MOLECULAR MECHANISM OF GLP-1 POTENTIATED INSULIN GRANULE EXOCYTOSIS

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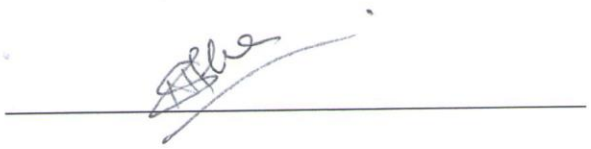
DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety.

I have duly acknowledged all the sources of information, which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



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SUMMARY

Inadequate insulin secretion is an important facet of Type II diabetes (T2D). Pancreatic beta cells release insulin in response to signals derived from glucose metabolism. While glucose is the major stimulus for insulin secretion, intracellular cyclic adenosine monophosphate (cAMP) signal generated by secretagogues like glucagon-like peptide 1 (GLP-1) is crucial in potentiation of insulin secretion. GLP-1 modulates exocytosis by coordinating protein kinase A (PKA)-dependent phosphorylation of proteins in exocytosis machinery and PKA-independent activation of cAMP-regulated guanine nucleotide exchange factor (GEF/EPAC) signaling. GLP-1 based drugs are being widely used to potentiate insulin secretion and improve metabolic control in diabetics; however, molecular mechanisms underlying GLP-1 potentiation of insulin secretion remain unclear. Synaptotagmin7 (Syt7), a principle calcium sensor for insulin secretion in beta cells, is a phosphorylation substrate of PKA. Phosphorylation of Syt7 is essential for GLP-1 potentiation of insulin release, given that phosphoinactive form of Syt7 fails to elicit an enhancement in insulin secretion in response to exendin-4, a potent GLP-1 receptor agonist. Using proteomic screening, Rabphilin3a (Rph3a) - a Rab effector protein was identified as a novel phosphorylation dependent Syt7 binding protein. By performing pull-down assays using phosphomimetic and phosphoinactive forms of GST-Syt7, it was further confirmed that Syt7 binds to Rph3a in a calcium and phosphorylation dependent manner. Treatment of insulin-secreting MIN6 cells with a cAMP analogue induces *in vivo* phosphorylation of these proteins and enhances

Rph3a-Syt7 interaction. Interestingly, knockdown of endogenous Rph3a in MIN6 cells results in diminished GLP-1 potentiated insulin release, while the overexpression of phosphomimetic Rph3a (S234E) in the primary mouse islets enhances GLP-1 potentiation of insulin secretion. This suggests that Rph3a is directly involved in GLP-1 dependent insulin release. Finally, motor protein Myosin-Va (MyoVa) has been demonstrated to coimmunoprecipitate together with Syt7 and Rph3a upon activation of PKA signaling. Furthermore, knockdown of MyoVa decreases GLP-1 dependent insulin release, which correlates with its known role in granule trafficking. Collectively, these findings provide mechanistic insights in supporting enhanced insulin secretory granule exocytosis following phosphorylation of both Syt7 and its interacting proteins by PKA.

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LIST OF ABBREVIATIONS

AC	Adenylyl cyclase
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CBB	Coomassie brilliant blue
CC	Coiled coil
CMV	Cytomegalovirus
DAG	Diacylglycerol
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
Doc2	Double C2
ds	Double stranded
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethylene glycol tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
Epac	Exchange factor activated by cAMP
FSK	Forskolin
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GEF	Guanine exchange factor

GK	Goto-Kakizaki
GLP-1	Glucagon-like peptide 1
GLUT	Glucose transporter
GSH	Reduced glutathione
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
GWAS	Genome wide association studies
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IACUC	International animal care and use committee
IGF-1	Insulin-like growth factor-1
IL-6	Interleukin-6
IP	Immunoprecipitation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KRH	Krebs-Ringer-HEPES medium
LB	Luria-bertani broth
MS	Mass spectrometry
nt	Nucleotide
OD	Optical density
PBS	Phosphate buffered saline
PC	Prohormone convertase
PCR	Polymerase chain reaction
PKA	Protein kinase A
PMSF	Phenylmethyl sulphonyl fluoride
ΔPrkar1a	Pancreas specific deletion of PKA regulatory subunit-1a
RBD	Rab binding domain

RIM	Rab3 interacting molecule
RNA	Ribonucleic acid
RNAi	RNA interference
RP	Reserve pool
Rph3a	Rabphilin 3a
RPMI	Roswell park memorial institute
RRP	Readily releasable pool
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of mean
shRNA	Short hairpin RNA
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein
SYT	Synaptotagmin
Stx1A	Syntaxin 1a
T1D	Type I diabetes
T2D	Type II diabetes
TE	Tris-EDTA
TIRFM	Total internal reflection fluorescence microscopy
VAMP	Vesicle associated membrane protein
VDCC	Voltage gated calcium channel
WT	Wild type

Chapter 1 INTRODUCTION

1.1 Overview

Diabetes mellitus, a global health problem affects more than 8% of the world population and by 2035, the number of diabetics is expected to rise as high as 592 million (International Diabetes Federation, 2014). Diabetes is a metabolic disorder characterized by chronic hyperglycemia resulting from defects in insulin secretion and its action. Insulin is a blood glucose-lowering hormone produced by pancreatic beta cells. The primary role of beta cell is to achieve metabolic homeostasis by coupling ambient glucose levels in blood with insulin secretion. Insulin lowers blood glucose level by stimulating glucose uptake from the peripheral tissues while inhibiting glucose production from liver. In type I diabetes, the pancreatic beta cells are progressively lost by autoimmune-mediate assault (Morgan, Leete, Foulis, & Richardson, 2014). Mostly common in children, it accounts for 5-10% of diabetics and is often treated with daily injections of insulin to control blood glucose level. On the contrary, Type II diabetes (T2D) is characterized by insulin resistance in the peripheral tissues and beta cell dysfunction and accounts for almost 90% of all diabetics. Insulin resistance is strongly related to visceral obesity and low-grade chronic inflammation (Tsatsoulis, Mantzaris, Bellou, & Andrikoula, 2013). While changes in diet and lifestyle are key drivers of diabetes pandemic, heritable factors also contribute to significant proportion of the disease (Hu, 2011). Standard treatment regimens thus include lifestyle changes together with drugs that stimulate release of endogenous insulin and/or increase peripheral insulin sensitivity (American Diabetes Association, 2014).

Although insulin resistance is a hallmark of T2D, in early stages of obesity, insulin resistance is often insufficient to cause hyperglycemia; as their pancreatic beta cell counteracts this by increasing insulin secretion and beta cell mass. Only when this adaptation fails, as a result of prolonged oxidative and endoplasmic reticulum stress and/or activation of pro-inflammatory pathways, diabetes ensues (Ahren, 2005; Prentki & Nolan, 2006). Thus, beta cell dysfunction remains at the core of diabetes determinant. Indeed, genome wide association studies (GWAS) also point to correlation between genetic variants associated with diabetes and defects in insulin secretion (Dupuis et al., 2010; Scott et al., 2012; Sladek et al., 2007; Strawbridge et al., 2011). Henceforth, understanding the molecular mechanism underlying defective insulin release not only provides a better understanding of the pathophysiology of diabetes mellitus but also provides impetus for developing better therapeutics for T2D.

Glucagon-like peptide (GLP-1), an incretin hormone lowers postprandial blood glucose by acting on beta cells to enhance insulin secretion in glucose-dependent manner. Thus, GLP-1 analogues are now widely used to treat patients with type II diabetes. However, besides lowering blood glucose, GLP-1 also regulates diverse physiological responses by increasing global cAMP level (Baggio & Drucker, 2007) which might result in various side effects when used as therapeutic agent. Besides, GLP-1 also has proliferative effect in beta cells which in long run can trigger pancreatitis or pancreatic cancer (Cure, Pileggi, & Alejandro, 2008; Denker & Dimarco, 2006). A challenge

henceforth is to understand the molecular targets downstream of GLP-1 in order to harness its blood glucose lowering effect and avoid other deleterious effect such as global cAMP elevation. Insights have come from studying the unique substrate of GLP-1 in the beta cells that may provide valuable insights and translatable information for drug design. In this thesis, I attempt to understand the molecular mechanism underlying GLP-1 potentiated insulin secretion, using recently identified GLP-1 substrate (Synaptotagmin-7) in beta cell. In this introduction section, I would like to elaborate on the general mechanisms of glucose and GLP-1 dependent insulin secretion and the molecular players involved in exocytosis of insulin granules.

