMT in these cells led to a modest increase in basal secretion (bars 1 or 2 vs. 5), but insulin secretion elicited by stimulatory glucose was significantly reduced in ICMT knocked down cells (bars 2 or 4 vs. 6). This data suggested that activation of ICMT is necessary for glucose-stimulated insulin secretion to occur. We then determined potential requirement for ICMT in insulin secretion elicited by a membrane depolarizing concentration of KCl. Data shown in Figure 3D suggested no significant effects of ICMT knockdown

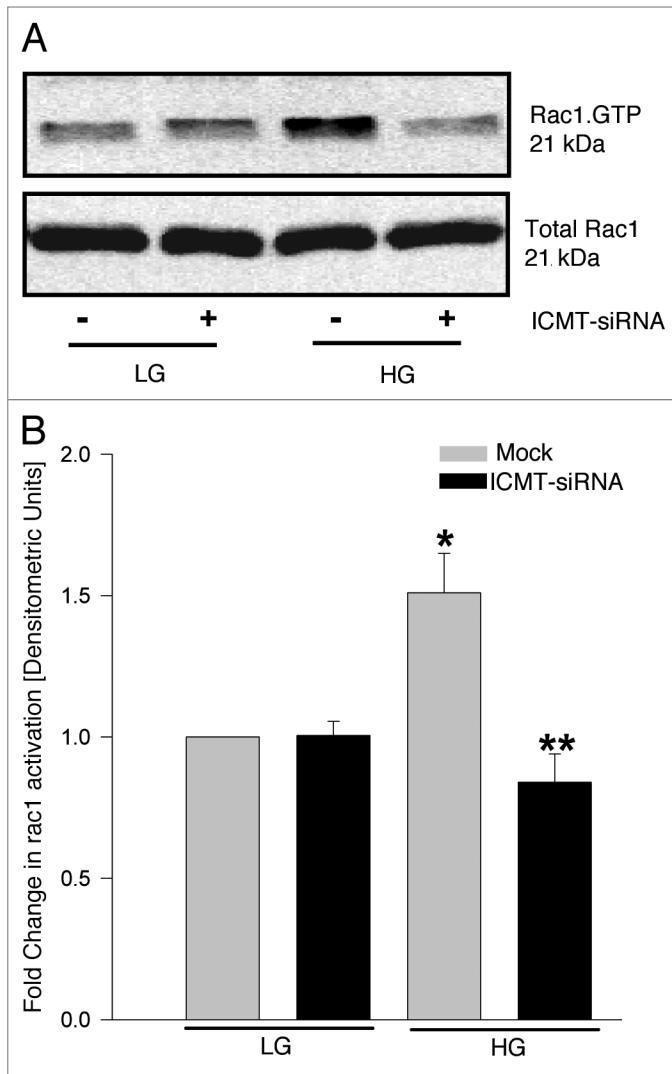


Figure 4. Depletion of endogenous ICMT markedly attenuates glucose-induced activation of Rac1 in INS 832/13 cells. INS 832/13 cells were transfected with ICMT-siRNA or mock transfected and cultured for 24 h. At confluence, cells were starved overnight and stimulated with either low (2.5 mM) or high (20 mM) glucose for 30 min. The extent of Rac1 activation in these cells was quantitated by PAK-PBD pull-down assay. Total and activated (Rac1.GTP) were determined by western blotting (A) and quantitated by densitometry (B). Data are expressed as fold change in Rac1 activation and are mean \pm SEM from three independent determinations. * $p < 0.05$ vs. mock transfected low glucose; ** $p < 0.05$ vs. mock transfected high glucose.

on KCl-induced insulin secretion. Together, data in **Figure 3C and D** suggest that glucose, but not KCl-evoked insulin secretion is mediated via activation of ICMT.

siRNA-mediated knockdown of ICMT attenuates glucose-induced Rac1 activation in INS 832/13 cells. Published evidence from several laboratories, including our own have suggested that activation of Rac1, a small G-protein, is a requisite step in the signaling events leading to glucose-insulin secretion.^{2,14,15} Furthermore, using inhibitors of post-translational geranylgeranylation (e.g., GGTI-2147) or a dominant negative mutant of the α -subunit of geranylgeranyl transferase, we have demonstrated a