1.2 Mechanisms of glucose-induced insulin secretion

Insulin is a peptide hormone produced exclusively by the beta cells in pancreatic islet. It is packaged and stored in dense core vesicles within the beta cells. Release of insulin in bloodstream thus requires fusion of these vesicles with the plasma membrane, which is a tightly regulated process. Increase in blood glucose concentration is the major trigger for insulin release from the vesicles. Beta cell serves as a mediator to couple changes in membrane potential brought about by glucose metabolism to exocytosis of insulin granules (Ashcroft & Rorsman, 1989). Under low glucose, a negative membrane potential (-70 mV) is maintained in the beta cell via a continuous outward flux of positively charged potassium ions (Ashcroft & Rorsman, 1989). After food intake, glucose is taken up by the beta cells via glucose transporters (GLUTs). While GLUT2 is the major isoform in rodent beta cell,

GLUT1 and GLUT3 predominate in human islets (McCulloch et al., 2011). Glucose is ultimately oxidized to generate high-energy intracellular adenosine triphosphate (ATP). The increased intracellular ATP/adenosine diphosphate (ADP) ratio within the beta cell inhibits potassium permeability by closing the ATP-sensitive potassium channels. This leads to an accumulation of positively charged ions inside the cells that triggers membrane depolarization and subsequent opening of voltage dependent calcium L-channels. Opening of calcium channels causes a rapid spike in free cytosolic calcium ions, thereby triggering exocytosis of insulin from the secretory granules (Ashcroft & Rorsman, 2012). Figure 1-1 shows the illustration of mechanisms involved in glucose dependent insulin secretion. While glucose triggers insulin granule exocytosis, various nutritional (free fatty acids and amino acids) and hormonal (GLP-1, adrenaline, cholecystokinin) secretagogues further potentiate insulin secretion in presence of threshold stimulatory level of glucose in the bloodstream.

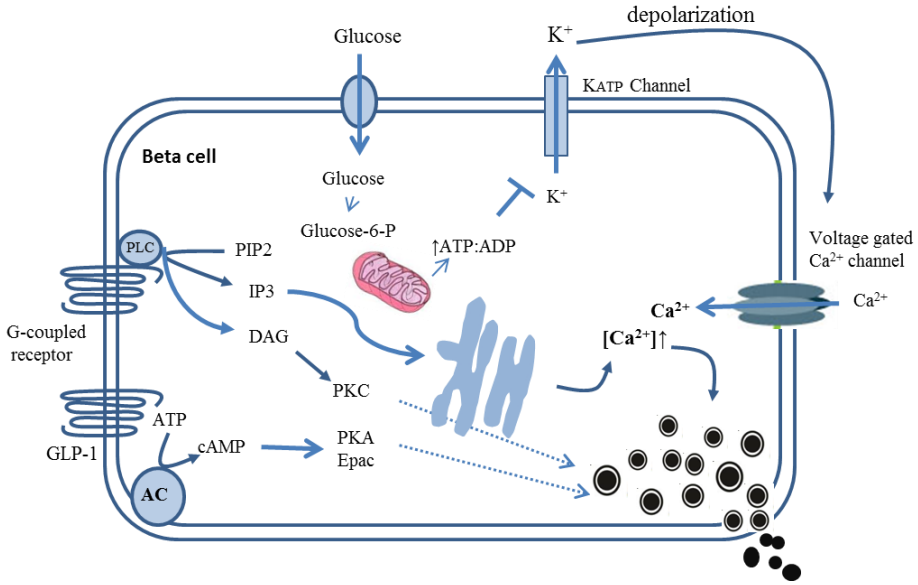

Figure 1-1 Mechanism of insulin secretion from pancreatic beta cells

Glucose enters pancreatic beta cells via glucose transporter (GLUT), gets metabolized to produce ATP. Rise in ATP/ADP ratio inhibits potassium channel resulting in membrane depolarization and opening of voltage gated calcium channel (VDCC). Sudden rise in calcium concentration inside the cell as a result of calcium influx triggers release of insulin from the secretory granules. Extracellular signals acting on G-protein coupled receptors in the beta cell membrane activate secondary messengers to further potentiate insulin release. (Adapted form (Ahren, 2009))

Although the molecular mechanisms of extracellular glucose sensing cumulating to insulin secretion has deepened remarkably in the recent years (Ashcroft & Rorsman, 2012), insulin secretion is a multifaceted process and the final steps in the insulin granule secretory process still remains elusive. Exocytosis of these granules from the secretory vesicle requires highly regulated multiple morphologically and functionally defined stages that includes recruitment of vesicles, its tethering and docking to the plasma membrane, priming to fusion machinery followed by calcium triggered

membrane fusion (Burgess & Kelly, 1987). Tethering involves recruiting the vesicles from the cytosolic depot to the plasma membrane. Once in the plasma membrane, the lipid bilayers of the vesicle membrane interact with the plasma membrane whereby the vesicle is assumed to be docked. Following docking, the vesicles are primed via an ATP-dependent step such that an increase in intracellular calcium can immediately result in fusion and granule release (Burgoyne & Morgan, 2003; Jahn & Fasshauer, 2012; Sudhof, 2013b).

1.3 Dynamics of insulin secretion

Glucose uptake causes a biphasic secretion of insulin. During the first minutes, insulin is rapidly secreted that accounts to about 2-3% of beta cell insulin content. This phase is followed by lower but long lasting secretion phase accounting for about 20% of total insulin content. Ultrastructural and biochemical studies together with electrophysiological measurements have revealed two distinct populations of insulin granules. The biphasic insulin response is thought to be mediated by these functionally distinct pools of insulin granules, the first phase being contributed by granules in the readily releasable pool close to the membrane followed by sustained phase resulting from mobilization of granules along the microtubules from the reserve pool alongside remodeling of filamentous actin (Rorsman & Renstrom, 2003; Z. Wang & Thurmond, 2009). This classical model has however been recently challenged by the observation in total internal reflective fluorescence microscopy (TIRFM) that the first phase also involves “newcomer” secretory granules suddenly appearing at the plasma membrane that undergo exocytosis

without any time delay pertaining to docking (Nagamatsu, Ohara-Imaizumi, Nakamichi, Kikuta, & Nishiwaki, 2006; Ohara-Imaizumi et al., 2007; Shibasaki et al., 2007). Many more studies report that newcomer granules contribute to both phases of insulin release (Yasuda et al., 2010; Zhu et al., 2015), questioning the requirement of stable docking for fusion. In fact, evidences also suggest that docking might even constrain granule fusion (Gomi, Mizutani, Kasai, Itohara, & Izumi, 2005; Kasai, Fujita, Gomi, & Izumi, 2008). Henceforth, mobility of granules to the membrane seems to be a critical factor for increasing probability of fusion and exocytosis (Allersma, Bittner, Axelrod, & Holz, 2006; Degtyar, Allersma, Axelrod, & Holz, 2007; Verhage & Sorensen, 2008).

1.4 Molecular machinery for insulin granule exocytosis

Regulated exocytosis involves multitude of events beginning with the synthesis of preproinsulin in the endoplasmic reticulum, followed by budding of immature insulin granules from the trans-golgi network, maturation of granules and the final fusion of release competent granules from the plasma membrane. The proteins in exocytosis machinery tightly regulate these discrete cascades of events. Similar to neurons and neuroendocrine cells (Seino & Shibasaki, 2005; Sudhof, 2004), the essential proteins in fusion machinery comprise of Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, calcium sensors and regulatory proteins. Their involvement and interaction in facilitating insulin granule exocytosis will be briefly described below.

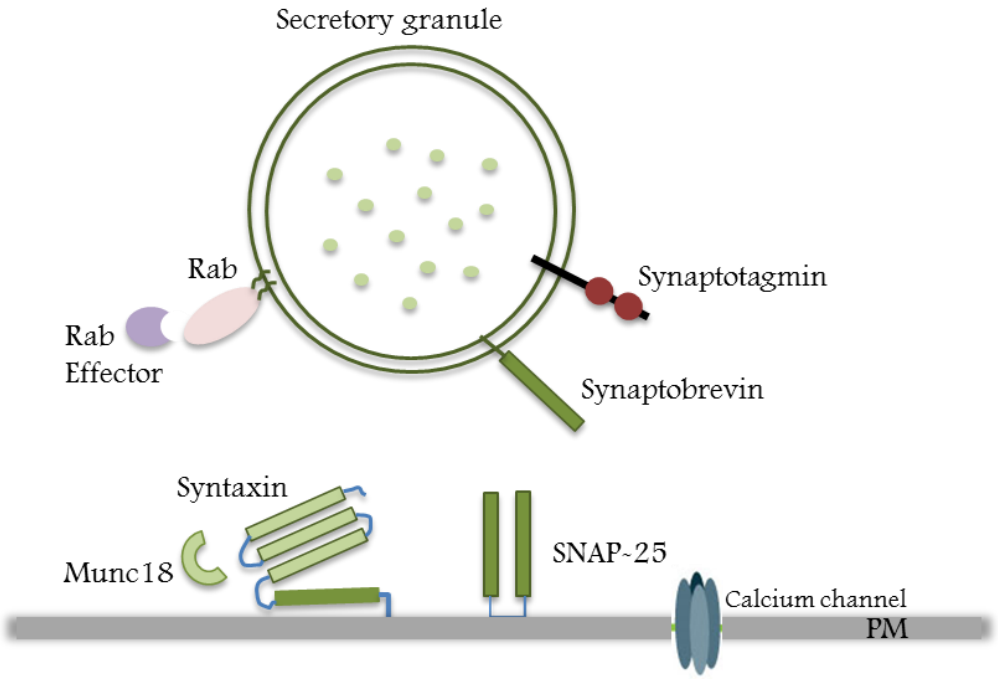

Figure 1-2 Molecular players involved in insulin granule exocytosis

SNARE complex comprising the syntaxin, SNAP-25 and synaptobrevin form the minimal machinery for fusion. Increase in calcium concentration inside the cell is sensed by C_2 domain protein-synaptotagmins. Numerous other conserved proteins like Munc proteins, Rab and their effectors aid in granule trafficking and fusion steps.