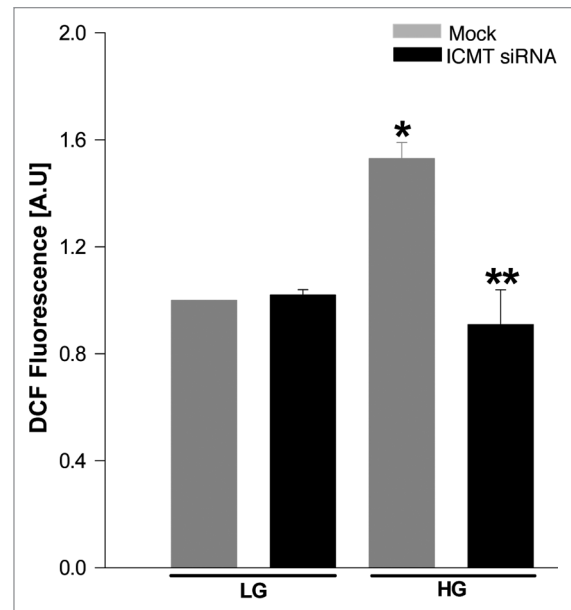


Figure 5. Glucose-induced ROS generation was attenuated in INS 832/13 cells following siRNA-mediated knockdown of ICMT. INS 832/13 cells transfected with ICMT-siRNA (or mock transfected) following which cells were stimulated with low glucose (2.5 mM) or high glucose (20 mM) for 1 h and were incubated with DCFHDA (10 μ M; 30 min) and harvested for quantitation of DCF fluorescence. Data expressed as DCF fluorescence and are mean \pm SEM from three independent determinations. * $p < 0.05$ vs. respective low glucose; ** $p < 0.05$ vs. high glucose in mock transfected cells.

requirement for post-translational geranylgeranylation in glucose-induced Rac1 activation and insulin secretion.¹⁶ Since Rac1 undergoes carboxymethylation, we investigated if silencing of ICMT affects glucose-induced Rac1 activation. Data shown in **Figure 4** demonstrated a significant increase in glucose-induced Rac1 activation (lane 1 vs. 3). siRNA-mediated knockdown of ICMT failed to exert any clear effects on basal Rac1 activation (lane 1 vs. 2), but significantly attenuated glucose-induced Rac1 activation (lane 3 vs. 4). Pooled data from multiple experiments are provided in **Figure 4B**. Together, these findings suggested a requirement for carboxymethylation for glucose-induced activation of Rac1.

siRNA-mediated knockdown of ICMT markedly inhibits glucose-induced reactive oxygen species generation in INS 832/13 cells. Emerging evidence from multiple laboratories appears to suggest novel second messenger roles for reactive oxygen species in glucose-stimulated insulin secretion.¹⁰ It has also been shown that reactive oxygen species generated via the activation of phagocyte-like NADPH oxidase (Nox) plays such regulatory roles in glucose-stimulated insulin secretion since pharmacological (e.g., apocynin or DPI) or molecular biological (e.g., siRNA or antisense for p47^{phox}) inhibition of Nox led to inhibition of glucose-stimulated insulin secretion.⁸⁻¹⁰ Since Rac1 represents one of the members of Nox holoenzyme,^{2,18} we investigated if siRNA-mediated knockdown of ICMT exerts any regulatory effects on glucose-induced generation of reactive oxygen species in INS 832/13 cells. Data in **Figure 5** suggested no