1.4.1 Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)

SNARE proteins comprising of syntaxin-1A and SNAP-25 at the plasma membrane and vesicle-associated membrane protein (VAMP) or synaptobrevin at the vesicular membrane represent the minimal machinery for vesicle fusion in all eukaryotic cells (Weber et al., 1998). The defining feature of the core components comprising the SNAREs is the presence of SNARE

motif which is composed of about sixty amino acids and has a high tendency to form coiled coil domains (Jahn & Sudhof, 1999; Rizo & Sudhof, 2002). Upon contact, SNAREs on opposed membranes form a tight four-helix bundle involving one SNARE motif from synaptobrevin, one from syntaxin and two SNARE motifs from SNAP-25. This pulls the opposing membranes together and the energy released during this assembly initiates membrane fusion (Sutton, Fasshauer, Jahn, & Brunger, 1998).

Various SNARE isoforms have been reported in pancreatic beta cells (Jewell, Oh, & Thurmond, 2010). The major SNARE isoforms involved in insulin secretion following glucose stimuli involves same SNARE isoforms as reported for synaptic vesicle exocytosis, namely, syntaxin-1, SNAP-25 and VAMP2 suggesting their conserved universal role in granule exocytosis. Other isoforms are suggested to play a role in providing specificity to vesicle targeting in various modes of insulin granule exocytosis such as recruitment of newcomer secretory granules and granule-granule fusion (Gaisano, 2012; Kasai et al., 2008; McNew et al., 2000; Shibasaki et al., 2007; Yasuda et al., 2010). In line with this, Zhu et al and authors demonstrated that syntaxin-1A co-immunoprecipitated abundant SNAP-25, SNAP-23 and VAMP-2 whereas syntaxin-2 and -3 immunoprecipitated abundant Munc18b under basal condition. However, when stimulated with high glucose and GLP-1, syntaxin-2 and -3 co-immunoprecipitated abundant SNAP-25, SNAP-23 and VAMP-8, indicating a preferential isoform binding following activation by different stimuli (Zhu et al., 2012).

The importance of the SNARE complex in insulin granule exocytosis has been documented in several prominent studies (Eliasson et al., 2008; Gerber & Sudhof, 2002; Kiraly-Borri et al., 1996; Wheeler et al., 1996). Most importantly, Syntaxin-1A and SNAP-25 proteins remain as clusters in close association with the insulin granules near the plasma membrane of beta cells to facilitate insulin granule exocytosis. These proteins are significantly reduced in Goto-Kakizaki (GK) rats, a non-obese Wistar rat substrain that develops T2D early in life (Nagamatsu et al., 1999) and in pancreatic islets of human T2D patients (Andersson et al., 2012; Ostenson, Gaisano, Sheu, Tibell, & Bartfai, 2006). In line with this, Syntaxin-1A null mice display fewer docked granules and consequently impaired first phase insulin release indicating the importance of syntaxin clusters in docking of insulin granules (Ohara-Imaizumi et al., 2007). Thus, SNARE proteins play significant role in insulin granule exocytosis and are important for modulating glucose homeostasis.

1.4.2 Calcium sensors

The fusion of vesicles to the plasma membrane and consequently the exocytosis of insulin granules is triggered by an elevation of the cytoplasmic Ca^{2+} concentration (Sudhof, 2012). In most cells that exhibit regulated exocytosis such as neuronal, endocrinal, neuroendocrinal and acrosomal cells, synaptotagmins have been identified as the primary calcium sensor proteins that couples increase in intracellular calcium ions to activation of exocytosis machinery for vesicle fusion. Synaptotagmins comprise an amino terminal

transmembrane region, a variable linker sequence and two C-terminal C_2 domains. The C_2 domains harbor five calcium coordinating residues such that C_2A and C_2B domains bind to three and two calcium ions respectively (Sudhof, 2002). Several synaptotagmin isoforms have been identified and calcium dependent phospholipid binding has been reported for eight synaptotagmins (synaptotagmin-1, -2, -3, -5, -6, -7, -9, -10). The remaining synaptotagmins either lack the aspartate residues in their C_2 domains that are required for binding calcium ions or are structurally too far apart to chelate to the calcium ions (Dai et al., 2004) and therefore cannot bind to calcium. Synaptoagmin-1, -2 and -9 are mainly expressed in brain and are involved in neurotransmitter release (Geppert et al., 1994; Sugita, Shin, Han, Lao, & Sudhof, 2002). Synaptotagmin-7 is abundant in neuroendocrine and endocrine cells where it regulates neuroendocrine and peptide hormone secretion (Gauthier et al., 2008; Gustavsson et al., 2008). Synaptotagmin-6 plays critical role in acrosomal exocytosis in sperm (Michaut et al., 2001; Tomes, 2015). Synaptotagmin-9 was recently identified to be involved in sex-specific regulation of follicle-stimulating hormone release (Roper, Briguglio, Evans, Jackson, & Chapman, 2015). Furthermore, multiple synaptotagmin isoforms might regulate distinct fusion events in same cells. For example, synaptotagmin-10 co-expresses with synaptotagmin-1 in olfactory bulb neurons and regulate insulin-like growth factor (IGF-1) secretion (Cao, Maximov, & Sudhof, 2011).

Pancreatic beta cells also express various isoforms of synaptotagmins but this varies within species or between primary and clonal beta cell lines (Gao, Reavey-Cantwell, Young, Jegier, & Wolf, 2000; Gauthier et al., 2008; Gustavsson & Han, 2009; Iezzi, Kouri, Fukuda, & Wollheim, 2004). However, in all cases, synaptotagmin-7 (Syt7) seems to be the most abundant isoform with a role in glucose stimulated insulin secretion (Gauthier et al., 2008; Gustavsson et al., 2009). Syt7 null-mutant mice exhibit impaired glucose tolerance and insulin secretion *in-vivo* and attenuated secretion from isolated islets despite normal insulin content (Gustavsson et al., 2008). Similar results were observed in clonal beta cell lines with decreased Syt7 expression following short hairpin (shRNA) knockdown (Gauthier et al., 2008). Taken together, Syt7 is a positive regulator of glucose-dependent insulin release.

Since, deletion of Syt7 reduced insulin secretion by only about 50%, this suggests involvement of other calcium-binding proteins. Synaptotagmin-9 is also highly expressed in mouse and rat islets however its affinity to calcium is extremely low (10-30 uM) (Sugita et al., 2002). Although, synaptotagmin-9 was reported to impair insulin release in rat islets (Iezzi, Eliasson, Fukuda, & Wollheim, 2005) and INS1E cell line (Iezzi et al., 2004) when silenced; no defects in insulin secretion was observed in the pancreas-specific synaptotagmin-9 knockout mice (Gustavsson et al., 2010). Role of synaptotagmin-3, -5, -6 and -10 isoforms in insulin secretion remains controversial. Synaptotagmin-3 (Brown et al., 2000; Gao et al., 2000; Gut et al., 2001) and synaptotagmin-5 (Gut et al., 2001; Iezzi et al., 2004) have been

shown to be a positive modulator of insulin release in insulin secreting cell lines. On the contrary, other studies demonstrate that recombinant calcium binding domains of synaptotagmin-3 is not effective in inhibiting calcium induced exocytosis in primary pancreatic islets (Gut et al., 2001). Moreover, specific role are yet to be assigned for synaptotagmin-6 and -10 in insulin secretion.

1.4.3 Regulatory proteins

In addition to core fusion proteins and calcium sensors, beta cells contain several regulatory proteins that orchestrate with fusion machinery and secretory granules to tightly regulate the exocytosis event. The major regulatory proteins include Rab proteins and its cytosolic effector molecules, Munc18 isoforms and motor proteins (Gerber & Sudhof, 2002; Ivarsson, Jing, Waselle, Regazzi, & Renstrom, 2005; Sudhof, 2013a).

1.4.3.1 Rab and effector proteins

Rab proteins constitute the large family of small GTPases that control various steps in membrane traffic, including vesicle formation, vesicle transport and membrane fusion via interaction with various cytosolic effector molecules. More than 60 isoforms of Rab proteins have been identified in humans (Stenmark, 2009; Zerial & McBride, 2001). Among those, Rab3 and Rab27 are implicated in final steps of regulated exocytosis in beta cells (Kasai et al., 2005; Yaekura et al., 2003). Rab3A null mice are glucose intolerant and exhibit loss of first phase insulin release (Yaekura et al., 2003) whereas Rab27

deficient mice exhibit reduced glucose dependent insulin secretion (Kasai et al., 2005) accompanied by delayed replenishment of readily releasable pool of insulin granules (Merrins & Stuenkel, 2008) suggesting isoform specific role of Rab3 and Rab27 in initial docking and replenishing of readily releasable vesicle pool respectively (Tsuboi & Fukuda, 2006).