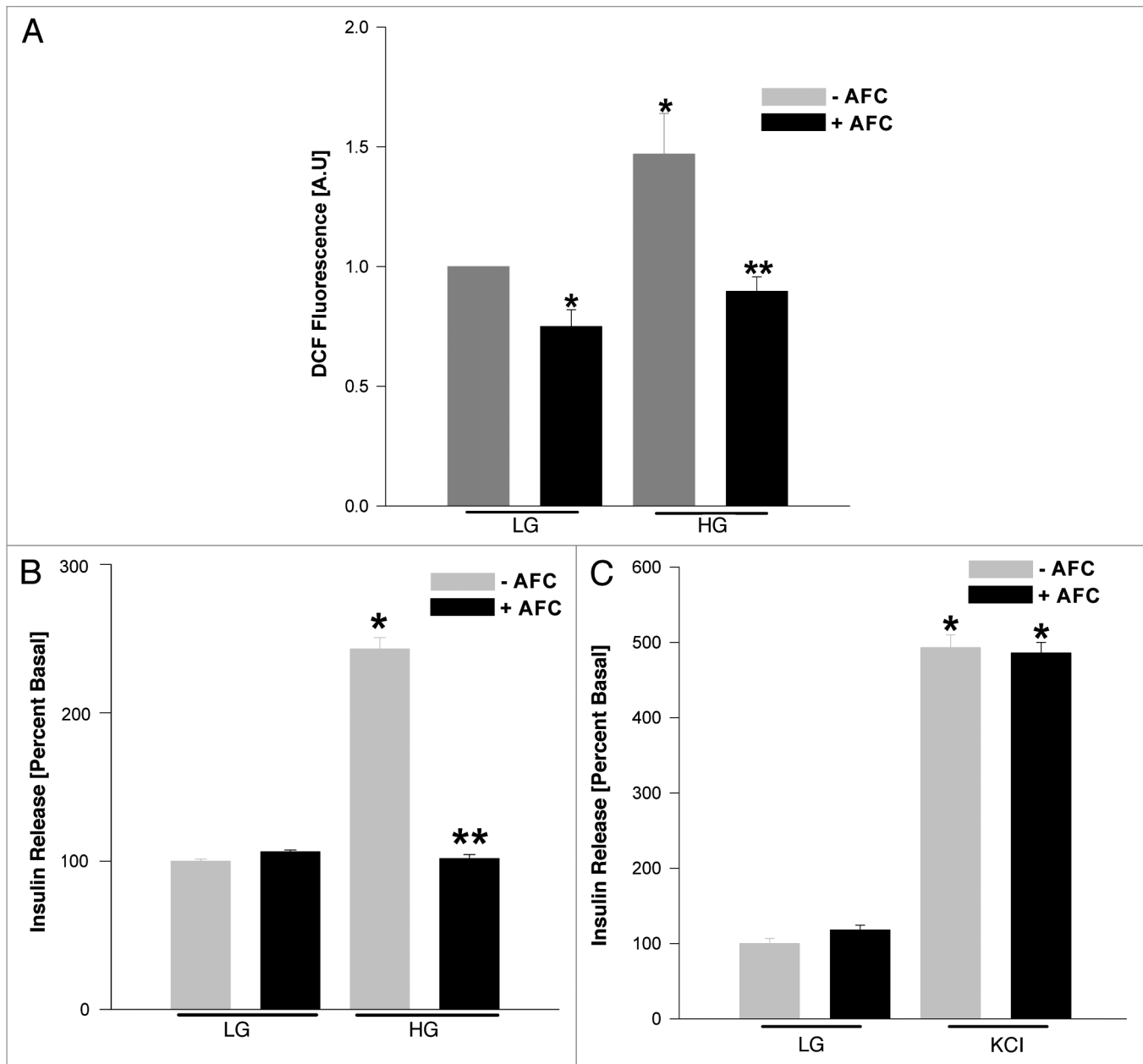


Figure 6. AFC, a competitive inhibitor of ICMT, attenuates glucose-induced ROS generation and insulin secretion in INS 832/13 cells. INS 832/13 cells were cultured overnight with low-glucose and low-serum medium and then incubated in KRB in the presence of diluent or AFC (100 μ M; 1 h) as indicated in the figure. Cells were further stimulated with either low glucose (LG; 2.5 mM) or high glucose (HG; 20 mM) for 1 h in continuous presence or absence of diluent or inhibitor. At the end of stimulation, ROS generation was determined by quantitating DCF fluorescence (A) as described in Figure 5. In a separate set of studies glucose- and KCl-stimulated insulin secretion was quantitated (B and C) under conditions described in Materials and Methods. Data in (B) are mean \pm SEM from three independent determinations. * $p < 0.05$ vs. low glucose without AFC; ** $p < 0.05$ vs. high glucose without AFC whereas data in (C) are mean \pm SEM from 12 determinations in each case. * $p < 0.05$ vs. low glucose without AFC and low glucose with AFC.

significant effects of ICMT knockdown on basal levels of reactive oxygen species in these cells (bar 1 vs. 2). However, glucose-induced generation of reactive oxygen species was markedly attenuated in cells in which expression of ICMT was knocked down (bar 3 vs. 4). Taken together, these data demonstrated that glucose-induced Rac1 activation (Fig. 4), generation of reactive oxygen species (Fig. 5) and insulin secretion (Fig. 3) are regulated by ICMT in INS 832/13 β -cells.

Acetyl farnesyl cysteine (AFC), a selective inhibitor of ICMT, attenuates glucose-induced generation of reactive oxygen species and insulin secretion in INS 832/13 cells. We next confirmed the above data accrued through the use of siRNA-ICMT by a pharmacological approach. In the following studies, we determined the effects of acetyl farnesyl cysteine (AFC), a selective inhibitor of ICMT^{4,5} on glucose-induced generation of reactive oxygen species and insulin secretion.