Rab proteins constantly switch between an inactive guanosine diphosphate (GDP)-bound form in the cytoplasm and an active guanosine triphosphate (GTP)-bound form on membranes. The Rab effectors selectively bind to the GTP-bound form and translate the signal from Rab proteins to regulate vesicle trafficking (Fukuda, 2008; Grosshans, Ortiz, & Novick, 2006). Although a large number of such effectors are present in beta cells, only few have been characterized for their role in insulin granule exocytosis. Granuphilin and Rabphilin3a (Rph3a) are Rab effectors associated with insulin granules (Brozzi, Diraison, et al., 2012). Knockout of granuphilin in mice islets results in increased accumulation of insulin containing vesicles close to the membrane but exhibits decreased fusion events (Gomi et al., 2005). While not directly addressed, this finding led to the hypothesis that docking could be a temporal brake for exocytosis to prevent incoming vesicles from being constitutively fused (Gomi et al., 2005; H. Wang et al., 2011). Rph3a on the other hand, involves in tethering of vesicles to plasma membrane in neuroendocrine cells, via its interaction with SNAP-25 (Deak et al., 2006; Tsuboi & Fukuda, 2005). However, its role in beta cells remains elusive due to its low abundance in islets. Moreover, overexpression studies in MIN6

suggested its role in PKA-dependent hormone secretion (Brozzi, Lajus, et al., 2012). Regardless, its role in insulin secretion will be investigated in this thesis.

Other important Rab effector proteins implicated in regulation of insulin secretion are Rab3-interacting molecule (RIM) (Iezzi, Regazzi, & Wollheim, 2000; Yasuda et al., 2010) and No C_2 domain (Noc2) proteins (Cheviet, Coppola, Haynes, Burgoyne, & Regazzi, 2004). While RIM was shown to interact with cAMP effector, exchange protein directly activated by cyclic AMP (Epac) to enhance cAMP-potentiated insulin secretion (Kashima et al., 2001), Noc2 was found to associate with cytoskeletal associated protein-zyxin to enhance insulin release (Cheviet et al., 2004; Kotake et al., 1997). Together these studies highlight the regulatory role of Rab proteins and their effectors in insulin secretion.

1.4.3.2 Munc18

Munc18, also known as **M**ammalian **Unc**oordinated-**18,** is a mammalian homologue of unc-18 proteins in Caenorhabditis elegans. Its indispensable role in regulated exocytosis is demonstrated by complete abolition of synaptic transmission in Munc18 knockout mice (Hata, Slaughter, & Sudhof, 1993; Verhage et al., 2000). Munc18 engages in granule fusion process by stabilizing Syntaxin-1A at the plasma membrane (Jahn, Lang, & Sudhof, 2003). Three isoforms of Munc18 have been identified; Munc18a, b and c. Munc18a and Munc18b pair with plasma membrane-localized syntaxin-1A

whereas Munc18c pairs exclusively with syntaxin-4. Islet β-cells express all three isoforms of Munc18 and thus regulate both syntaxin-1A and syntaxin-4 based SNARE complexes (Wheeler et al., 1996). Different regulatory proteins like Munc13 (Kwan et al., 2006) and Double C_2 protein (Doc2) (J. Li et al., 2014) further interact with Munc18 to catalyze priming and assembly of fusion machinery.

Munc18a is thought to mediate exocytosis of pre-docked granules that underlie first-phase secretion (Oh, Kalwat, Kim, Verhage, & Thurmond, 2012). Likewise, Munc18b activate and induce the formation of syntaxin-3 SM/SNARE complexes that mediate additional exocytosis of pre-docked granules as well as regulate granule-granule fusion in rat pancreatic beta cells (Lam et al., 2013).

1.4.3.3 Motor proteins

In the basal state, cortical actin networks hinder granule translocation from the cytosol to the plasma membrane. Following extracellular stimuli, motor proteins, kinesin and myosin transport the granules towards the plasma membrane where the relevant SNARE proteins engage to facilitate granule release (Burchfield, Lopez, Mele, Vallotton, & Hughes, 2010; Varadi, Ainscow, Allan, & Rutter, 2002). While both kinesin and myosin are important for vesicle transport, Myosin-Va (MyoVa), an actin based motor protein, involves in final steps of granule transport via its interplay with F-actin. Mutation of MyoVa gene causes Griscelli syndrome in humans (Pastural

et al., 1997) and lethal phenotype in mice (X. Wu, Bowers, Wei, Kocher, & Hammer, 1997), both characterized by pigment dilution and neurological defects caused by impaired vesicle transport in melanosomes and neuronal cells respectively.

MyoVa is composed of two identical heavy chains that dimerize in their coiled coil (CC) domain. In peripheral tissues such as adipocytes, MyoVa has been identified as insulin stimulated AKT2 substrate regulating ante-retrograde vesicle trafficking (Yoshizaki et al., 2007). MyoVa is enriched in insulin containing secretory granules and is involved in transport of granules from reserve pool to readily releasable pool to facilitate sustained insulin secretion (Brozzi, Diraison, et al., 2012). Consistent with this idea, reduced expression of MyoVa in pancreatic beta cells exhibited reduced expression of insulin containing granules in the TIRF zone along with disrupted transport and attachment behavior (Ivarsson et al., 2005; Varadi, Tsuboi, & Rutter, 2005). A study demonstrated that MyoVa directly interacts with docking proteins, Rph3a and Granuphilin to facilitate nutrient-stimulated hormone release (Brozzi, Lajus, et al., 2012). However, experiments directly addressing the immediate role of MyoVa in secretagogue-dependent insulin release are missing. Given the importance of motor proteins in granule transport, further investigations would warrant better understanding of vesicle dynamics and their regulation in physiology and pathophysiology.

1.5 Potentiation of insulin secretion by GLP-1

Under physiological settings, glucose plays a major role in triggering insulin release; however other nutritional cues and circulating hormones can also modulate the classical glucose-stimulated insulin secretion cascade. Of special interest is the incretin hormone, glucagon-like peptide-1 (GLP-1), that acts on beta cells to stimulate insulin secretion in a glucose-dependent manner (Baggio & Drucker, 2007). GLP-1 effect on insulin secretion was recognized when it was observed that compared to intravenous glucose load, isoglycemic oral load increases insulin secretion by almost 50-70% (Elrick, Stimmler, Hlad, & Arai, 1964). This phenomenon was later deemed as incretin effect.

GLP-1 is derived from tissue-specific post-translational processing of precursor proglucagon gene. These proglucagon mRNA transcripts are expressed in the pancreatic alpha cells, intestinal L-cells and some hypothalamic neurons (Larsen, Tang-Christensen, Holst, & Orskov, 1997; Mojsov et al., 1986; Novak, Wilks, Buell, & McEwen, 1987). The differential post translational processing of proglucagon is dependent upon the tissue-specific expression of prohormone convertase (PC) isoforms. In intestine and some hypothalamic neurons, enzyme PC1/3 acts on proglucagon to release GLP-1, in addition to GLP-2, glicentin and oxyntomodulin. In contrast, alpha cell expresses PC2 that cleaves the proglucagon precursor molecule to glucagon, glicentin-related pancreatic peptide and the major proglucagon fragment containing both GLP-1 and GLP-2 (Holst, 1997). Although adult alpha cell is thought to produce little GLP-1, induction of diabetes in rodents

leads to increased production of GLP-1 together with increased alpha cell expression of enzyme PC1/3. Moreover, this switch is beneficial to lower blood glucose levels and promote islet survival (Wideman, Covey, Webb, Drucker, & Kieffer, 2007). GLP-1, however has a very short half-life of approximately two minutes due to its rapid degradation by enzyme, dipeptidyl peptidase 4 (DPP4). DPP4 is a serine protease that is widely expressed in multiple tissues and circulates systemically as a soluble protein (Baggio & Drucker, 2007).

Oral nutrients such as glucose and fat are primary physiological regulators of GLP-1 secretion. The role of proteins in GLP-1 secretion is controversial with most reports suggesting relatively small or no contribution except glutamine, a highly abundant non-essential amino acid that has been demonstrated to stimulate GLP-1 release (Greenfield et al., 2009; Tolhurst et al., 2011). Consistent with the proximity of L-cells to neurons and microvasculature of intestine, GLP-1 secretion is also affected by neural and hormonal (acetylcholine and gastrin-releasing peptide) signals (Anini, Hansotia, & Brubaker, 2002; Brubaker, 1991; Roberge, Gronau, & Brubaker, 1996). One intriguing factor triggering GLP-1 secretion is Interleukin (IL)-6, an inflammatory cytokine. Diabetes and obesity are usually accompanied by activation of innate immune system reflected by increased level of inflammatory markers including elevated level of interleukins (Donath & Shoelson, 2011). Interestingly, interleukins are also released in response to exercise and it was demonstrated that elevated IL-6 post-exercise stimulated

GLP-1 secretion from intestinal L-cells as well as pancreatic alpha cells in mice leading to improved glucose homeostasis. Complementing this finding, both acute and chronic administration of IL-6 stimulated GLP-1 secretion both *in vitro* and *in vivo,* whereas neutralization of IL-6 reduced pancreatic GLP-1 content and deteriorated glycemic control (Ellingsgaard et al., 2011).

GLP-1 has numerous biological actions (Drucker, 2015). In beta cell, it binds to GLP-1 receptor (GLP-1R) to directly enhance insulin secretion. Accordingly, genetic ablation of GLP-1R in mice results in glucose intolerance and impaired insulin secretion in response to oral and intraperitoneal glucose challenge (Lamont et al., 2012). Besides potentiating insulin release, GLP-1 also inhibits glucagon release from the alpha cells, increase beta cell proliferation and inhibit its apoptosis (Drucker, 2013; Egan, Bulotta, Hui, & Perfetti, 2003). Aside from its predominant effects on pancreas, GLP-1 also exhibits numerous extra-pancreatic effects. Infusion of GLP-1 inhibits gastric emptying and motility thereby slowing the rate of post-prandial nutrient absorption (Schirra et al., 1996). It also potently inhibits food intake through activation of peripheral and central GLP-1 receptors (Knauf et al., 2008; Turton et al., 1996). Moreover, GLP-1 also stimulates hepatic glycogen synthesis and inhibits both hepatic gluconeogenesis and muscle glucose utilization (Ayala et al., 2009; Knauf et al., 2005).

Given its role in maintaining glucose homeostasis, GLP-1 serves as attractive target for treatment of T2D. Most importantly, GLP-1 signaling in beta cells

manifest only after triggering signal has been produced by high blood glucose. The hierarchy between the triggering action of glucose and amplifying action of GLP-1 ensures that insulin is not secreted inappropriately under low glucose condition. Since commonly used drugs for treatment of diabetes like sulphonylureas directly target the ATP-dependent potassium channel complex and often result in hypoglycemia, therapies based on GLP-1 (long acting GLP-1 analogues or inhibitors of GLP-1 degrading enzyme DPP4) make a more attractive target for diabetes treatment due to their dependence on glucose for amplification of insulin release (Ahren, 2009; Drucker, 2013). In fact, two new classes of drugs have been approved for lowering blood glucose in T2DM: an incretin mimetic (long lasting agonist of GLP-1 receptor) and an incretin enhancer (DPP4 inhibitor) (Gallwitz, 2005; Wilding & Hardy, 2011). Continuous GLP-1 treatment in T2DM can normalize blood glucose, improve beta cell function and restore beta cell glucose competence (G. G. t. Holz, Kuhtreiber, & Habener, 1993; Zander, Madsbad, Madsen, & Holst, 2002). Nonetheless, despite its beneficial effects, chronic activation of GLP-1R can have various side effects, thus a better understanding of GLP-1 action can lead to novel GLP-1 related treatment for T2D.

1.6 Molecular targets of GLP-1 signaling pathway

GLP-1 signaling is transduced through binding of GLP-1 to its cognate receptor in the beta cell membrane. GLP-1R is a ligand-specific, seven-transmembrane domain G-protein coupled receptor. Binding of GLP-1 to the receptor facilitates the release of activated Ga_s subunit of the trimeric G-

protein complex, which then activates plasma membrane-bound adenylyl cyclase to produce cyclic adenosine monophosphate (cAMP) (Doyle & Egan, 2007; Thorens, 2004). cAMP serves as a secondary messenger that transduces extracellular signals from hormones and neurotransmitters to control diverse range of cellular processes. In beta cells, GLP-1R induced cAMP potentiates insulin secretion via two primary downstream effectors: Protein Kinase A (PKA) and cAMP-regulated guanine nucleotide exchange factor (cAMP-GEF), Epac (Seino & Shibasaki, 2005). Extensive studies over the years have led to identification of substrates for both PKA-dependent and PKA-independent/Epac pathway (G. Holz, Chepurny, Leech, Song, & Hussain, 2014; Seino & Shibasaki, 2005) however; many aspects of the mechanism of potentiation and its regulation remain obscure.

PKA independent pathway involves Epac signaling (de Rooij et al., 1998; Ozaki et al., 2000). Two isoforms of Epac protein have been identified, Epac1 and Epac2. Both isoforms are expressed in beta cells; however, Epac2 plays a dominant role in GLP-1 induced insulin secretion (Leech, Holz, Chepurny, & Habener, 2000). Epac2 contains a guanine nucleotide exchange factor (GEF) domain that activates small G-proteins, Rap1 and Rap2, when bound to cAMP (Bos, 2006). Rap proteins bind to phospholipase C (PLC) to stimulate its catalytic activity. PLC catalyzes hydrolysis of phosphatidylinositol 4.5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate. (IP3). While DAG stimulates protein kinase C signaling, IP3 mobilizes calcium from intracellular stores like endoplasmic reticulum. Together, these

actions result in highly localized calcium signal that promotes granule exocytosis (Branham et al., 2009). Consistent with this, Epac2 knockout mice exhibit reduced potentiation in first phase insulin secretion in response to GLP-1 agonist (Song, Mondal, Li, Lee, & Hussain, 2013). Furthermore, Epac2 also associates directly with RIM2 and piccolo to enhance exocytosis by promoting insulin granule dynamics (Fujimoto et al., 2002; Shibasaki et al., 2007). Of interest, sulphonylurea is a widely used antidiabetic drug and this was shown to directly interact with Epac to potentiate insulin secretion (Zhang et al., 2009), thus highlighting the possibility of Epac targeting in diabetes therapy.

While the signaling involving PKA-independent/Epac pathway is more defined, the downstream targets of PKA-dependent pathway have not been fully understood. PKA consists of two catalytic and two regulatory subunits; together they form an inactive tetrameric holoenzyme. Binding of cAMP to the regulatory subunit allows dissociation of the complex, thus enabling catalytic subunits to phosphorylate substrate proteins. Recent studies have reported that PKA phosphorylates various proteins associated with the insulin granule secretory process to directly modulate exocytosis. Some of the known PKA substrates in beta cells are listed in **Table 1-1**.

Table 1-1 PKA substrates identified in beta cells

Proteins	Site	Validation	Reference
Snapin	Ser50	Mouse	(Song et al., 2011)
Syt7	Ser103	Mouse	(Wu et al., 2015)
MyRIP	-	MIN6	(Brozzi et al., 2012)
Rabphilin	Ser234	-	(Brozzi et al., 2012)
Rip11	Ser307, Ser357	MIN6	(Sugawara, et al., 2009)

Snapin is a SNAP-25 binding protein and is phosphorylated by PKA at Ser-50 (Chheda, Ashery, Thakur, Rettig, & Sheng, 2001; Ilardi, Mochida, & Sheng, 1999). Snapin as a target of PKA was initially identified in neurons with essential role in vesicle exocytosis via direct interaction with SNAP-25 (Chheda et al., 2001). Moreover, importance of snapin phosphorylation in GLP-1-potentiated GSIS in pancreatic beta cells was demonstrated using mouse model with pancreas specific deletion of PKA regulatory subunit-1a (Δ prkar1a) (Song et al., 2011). Prkar1a is abundantly expressed in pancreatic islets (Petyuk et al., 2008) and deletion of this subunit allows constitutive expression of PKA. Interestingly, snapin phosphorylation was reduced in diabetic islets and expression of phosphomimetic snapin mutant (S50D) in these islets could restore glucose stimulated insulin secretion (Song et al., 2011). This finding provides a new perspective on how post translational modification can be targeted for therapy in diabetics.

Recently, Syt7 was identified as a target of PKA. PKA phosphorylates Syt7 at Ser103 (B. Wu et al., 2015). This finding indicates that Syt7, besides being a major calcium sensor in beta cells, also regulates PKA-dependent insulin granule exocytosis. In support of this, islets from Syt7 knockout exhibits impaired GLP-1 dependent insulin secretion, whereas overexpression of mutant Syt7 that mimics phosphorylated Syt7 state (i.e. S103E) in the knockout islets completely rescued GLP-1 dependent impaired insulin release (B. Wu et al., 2015). These findings indicate that Syt7 can be directly activated by GLP-1 and likely serve to enhance GLP-1 dependent insulin secretion.

Given that PKA signaling is tightly controlled in beta cells, its temporal and spatial regulation requires specialized subcellular compartmentation by scaffolding proteins. MyRIP is one such scaffolding protein for PKA. In beta cells, GLP-1 signaling leads to formation of complex involving MyRIP, MyoVa and Rab27 that enhances insulin granule exocytosis in response to GLP-1 treatment by allowing phosphorylation of Rph3a (Brozzi, Lajus, et al., 2012). While Rph3a has been extensively studied in neurons (Deak et al., 2006; Joberty, Stabila, Coppola, Macara, & Regazzi, 1999; Lonart & Sudhof, 1998; Schluter et al., 1999) and neuroendocrine cells (Abdel-Halim et al., 1996; Tsuboi & Fukuda, 2005; Tsuboi, Kanno, & Fukuda, 2007; Tsuboi, Kitaguchi, Karasawa, Fukuda, & Miyawaki, 2010), so far only one study (Brozzi, Lajus, et al., 2012) has indirectly indicated its role in granule exocytosis in insulin secreting MIN6 cells. However, given its role in docking of granules, we can envisage its positive role in granule exocytosis. Besides Rph3a, a Rab11 effector-Rip11 was also reported to be a

direct substrate of PKA that potentiates GLP-1 dependent insulin release in MIN6 cells (Sugawara, Shibasaki, Mizoguchi, Saito, & Seino, 2009).