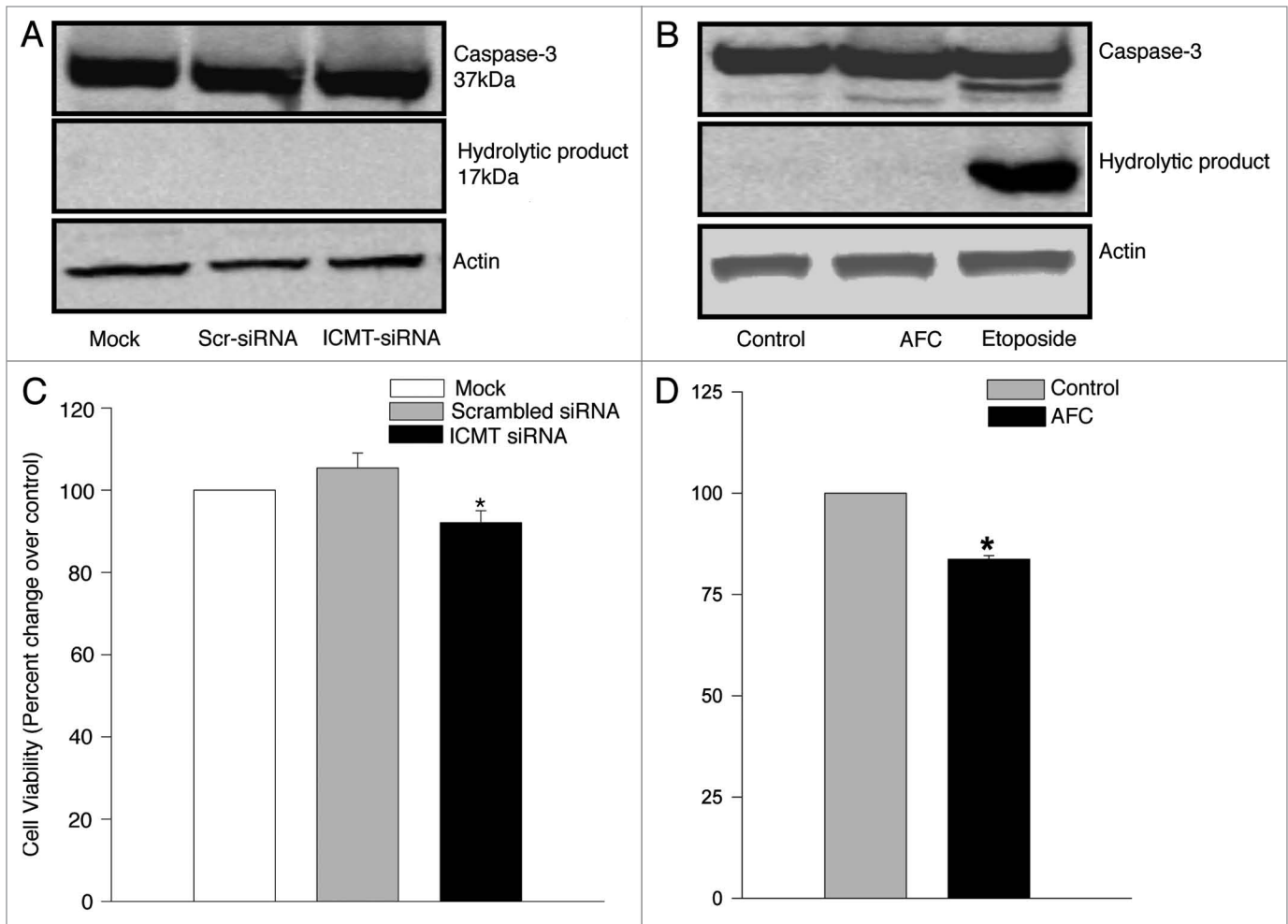


Figure 7. ICMT inhibition does not affect cell viability. (A) INS 832/13 cells were mock transfected or transfected either with ICMT siRNA or scrambled siRNA (100 nmol, 24h) or treated with AFC [(B) 100 μ M, 1h]. Activated caspase 3 in the lysates was determined by western blot analysis using an antiserum that identifies both the native procaspase and degradative product of caspase 3 using etoposide as a positive control as described under Methods. A representative blot for [(A) $n = 2$ determinations] and for [(B) $n = 3$ determinations] is shown here. Actin was used as a loading control. (C) INS 832/13 cells were mock transfected or transfected either with ICMT siRNA or scrambled siRNA (100 nmol, 24h). Cell viability in transfected cells was determined by MTT reduction method as described above. Data are means \pm SEM from two independent experiments yielding identical results with $n > 12$ in each group and expressed as percent change over control. * represents $p < 0.05$ compared with mock or scrambled siRNA transfected cells. (D) INS 832/13 cells treated with AFC (100 μ M, 1h) were incubated with MTT (5 mg/mL, 4h) as described in Materials and Methods. Cell viability was determined by quantitating reduction of MTT by metabolically active cells at 570 nm. Data are means \pm SEM from two independent experiments yielding identical results with $n > 12$ in each group and expressed as percent change over control. * represents $p < 0.05$ compared with control.

Data shown in **Figure 6A** indicated a modest, but significant inhibition in basal level of reactive oxygen species in these cells following exposure to AFC (**Fig. 6**; bar 1 vs. 2). However, increase in the level of reactive oxygen species seen in the presence of stimulatory glucose was significantly inhibited by AFC (**Fig. 6**; bar 3 vs. 4). Furthermore, insulin secretion elicited by stimulatory (**Fig. 6B**; bar 3 vs. 4), but not basal glucose (**Fig. 6B**; bar 1 vs. 2), was markedly attenuated by AFC. In addition, in a manner akin to siRNA-ICMT effects, we observed no significant effects of AFC on KCl-induced insulin secretion (**Fig. 6C**). Together, our above described findings confirm that glucose-, but not KCl-mediated effects on insulin secretion require activation of ICMT. Furthermore, along these lines, we also noticed a significant inhibition of glucose-induced activation of Rac1 by AFC under the conditions it inhibited glucose-induced generation of reactive

oxygen species ($\sim 41 \pm 10\%$ inhibition by AFC; mean \pm SEM from three pull down assays; $p < 0.05$ vs. diluent) and insulin secretion (additional data not shown). Together, these data further confirm our siRNA-ICMT findings and support our hypothesis that ICMT-mediated carboxymethylation of specific proteins (e.g., Rac1) plays a positive modulatory role in the cascade of events leading to glucose-induced generation of reactive oxygen species and insulin secretion in INS 832/13 cells.