By far, GLP-1 modulates granule exocytosis by PKA-dependent and PKA-independent mechanism; however, it remains unclear whether cAMP signaling results in preferential coupling of extracellular signaling to either PKA- dependent or independent pathway. A-kinase anchoring proteins (AKAP) have been shown to anchor both PKA (Lester, Langeberg, & Scott, 1997; Welch, Jones, & Scott, 2010) and Epac (Hong, Lou, Gupta, Ribeiro-Neto, & Altschuler, 2008; Nijholt et al., 2008) separately, which might indicate independent actions. However, Snapin on the other hand is phosphorylated by PKA and phosphorylation enhances its interaction with Epac, indicating convergence of PKA-dependent and independent cAMP signaling (Song et al., 2011). Conceivably, concerted actions of both these pathways enable GLP-1 to potentiate insulin secretion.

1.7 Aims and Objectives

Protein phosphorylation is emerging as an important regulatory mechanism that couples signaling cascades generated from extracellular and intracellular signals to vesicle transport machinery. GLP-1 is a potent incretin hormone that accelerates stimulus secretion coupling in pancreatic beta cells via PKA-dependent phosphorylation of target proteins in the exocytosis machinery. Of interest, several studies have demonstrated independent targets of PKA in beta cells and their roles in the potentiation of insulin secretion. However, the downstream events following phosphorylation of target proteins culminating in enhanced insulin granule exocytosis is not clear. Thus, the overarching objective of the current study is *to identify and characterize the role of phosphorylation dependent Syt7 binding proteins in insulin secreting cells.* This will further contribute to the understanding of the mechanistic machinery underpinning GLP-1 mediated enhanced insulin granule exocytosis following PKA-dependent phosphorylation. To address this, the aims of this project are:

- To identify phosphorylation dependent Syt7 interacting proteins (addressed in Chapter 3.2);
- To characterize their functional role in GLP-1 mediated insulin secretion (addressed in Chapter 3.3 and 3.4);
- To delineate the mechanism by which Syt7 and interacting protein mediates GLP-1 potentiation of insulin release (addressed in Chapter 3.5).

Chapter 2 MATERIALS AND METHODS

2.1 Animal studies

The Syt7 mutant mice were generated as described before (Maximov et al., 2008). Homozygous Syt7 and wild type littermates used in this project were obtained using a heterozygous breeding strategy. All animal-related studies were approved by A*STAR Institutional Animal Care and Use Committees (IACUC # 110683).

2.2 Cell Culture

HEK293T cells were cultured in DMEM medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 50 U/ml penicillin, and 50 µg/ml streptomycin (GIBCO, USA). MIN6 cells were cultured in high glucose DMEM medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 1 mM glutamate, 50 µM β-mecaptoethanol, 50 U/ml penicillin, and 50 µg/ml streptomycin (GIBCO, USA). INS1E cells were kindly provided by Dr. C. Wollheim (Geneva, Switzerland). INS1E cells were grown in RPMI 1640 medium (GIBCO, USA) containing 11 mM glucose and 2 mM L-glutamine supplemented with 10 mM HEPES (pH 7.5), 10% (v/v) heat-inactivated fetal bovine serum, 1 mM Na-pyruvate, 50 µM β-mecaptoethanol, 50 U/ml penicillin, and 50 µg/ml streptomycin (Merglen et al., 2004). Cells were maintained at 37°C, 5% CO_2, 95% O_2 humidified chamber.

2.3 DNA constructs

Primers were designed to amplify the entire coding sequence of corresponding genes with myc or flag tag epitope at the N-terminus. When required, the primers also incorporated restriction endonuclease sites, to simplify the cloning process. Directional cloning was used when possible so each primer pair incorporated two different restriction enzyme sites to ensure that the gene would be inserted into the vector in right orientation. The primers were then used in PCR reaction and DNA obtained purified by gel extraction. The primers used in the study are listed in Table 2-1. The phosphorylated site mutation was obtained using Quickchange II site-directed mutagenesis kit (Stratagene, USA).

For all myc-epitope tagged vectors, the c-myc sequence (peptide: MEQKLISEEDL; nucleotide: ATG GAA CAA AAA CTT ATT TCT GAA GAA GAT CTG) was placed in frame immediately upstream of coding sequence. Similarly for flag-epitope tagged vectors, flag sequence (peptide: DYKDDDDK; nucleotide: ATG GAC TAC AAG GAC GAT GAC GAT AAG) was placed at the N-terminus to be in-frame of the coding sequence. For these N-terminal tags, the transcription start site (ATG) was placed at the beginning of the epitope sequence deleting the endogenous start codon.

Table 2-1 List of primers used for polymerase chain reaction (PCR) cloning and quantitative PCR (qPCR)

Plasmids	**Sense/ Antisense primer sequence (5'-3')**	**Strategy**
pGEX-KG-rSyt7-41-403	ATCTATCTAGACGCCACCTG TCAGCGCAAACTGGGCAAA	PCR→XbaI cut, ligate into pGEX-KG
PCMV5-Flag-mRph3a-WT-1-681	ATCTAGGTACCCAATGACTG ACACTGTGGTGAAC ATCTATCTAGAGGTGGCGCT AATCACTGGACACGTGGTTC TCGTT	PCR→KpnI+XbaI cut, ligate into pCMV5-KpnI+XbaI
PCMV5-Flag-mRph3a RBD-1-325	ATCTAGGTACCCAATGACTG ACACTGTGGTGAAC ATCTATCTAGAGGTGGCGCT AGCCCCTGGATAGCCAGGGT CACTT	PCR→KpnI+XbaI cut, ligate into pCMV5-KpnI+XbaI
PCMV5-Flag-mRph3a C_2A-326-525	ATCTAGGTACCCAATGGTCG CCCCAGCCCGAGAGGA ATCTATCTAGAGGTGGCGCA CTCATAGAGAGCCATGCCAC GGGCCGA	PCR→KpnI+XbaI cut, ligate into pCMV5-KpnI+XbaI
PCMV5-Flag-mRph3a C_2B-526-681	ATCTAGGTACCCAATGGAGG AGCAGGTAGAGCGGAT ATCTATCTAGAGGTGGCGCT AATCACTGGACACGTGGTTC TCGTT	PCR→KpnI+XbaI cut, ligate into pCMV5-KpnI+XbaI

PCMV5-Myc rSyt7-S103A	AACGGAGCCCCGTTCCGCTG TCTCGGACCTCGTCAA TTGACGAGGTCCGAGACAGC GGAACGGGGCTCCGTT	SDM, Phosphodefective mutant
PCMV5-Myc rSyt7-S103E	AACGGAGCCCCGTTCCGAAG TCTCGGACCTCGTCAA TTGACGAGGTCCGAGACTTC GGAACGGGGCTCCGTT	SDM, Phosphomimetic mutant
PCMV5-Flag-mRph3a-S234A	CCCACGCGTAGGGCCGCTGA GGCACGGATGAGT ACTCATCCGTGCCTCAGCGG CCCTACGCGTGGG	SDM, Phosphodefective mutant
PCMV5-Flag-mRph3a-S234E	CCCACGCGTAGGGCCGAAG AGGCACGGATGAGT ACTCATCCGTGCCTCTTCGG CCCTACGCGTGGG	SDM, Phosphomimetic mutant
qPCR GAPDH	CAAGGTCATCCATGACAACT TTG GGCCATCCACAGTCTTCTGG	
qPCR Rph3a exon junction 1702/1703	CCAAGACAACAGCAACCTG CTTGTTGGACTTGCTGGCTC	

qPCR Rph3a exon junction 850/851	TGTGTCGTGTGTGAAGACTG GCTTCCAGACCTCTCTCTGC	
qPCR MyoVa	CGATTTGCTGGATGAGGAAT ACAGTTTTTGGGCCCATGT	

2.4 Glutathione S-transferase (GST) protein expression and purification

The cytoplasmic domain of Syt7 DNA sequences encoding the linker and C_2 domains (amino acid 41 to amino acid 403) was amplified from wild type and phosphomutant PCMV5-Syt7 plasmid. Primers were designed to introduce Xba1 overhangs on the ends. The amplified product was ligated into pGEX-KG vector linearized with Xba1 restriction enzyme. Insertion of the fragment was confirmed by restriction digestion and sequencing. Correct plasmids were then transformed into BL21 (DE3) competent cells for protein expression.

2.4.1 Optimization of recombinant protein expression

To ensure high quality protein production, at least four clones for each plasmid were grown in small scale in Luria-Bertani broth (LB broth) with ampicillin (0.1 mg/ml) till OD_{600} of 0.7 was attained, at which point isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce protein production. Once the clone with highest protein expression was identified, further optimization was performed for the expression conditions. The expression of

proteins can vary greatly depending upon the concentration of IPTG used, time of shaking or the incubation temperature. To optimize these parameters, selected clones were shaken either for 6 h or overnight with 0.1 mM or 1 mM IPTG at both 25 °C and 37 °C. After induction, bacterial pellets were resuspended in 1x SDS sample buffer and analyzed by western blot followed by Coomassie brilliant blue (CBB) staining.

2.4.2 Large Scale expression and purification

Once the optimal conditions were determined, large-scale fusion protein purification was performed. PGEX-Syt7 wild type and mutant plasmids were transformed into BL21 Escherichia coli and induced with IPTG for 6 h at 25 °C. The bacterial suspension was centrifuged at 4000 rpm for 20 mins and the pellets were frozen at -80 degree. Following day, bacteria pellets were suspended in suspension buffer (40 mM TRIS, pH 8.2, 200 mM NaCl) with 1% triton containing 1 mM phenylmethyl sulphonyl fluoride (PMSF) and protease inhibitor cocktail tablet (Roche, Germany) and sonicated on ice using ultrasonic processor Vibra-Cell VCX130 (Sonics & Materials, Inc.) with four cycles of a 30 s pulse followed by 30 s break at 30% amplitude output for 2 mins. The lysate was cleared by centrifugation at 14,000 rpm at 4 °C for 30 mins. The supernatant was then incubated with pre-equilibrated glutathione sepharose 4B beads with 1 mM dithiothreitol (DTT) overnight at 4 °C.

Following day, the beads were washed twice in suspension buffer, twice in suspension buffer with 50 mM $CaCl_2$, three times in Tris-EDTA (TE) buffer

with 1 M NaCl followed by three washes in PBS. The extensive high salt wash was performed to remove nucleic acid contaminants from bacteria that have previously been shown to cause oligomerization or affect its interaction with other molecule (Ubach et al., 2001). The beads were stored in PBS containing 1 mM PMSF and 10 mM NaN_3 at 4 °C. Beads were freshly prepared prior to start of experiments and run on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel to check for any degradation and visualized using CBB dye.

2.5 Preparation of brain homogenate

Forebrain was collected from Syt7 knockout mouse and homogenized on ice in Buffer A (20 mM HEPES pH 7.4, 100 mM NaCl, 1 mM EDTA, 10 mM NaF, 1% Triton-X-100) containing 1 mM PMSF and protease inhibitor cocktail tablet (Roche). The homogenate was rotated in cold room for 2 h and the insoluble materials were removed by centrifugation at 14000 rpm at 4 °C for 30 mins. The total brain extracts was pre-cleared by further incubation of the supernatant with mixture of 0.125 ml of pre-swelled glutathione agarose and 0.125 ml of GST beads for 2 h in cold room. The cleared lysate was collected by centrifugation at 800 g for 2 mins.

2.6 GST pull down

Pre-cleared brain/MIN6 lysate was incubated with freshly prepared beads containing approximately 5 μg of immobilized GST fusion proteins with 3.5 mM $CaCl_2$ or 5 mM EGTA overnight in cold room. Following this, beads

were washed five times with buffer A with five-min incubation between each wash. The bound proteins were then eluted using 2x SDS-PAGE sample buffer and loaded on 10% SDS PAGE gel and resulting protein bands were visualized by coomassie staining. For mass spectrometric (MS) analysis, the proteins were eluted from the beads by boiling in fresh NuPAGE® LDS sample buffer (Invitrogen™) for 5 mins or by incubating with freshly prepared 8 M urea buffer (containing 0.1 M ammonium bicarbonate) for 30 mins at room temperature with gentle agitation.

For verification of protein binding, HEK293T cells were transfected with plasmid encoding tagged constructs and harvested after 48 h. Cell were lysed in 1.5 ml lysis buffer A containing protease inhibitors. Cleared supernatant was incubated with approximately 2 μg GST fused Syt7 protein immobilized on glutathione beads for 1 h at 4 °C with gentle rotation. Beads were then washed three times with same buffer and bound proteins were analyzed by immunoblotting with tagged antibodies. Beads bound to GST only were used as negative control.

2.7 Western Blotting

Samples were either freeze-thawed or sonicated (Labsonic, Sartorius, Germany) to ensure total lysis of cells. Total protein lysates were collected by centrifugation at 14,000 rpm for 20 mins at 4 °C. Protein concentration was determined by Bradford assay (Bio-Rad). Equal amount of cell lysates were denatured using 5X detergent sodium dodecyl sulphate (SDS) at 100 °C for 10

mins. Following denaturation, samples were resolved on 10% SDS-PAGE gel and transferred to nitrocellulose membrane (Invitrogen, USA) and blocked with 5% non-fat dry milk in PBS with 0.1% Tween-20 (PBS-T) for 1 h at room temperature. The membranes were incubated in optimized primary antibody concentration for either 1 h at room temperature or overnight at 4 °C before incubating in horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, UK) for 1 h at room temperature. Protein bands were visualized with enhanced chemiluminescence detection kit (GE Healthcare, UK). Beta tubulin (1:5000, Sigma) was used as a control to verify equal protein loading. The antibodies used in the study are listed in Table 2-2 below.

Table 2-2 List of antibodies used

Antibodies	**Isotype**	**Source**
Anti-FLAG	Mouse	Sigma
Anti Myc, clone 9E10	Mouse	Santa-Cruz
Anti-Syt7	Rabbit	Synaptic system
Anti-Rph3a	Rabbit	Synaptic system
Anti-Rph3a	Mouse	ECM bioscience
Anti-P-Rph3a	Rabbit	Abnova
Anti-SNAP23	Rabbit	Synaptic system
Anti-MyoVa	Rabbit	Cell Signaling
Anti-Phosphoserine	Rabbit	Sigma
Anti-Tubulin	Mouse	Sigma

2.8 Coimmunoprecipitation

HEK293T cells were transfected with plasmid encoding flag-tagged Rph3a along with myc-tagged Syt7 mutants using lipofectamine transfection following manufacturer's protocol. After 48 h of transfection, cells were gently rinsed in ice cold PBS and lysed in Buffer A containing 1 mM PMSF and protease inhibitor cocktail tablet (Roche). The lysates were then rotated in cold room for 30 min followed by centrifugation at 14000 rpm for 20 min at 4 °C. The supernatant was precleared using magnetic beads for an hour and the cleared supernatant was used for immunoprecipitation with anti-myc conjugated magnetic beads. Bound complexes were washed extensively with same buffer and eluted with 2x SDS loading dye. The eluate were loaded on 10% SDS PAGE gel and probed for myc or flag antibody.

For endogenous coimmunoprecipitation, MIN6 cells were grown to confluency (around 2 X 10^7 cells on 10-cm plate). On the day of treatment, calls were fasted for 2 h in Krebs-Ringer medium (KRH) containing 130 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 2.56 mM $CaCl_2$, supplemented with 1% bovine serum albumin (BSA) (w/v) and 3 mM D-glucose and buffered with 20 mM HEPES-NaOH to pH 7.4 followed by treatment with either DMSO or 10 µM foskolin for 30 min. Forskolin activates adenylate cyclase and increases the intracellular concentration of cAMP thereby activating PKA signaling pathway. Similar to GLP-1, treatment of MIN6 cells with the aforementioned concentration of forskolin for 30 min results in phosphorylation of Syt7. Treated cells were rinsed in ice cold PBS

and harvested in 1.5 ml lysis buffer A containing protease and phosphatase inhibitors. The lysates were rotated end to end in cold room for 30 min, followed by centrifugation at 14000 rpm for 15 min. The supernatant were then pre-cleared using 25 µl magnetic beads for 1 h to reduce any non-specific binding of proteins to the beads. About 2 µg cleared supernatant was then rotated end to end with primary antibody overnight at 4 °C. On the following day, 25 µl magnetic beads were added to the lysate and rotated for another 3 h at 4 °C. Beads were washed three times with same buffer with five mins incubation in between the washes and then eluted using 2x SDS loading dye. Immunoprecipitates were then analyzed by immunoblotting with respective antibodies. Beads containing equivalent amount of IgG antibody was used as control.

2.9 RNA Isolation and Real-time-polymerase chain reaction (PCR) Analysis

Total RNA was isolated from INS1E or MIN6 cells or islets using an RNeasy kit (QIAGEN, Germany). Briefly, the cells were harvested in RLT lysis buffer and transferred to a Qiashredder column for complete lysis. Equal volume of 70% ethanol was added to the lysate and transferred into the RNAeasy spin column. DNA contamination was removed by on-column DNase treatment (QIAGEN). After subsequent washing steps, purified RNA was eluted from the column and the concentration determined by Nanodrop (Thermo Scientific, USA).

To determine the mRNA expression levels, 1 μg of total RNA was reverse transcribed using RevertAID cDNA conversion kit (Fermentas, USA) according to the manufacturer's instructions. A standardized amount of 10 ng of cDNA was used for each PCR. Transcript levels were determined by quantitative RT-PCR using SYBR Green PCR master mix (Applied Biosystems, USA). For amplification, cycle parameters were adjusted to 1 cycle at 95 °C for 10 mins followed by 40 cycles of tandem 95 °C for 30 sec and 60 °C for 1 min. To prevent amplification of genomic DNA, intron-spanning primers were used (Table 2-1). The relative mRNA expression was calculated using comparative Ct method. Normalization was done to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene.

2.10 Cell Transfection

HEK293T cells were cultured in 10 cm plate at a density of $2x10^6$ cells a day before transfection. At the time of transfection, the cells were approximately 70% confluent. Cells were subsequently transfected transiently using Lipofectamine 2000 reagent (Invitrogen, USA), according to the manufacturer's instructions. Briefly, plasmids and Lipofectamine 2000 were mixed together (4 μg DNA to 10 μl Lipofectamine) in Opti-MEM I reduced serum medium (Invitrogen) and complexes formation was allowed at room temperature for 20 mins before adding onto the cells. Transfection media was replaced with normal culture media after 6 h and cells were cultured for 2 days before harvesting for subsequent experiments.