Inhibition of ICMT does not affect cell viability. We next investigated potential cytotoxic effects, if any, of ICMT knock-down (via siRNA-ICMT) or inhibition of ICMT activity (by AFC) on INS 832/13 cells. We asked this question to be sure that either inhibition in Rac1 activation, reactive oxygen species generation or insulin secretion seen under these conditions are not due to potential loss in cell viability or cell demise following

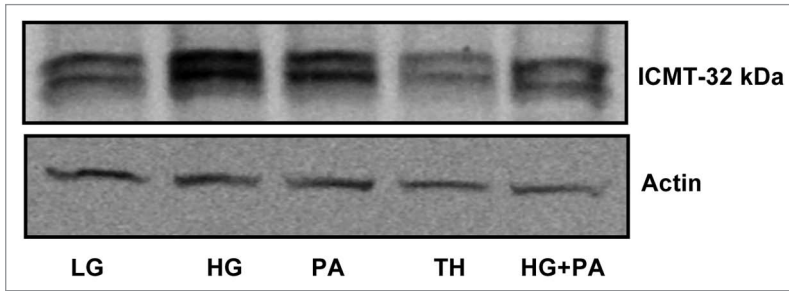


Figure 8. Expression of ICMT in lysates of INS 832/13 cells under the duress of gluco-, lipo-, glucolipototoxicity and endoplasmic reticulum stress. INS 832/13 cells were plated in six-well plates, grown to 70% confluence and treated with low glucose (LG, 2.5 mM, 48 h), high glucose (HG, 50 mM, 48 h), palmitic acid (PA, 300 μ M; 48 h), HG plus PA (48 h) and thapsigargin (TH, 0.5 μ M, 9 h). ICMT expression was determined by western blotting. A representative of two blots is shown here. Actin was used as a loading control.

inhibition of ICMT expression and/or activity. We addressed this by two independent experimental approaches. In the first, we quantitated activation of caspase-3, a hallmark of cellular apoptosis, in both siRNA-ICMT transfected cells and AFC-treated cells. In the second approach, we quantitated the metabolic viability of siRNA-ICMT transfected or AFC-treated cells using the MTT assay. Data shown in **Figure 7A and B** indicated no caspase 3 activation following siRNA-ICMT transfection or AFC treatment. However, a significant activation of caspase 3 was seen in INS 832/13 cells treated with etoposide, which causes apoptosis in cells via caspase 3 activation. Together, these data in **Figure 7A and B** suggest no cell death in INS 832/13 cells following inhibition of expression and activity of ICMT. In addition, we observed only a modest inhibition in cell viability as assessed by the MTT in cells following ICMT knockdown via siRNA-ICMT (**Fig. 7C**) or AFC treatment (**Fig. 7D**). Together, these findings suggest that the observed inhibition of glucose-induced Rac1 activation, generation of reactive oxygen species and insulin secretion following inactivation of ICMT are specific and do not involve cytotoxic mechanisms.

Alterations in ICMT expression in in vitro models of gluco-, lipo-, glucolipototoxicity and endoplasmic reticulum stress. A growing body of evidence implicates that long-term exposure of β -cells to saturated fatty acids (i.e., lipotoxicity), glucose (i.e., glucotoxicity) or both (i.e., glucolipototoxicity) leads to severe metabolic dysfunction and eventual demise of the β -cell.¹⁹ Furthermore, exposure of these cells to thapsigargin, leads to endoplasmic reticular stress via depletion of calcium pools culminating in cellular dysfunction.^{20,21} Therefore, in the last series of these studies we investigated potential alterations in the expression of ICMT in INS 832/13 cells following exposure to palmitate, glucose or thapsigargin. Data shown in **Figure 8** indicated a significant increase in the expression of ICMT in cells exposed to gluco-, lipo- or glucolipototoxic conditions. However, no detectable changes were seen in the expression of ICMT protein in thapsigargin-treated cells.

Discussion

Several earlier studies have implicated activation of small G-proteins (e.g., Arf6, Cdc42 and Rac1) in physiological insulin secretion. Such conclusions were drawn from studies involving the use of Clostridial toxins, dominant negative mutants, siRNAs and inhibitors of post-translational modifications, including prenylation, carboxymethylation and palmitoylation (reviewed in ref. 2). To the best of our knowledge, the current study provides the first evidence to implicate carboxymethylation of Rac1 in the

signaling cascade leading to glucose-induced ROS generation and insulin secretion. We have presented supporting evidence via two distinct approaches, namely siRNA-mediated knockdown or selective pharmacological inhibition of ICMT, which mediates the carboxymethylation of these signaling proteins.

At least two distinct carboxylmethyl transferases have been identified in insulin secreting cells. The first one is involved in methylating the carboxy terminal leucine (Leu-309) of the catalytic subunit of protein phosphatase 2A; such a signaling step has been implicated in subunit interaction and catalytic activation of the enzyme.²² The second enzyme, which is the focus of the current study, is the ICMT. In a previous study, Li and associates characterized the ICMT in insulin-secreting cells and normal rat islets.⁶ Such an activity was monitored by quantitating the degree of methylation of AFC by the islet ICMT in the presence of [³H] S-adenosylmethionine as the methyl donor. Subcellular fraction assays revealed that this enzyme activity is enriched in the endoplasmic reticulum.⁶ Along these lines, using the pharmacological approaches, we have demonstrated that glucose promotes the carboxymethylation of Cdc42, another small G-protein involved in cytoskeletal remodeling and glucose-stimulated insulin secretion.⁴ It was also demonstrated that the G γ -subunits also undergo carboxymethylation in a glucose-sensitive manner in clonal β -cells, normal rat islets and human islets.⁵ Not much has been reported since then with regard to potential functional consequences of carboxymethylation in islet function primarily due to lack of experimental tools (e.g., siRNA) to selectively deplete the expression of ICMT in isolated β -cells. Indeed, data from the current investigation further reinforce our original hypothesis that in addition to prenylation, carboxymethylation of specific G-proteins (e.g., Rac1) plays regulatory roles in physiological insulin secretion. Such regulatory effects may, in part, be due to the ability of methylated Rac1 to increase the activation of Nox and associated generation of reactive oxygen species.

Data accrued in the current studies implicate carboxymethylation as one of the requisite signaling steps for glucose-induced activation by Rac1 in a stimulated β -cell. Moreover, the carboxymethylation of Rac1 appears to be necessary for glucose-induced Nox activation and generation of reactive oxygen species. In this context, using reconstituted systems and the C-terminal Rac1 peptides, Kreck and coworkers have provided experimental support to implicate participatory roles for Rac1 in cell-free activation and assembly of NADPH-oxidase.²³ Compatible with these findings are our recent data to implicate inhibition of glucose- or mitochondrial-fuel-induced Nox activation and generation of reactive oxygen species in INS 832/13 cells and normal rat islets by inhibitors

of protein prenylation. These studies thus provided evidence for requisite roles for prenylation in the functional regulation of Nox in the islet β -cell.¹¹ Data from the current investigation indicate that in addition to prenylation, the carboxymethylation of specific G-proteins may be necessary for optimal regulation of Nox by glucose. More importantly, our current findings also suggest that carboxymethylation is necessary for glucose-induced activation of Rac1, since pretreatment of isolated β -cells with AFC or selective depletion of ICMT by siRNA markedly attenuated glucose-induced Rac1 activation. These findings are in agreement with recent findings of Cushman and Casey demonstrating inhibition of EGF-induced Rho A and Rac1 activation by cysmethynil, a selective inhibitor of ICMT, in MDA-MD-231 cells.²⁴ Together, based on the above discussion it is concluded that both prenylation and carboxymethylation of Rac1 are necessary for glucose-induced Nox-mediated ROS generation and insulin secretion. It is important to note that palmitoylation of cysteine residues upstream to prenylated and carboxymethylated Rac1 may not be involved in this signaling cascade at least based on recent studies from Roberts and associates who reported no known consensus palmitoylation motifs for Rac1,²⁵ although this remains to be verified experimentally in the islet β -cell.

Emerging evidence appears to implicate a significant contributory role for Nox in the generation of oxidative stress and the onset of mitochondrial dysfunction in multiple cell types, including the islet β -cell. For example, it has been shown that chronic exposure of isolated β -cells to high concentrations of saturated fatty acids (e.g., palmitate; lipotoxicity), glucose (i.e., glucotoxicity) or both (i.e., glucolipotoxicity) or a mixture of cytokines (e.g., IL-1 β , TNF α and IFN γ) culminates in increased oxidative stress, mitochondrial dysfunction and apoptosis in these cells.²⁶⁻²⁸ Inhibition of protein prenylation of Rac1 by pharmacological approaches (e.g., GGTI-2147) or Rac1 activation by Tiam1, a known guanine nucleotide exchange factor for Rac1 (using NSC23766) markedly attenuated metabolic dysfunction of the β -cell.^{27,28} Along these lines, data described herein suggest a significant increase in the expression of ICMT under glucolipotoxic conditions. Whether such an increase in the expression translates into increased ICMT activity remains to be verified. Nonetheless, it may be likely that use of selective inhibitors of carboxymethylation might prove to be valuable in preventing oxidative stress induced under the duress of glucolipotoxicity and/or cytokines. These are being studied in our laboratory currently. Based on the data accrued in the current studies we conclude that ICMT regulates glucose-induced Rac1 activation, generation of reactive oxygen species and insulin secretion in pancreatic β -cells.

Materials and Methods

Materials. siRNA-ICMT (Cat # 43907710) and scrambled siRNA (negative control; Cat # 4390843) were from Ambion. AFC was from Cayman Chemical (Cat # 63270). ECL reagent was from GE Healthcare (Cat # RPN2132). HiPerFect transfection reagent was obtained from Qiagen (Cat # 301705). The rat insulin ELISA kit was from American Laboratory Products (Cat # 80-INSRTH-E01). Rac1 activation assay kit was from

Cytoskeleton (Cat # BK035). ICMT antiserum was from Santa Cruz Biotechnology, Inc., (Cat # Sc-130150). DCHFDA (Cat # 35845), thapsigargin (Cat # T9033) and etoposide (Cat # E1383) were from Sigma Aldrich. Alexa-fluor 488 anti-rabbit secondary antibody (Cat # A11008) and Hoechst dye (Cat# 3570) was from Invitrogen molecular probes. Cell proliferation kit (MTT, Cat # 11465007001) was purchased from Roche diagnostics.

Insulin-secreting cells. INS 832/13 cells were provided by Dr. Chris Newgard (Duke University Medical Center, Durham, NC) and were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum supplemented with 100 IU/ml penicillin and 100 IU/ml streptomycin, 1 mM sodium pyruvate, 2-mercaptoethanol (50 μ M) and 10 mM HEPES (pH 7.4). The medium was changed twice weekly and cells were trypsinized and subcloned weekly.

Isolation of total particulate and soluble fractions from INS 832/13 cells. INS 832/13 cells were homogenized in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na₃VO₄, 1 mM NaF and protease inhibitor cocktail) and were centrifuged at 105,000x g for 1 h to separate total particulate and soluble fractions. Proteins from individual fraction were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The blots were then probed with antibody raised against ICMT (1:500 dilution) and with rabbit secondary antibody conjugated to horseradish peroxidase. Immune complexes were then detected using the enhanced chemiluminescence kit.

Immunofluorescence studies. INS 832/13 cells were plated onto coverslips and incubated with (2.5 or 20 mM) glucose for 45 min at 37°C followed by washing in PBS and fixed with 4% paraformaldehyde solution for 15 min at room temperature. They were then permeabilized with 0.2% Triton X-100 for 15 min at room temperature. After blocking with 1% BSA for 1 h, the cells were further incubated with primary antibody ICMT (1:150) in 0.1% BSA solution for 1 h. After extensive washes, the cells were further incubated with secondary antibody Alexa-fluor 488 anti-rabbit (1:1,000) in 0.1% BSA solution for 1 hr at 37°C. Hoechst dye was used to stain for nuclei. The coverslips were then mounted on glass slides containing mounting media (DAKO corporation; Carpinteria, CA) and visualized under an Olympus IX71 inverted fluorescence microscope using a x100 oil-immersion lens.¹⁷

siRNA-mediated knockdown of ICMT. Endogenous expression of ICMT was knocked down by transfecting INS 832/13 cells with ICMT-siRNA. In brief, INS 832/13 cells were plated on 24-well plates and transfection with ICMT-siRNA was performed at 50–60% confluence at a final concentration of 100 nM using HiPerFect transfection reagent. Further, to assess specificity of siRNA, cells were transfected in parallel with non-targeting siRNA (scrambled siRNA; 100 nM). Transfected cells were cultured in complete growth medium for 24 h and efficiency of ICMT knockdown was determined by western blot analysis.

Caspase 3 activity. Activation of caspase-3 was assessed in cells either transfected with ICMT siRNA or in cells treated

with AFC. Cells were harvested and homogenized in sample buffer (0.5% Nonidet P-40, 20 mM HEPES, pH 7.4, 100 mM NaCl and 20 mM DTT and PIC). And ~30 µg of proteins were resolved by SDS-PAGE (12%) and immunoprobed for caspase-3. Activation of caspase-3 is evidenced by the presence of a hydrolytic product (~17 kDa). Etoposide (60 µM, 6 h) was used as a standard for apoptotic cell death.

Cell viability assay. INS 832/13 cells were either treated with AFC (100 µM, 1 h) or transfected with ICMT-specific or scrambled siRNA as described above. Cell viability was determined by incubating AFC treated or ICMT-siRNA transfected cells with 10 µL of stock MTT [4(5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] for 4 h at 37°C. Following dissolution of formazan crystals in solubilization solution, the absorbance was measured at 570 nm using ELISA plate reader.

Insulin release studies. siRNA-ICMT or scrambled siRNA transfected cells or AFC-treated cells were cultured overnight in low serum-low glucose-containing media. They were stimulated further either with low or high glucose or potassium chloride in Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4 for periods of time indicated in the text. In studies involving KCl-induced insulin secretion, we noticed that INS 832/13 cells were not responsive to 40 mM KCl in releasing insulin. However, higher KCl concentrations [60 mM] were found to elicit robust insulin release. Therefore, in KCl-stimulated insulin secretion studies, cells were incubated with 60 mM KCl in an osmolality balanced KRB medium.¹⁷

Rac1 activation assay. The extent of Rac1 activation (i.e., GTP-bound form) was determined using a commercially available kit (Cytoskeleton, Denver, CO).^{16,27,28} ICMT-siRNA transfected or AFC (100 µM) treated INS 832/13 cells were incubated with low (2.5 mM) or high glucose (20 mM) for 30 min at 37°C. Cell lysates were clarified by centrifugation and p21-activated kinase binding domain (PAK-PBD)-beads were added to the supernatant and mixed gently at 4°C for 1 h. The beads were then centrifuged at x4,000 g for 5 min, rinsed with wash buffer (25 mM Tris, pH 7.5, 30 mM MgCl₂, 40 mM NaCl and 150 mM EDTA), and then reconstituted in Laemmli buffer.

Proteins were separated on 12% SDS-PAGE and immunoblotted for activated Rac1.

NADPH oxidase activity assay. This was carried according to the method we described recently in reference 27 and 28. In brief, INS 832/13 cells were plated in six-well plates, grown to subconfluence and then either transfected with ICMT-siRNA or treated with AFC (100 µM; 1 h). The cells were washed with PBS and further incubated with 2',7'-dichloro-dihydrofluorescein diacetate (DCHFDA, 10 µM) for 30 min at 37°C. Cells were then harvested and centrifuged. The pellet was resuspended in PBS and protein concentration was determined using Bradford's assay. Equal amount of proteins were taken and fluorescence was measured at excitation and emission wavelengths of 485 and 530 nm respectively (using Perkin Elmer fluorimeter, Waltham, MA).

ICMT expression profile. INS 832/13 cells were plated in six-well plates, grown to 70% confluence and treated with low glucose (2.5 mM), high glucose (50 mM), palmitic acid (300 µM), palmitic acid plus high glucose for 48 h or thapsigargin (0.5 µM) for 9 h. Cells were then harvested and centrifuged. The pellet was resuspended in buffer solution (0.5% Nonidet P-40, 20 mM HEPES, pH 7.4, 100 mM NaCl, 20 mM DTT and protease inhibitor cocktail). Equal amount of proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The blots were then probed with antibody raised against ICMT (1:500 dilution) and with rabbit secondary antibody conjugated to horseradish peroxidase. Immune complexes were then detected using the enhanced chemiluminescence kit.

Statistical analysis. Data are presented as mean ± SEM. Statistical significance differences between values were evaluated by Student's t-test or ANOVA where appropriate. p < 0.05 was considered to be statistically significant.

Acknowledgements

The research work described in this article is supported (to A.K.) by the National Institutes of Health (RO1 74921) and the Department of VA MERIT Review award. A.K. is also the recipient of a Senior Research Career Scientist Award from the Department of VA.

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ABSTRACT**SMALL GTP-BINDING PROTEINS IN INSULIN SECRETION**

by

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August 2011

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Type 2 diabetes mellitus is marked by a substantial beta-cell failure which is characterized by defective insulin secretion and resistance to insulin. Understanding the molecular events leading to Glucose-stimulated insulin secretion [GSIS] might serve as therapeutic potential towards diabetes. GSIS involves interplay between small G-proteins and their regulatory factors. Herein, I tested the hypothesis that Arf nucleotide binding site opener [ARNO], a guanine nucleotide exchange factor [GEF] for the small G-protein Arf6, mediates the activation of Arf6, and that ARNO/Arf6 signaling axis, in turn, controls the activation of downstream effectors. Salient features of my study are: [i] ARNO/Arf6 is expressed in clonal β -cells, rodent islets and human islets; [ii] overexpression of inactive mutants of ARNO or Arf6 or siRNAs of Arf6 or ARNO reduces both GSIS and membrane depolarization induced insulin release in clonal β -cell line; [iii] secinH3, a selective inhibitor of ARNO/Arf6 signaling pathway, also inhibits

GSIS in INS 832/13 cells and rodent islets; [iv] insulinotropic concentration of glucose stimulates Arf6 activation; [v] glucose-induced Arf6 activation is inhibited by secinH3 or siRNA-ARNO, suggesting a critical involvement of ARNO/Arf6 in insulin secretion; and [vi] glucose promotes association between ARNO and Arf6 as evidenced by co-immunoprecipitation and confocal microscopic studies. These findings provide the first evidence to implicate novel roles for ARNO/Arf6 in insulin secretion.

There are many factors that contribute to GSIS including various enzymes, small G-proteins and actin remodelers. As a step towards elucidating the ARNO/Arf6 signaling cascade, I identified potential downstream effectors that were regulated by ARNO/Arf6 upon glucose stimulation. I identified potential effectors using an ARNO-selective inhibitor [e.g., secinH3] and determined regulatory roles for Arf6/ARNO in promoting phospholipase D [PLD], extracellular-regulated kinases [ERK 1/2], Rac1/Cdc42, NADPH oxidase [Nox], reactive oxygen species [ROS], dynamin-1 and cofilin [actin-severing protein] signaling steps in isolated beta-cells.

Lastly, this work demonstrates dysfunction of Arf6 and Rac1 in beta-cells exposed to glucotoxicity and their abnormal response to physiological concentrations of glucose. This evidence indicates that defective insulin secretion seen in progressive beta-cell failure even after normalization might be at the level of abnormal functioning of small G-proteins. Together my data suggest Arf6/ARNO → PLD → Rac1 → Nox → cofilin signaling cascade regulate the exo-endocytotic pathway leading to GSIS.

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- 1) Increased phagocyte-like NADPH oxidase and ROS generation in type-2 diabetic ZDF rat and human islets: Role of Rac1- JNK1/2 signaling pathway in mitochondrial dysregulation in the diabetic islet. Syed I, Kyathanahalli CN, **Jayaram B**, Rhodes CJ and Kowluru A. Diabetes 2011 [In revision].
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- 3) Arf nucleotide binding site opener [ARNO] promotes the sequential activation of Arf6, Cdc42 and Rac1 and insulin secretion in INS 832/13 β -cells and rat islets. **Jayaram B**, Syed I, Kyathanahalli CN, Rhodes CJ, Kowluru A. Biochem Pharmacol 2011; 81(8): 1016-27.
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- 5) Regulation of glucose- and mitochondrial fuel-induced insulin secretion by a cytosolic protein histidine phosphatase in pancreatic beta-cells. Kamath V, Kyathanahalli CN, **Jayaram B**, Syed I, Olson LK, Ludwig K, Klumpp S, Kriegstein J and Kowluru A. Am J Physiol Endocrinol Metab 2010; 299(2): E276-86.