2.11 Lentiviral ShRNA constructs for knockdown studies

For loss of function studies, shRNA mediated gene silencing approach was used. Specific shRNA sequences were expressed either in pLenti-L309 (Sudhof T, Standford, USA) or in PLKO.1 (Addgene) vector. For Rph3a gene silencing, shRNA and scrRNA sequences were cloned downstream of H1 promoter in L309 vector backbone using XhoI/XbaI restriction sites. For MyoVa knockdown, shRNA and scrRNA sequences were cloned downstream of U6 promoter in PLKO.1 vector using AGEI/EcoRI restriction sites. The corresponding scrRNA and shRNA target sequences are shown in table 2-3. The sense sequences are highlighted in bold and underlined; antisense sequences are underlined. The loop sequences are denoted in small caps and the terminator signals are denoted in red.

Table 2-3 ShRNA constructs used in the study

Genes	shRNA sequence (5'-3')
Rph3a	TCGACCC**GCATTACAGAGGAGGACAT**TTCAAGAGAATGTCCTCCTCTGTAATGC TTTTTTGGAAAT
Scramble L309	TCGACCC**GCCATGGACGACAACACAA**TTCAAGAGATTGTGTTGTCGTCCATGGC TTTTTTGGAAAT
MyoVa	CCGG**CGCTACAAGAAGCTCCATATT**CTCGAGAATATGGAGCTTCTTGTAGCG TTTTTG
Scramble PLKO.1	CCGG**CCGCAGGTATGCACGCGT**CTCGAGACGCGTGCATACCTGCGGTTTTTG

2.12 Lentivirus Packaging and Infection

Lentiviruses were produced by co-transfecting lentiviral expression vector and packaging plasmids into HEK293T cells using Lipofectamine 2000 reagent (Invitrogen, USA). Viral supernatant was collected 48 h after transfection,

centrifuged at 1000 g for 5 mins and filtered with 0.45 μm filter. The viruses were concentrated by ultracentrifugation at 26,000 RPM for 2 h at 20 °C (Beckman Coulter Optima-L-100 XP, SW41 swinging rotor). Cultured cells were infected with concentrated viruses in the presence of 8 μg/ml polybrene to enhance the efficiency of viral infection. Culture media containing the virus was replaced with fresh media 24 h after infection. Knockdown efficiency was assessed by qRT-PCR and western blot for mRNA and protein abundance respectively 72-96 h after viral transduction.

2.13 Adenoviral overexpression

Recombinant adenovirus encoding Rph3a was prepared using the AdEasy system. A DNA fragment encoding wild type and phosphomutant Rph3a was generated using PCR with primers containing Kpn1 and Xba1 restriction site (Table 2-1) and ligated into p-Adshuttle-CMV under the control of the human cytomegalovirus promoter. Insertion of genes were confirmed by restriction digest and sequencing. The shuttle vectors containing wild type and phosphomutant Rph3a were then linearized using PmeI restriction enzyme and transformed into recombinant proficient (RECA+) BJ21 competent cells containing pAdEasy-1 backbone vector to generate recombinant adenoviral plasmid. Successful recombination event produced tiny colonies in agar plate, which were linearized using PacI restriction enzyme. The linearized recombinant DNA was then ethanol immunoprecipitated and directly transfected into mammalian packaging cell line (HEK293T cells) to produce viral plaques. The transfected cells were readily monitored through green

fluorescence. Ten days after transfection, the adenoviral particles were harvested from the cells by repeated freeze thaw lysis method. Further propagation was done in HEK293T cells and harvested every 48 h till a concentrated pool of virus was obtained. Viral particles so obtained were aliquot and stored at -80 ºC. For overexpression studies, freshly picked islets were transduced with appropriate multiplicity of infection (MOI) of virus for 24-48 h prior to functional assay.

2.14 Glucose-stimulated insulin secretion (GSIS)

Min6 cells were cultured in 12-well plates until 80-90% confluence ($2x10^5$ cells/ well). Before the experiments, Min6 cells were fasted for 2 h in Krebs-Ringer medium (KRH) containing 130 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 2.56 mM $CaCl_2$, supplemented with 1% bovine serum albumin (BSA) (w/v) and 3 mM D-glucose and buffered with 20 mM HEPES-NaOH to pH 7.4. The cells were then washed and preincubated with same buffer for 30 mins. Next, cells were incubated with 500 µl KRH containing 3 mM glucose for 30 mins followed by stimulation with KRH containing 16.7 mM glucose or 10 µM forskolin to stimulate insulin secretion. Supernatants were retained after each stimulus and the amount of insulin secreted was estimated by Insulin Enzyme-linked Immunosorbent Assay kit (ELISA) (Mercordia, Sweden) following manufacturer's guidelines. Total protein content was determined by Bradford assay (Biorad, USA).

2.15 Islet isolation and secretion assay

Islets were isolated from heterozygous Syt7 mutant mice for adenoviral overexpression of Rph3a plasmid. Briefly, mice were sacrificed by cervical dislocation and the abdominal cavity was opened. The pancreatic duct was cannulated and 3 mL of 2 mg/mL collagenase P (Roche, Switzerland) was injected into the common bile duct for pancreatic digestion. The pancreas was then excised and incubated at 37 ºC for 15 min, followed by gentle shaking. The samples were then centrifuged at 900 G for 1 min at 4 ºC, washed with HBSS and filtered through gauze. The islets were then handpicked under an upright stereomicroscope and cultured overnight in RPMI 1640 medium supplemented with 11 mM glucose, 10% Fetal bovine serum (FBS), 1% penicillin and streptomycin. Subsequent experiments were handled in KRH supplemented with 1 mg/ml BSA, 3 mM D-glucose and 20 mM HEPES at pH 7.4 (KRH buffer). For static insulin secretion assay, islets were starved in 3 mM glucose KRH buffer for 60 mins followed by sequential incubation in 3 mM glucose KRH buffer for 30 mins and KRH buffer containing various stimuli for another 30 mins. Incubation buffer was collected following each stimulus and the insulin released was measured using Mouse Insulin ELISA kit (Mercodia, Sweden). Following stimulation, islets were lysed by sonication in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1mM EDTA, 0.1% NP-40, supplemented with protease and phosphatase inhibitor) and the total insulin and protein content were measured.

2.16 Statistical Analysis

Data were presented as mean ± SEM. Statistical analyses were made using two-tailed Student's test or one-way ANOVA for group comparisons. The level of statistical significance was denoted in figures as *$p < 0.05$ or **$p < 0.01$.

Chapter 3 RESULTS

3.1 Phosphorylation of Syt7 by PKA enhances GLP-1 dependent potentiation of insulin secretion

Exocytosis of insulin-containing secretory granules in response to extracellular stimuli is tightly coupled with sensing of high local concentration of calcium in the vicinity of membrane, which is facilitated by calcium sensing protein. Syt7 is a major calcium sensor in both primary beta cells and clonal cell lines and plays a crucial role in glucose stimulated insulin secretion (GSIS). In islets, deletion of Syt7 resulted in more than 50% reduction in both first and second phase of insulin secretion (Gustavsson et al., 2008). Consequently, Syt7 has been identified as the predominant regulator of insulin secretion. While Syt7 appears to be an essential calcium sensor in mediating GSIS, it was also identified as a phosphorylation substrate of PKA in beta cells. Treatment of HEK293T cells overexpressing wild type Syt7 with forskolin or GLP-1 analogue, exendin-4 resulted in robust increase in apparent size of Syt7 when analyzed by western blotting suggesting post-translational modification (Figure 3-1B, third lane). This shift in molecular weight was abolished when the cells were treated with PKA inhibitor, H-89 (Light et al., 2002) indicating PKA dependent signal transduction pathway by which forskolin or GLP-1 exerts their effect. Using series of truncation and point mutants, the phosphorylation site was identified to be at Ser103 in the linker region (Figure 3-1A and B). Interestingly, Syt7 mice have defective GLP-1 mediated insulin secretion which could be rescued by adenoviral overexpression of wild type Syt7 but not phophodefective Syt7 mutant (Ser103--> Ala) suggesting the

importance of phosphorylation in GLP-1 mediated potentiation of insulin granule exocytosis (B. Wu et al., 2015). These findings are relevant for several reasons. Firstly, Syt7 seems to be the key target modulating both glucose-dependent and PKA-dependent insulin secretion. Secondly, it provides important evidence on regulation of synaptotagmin by post-translational modification. Finally, it expands the scope of synaptotagmin from being a critical calcium binding protein to regulator of exocytosis. At this point, while phosphorylation of Syt7 seems to be essential for GLP-1 potentiation of insulin secretion, the mechanism behind this potentiation is yet to be understood.

In this thesis, using biochemical and molecular approaches to identify phosphorylation-dependent binding partner of Syt7, I strive to understand the molecular basis of GLP-1 action on insulin secretion.

3.2 Proteomic screening of phosphorylation dependent Syt7 interacting proteins

The ability of beta cells to respond quickly to varying flux of intra- and extracellular cues relies on the extensive network of protein-protein interactions thereby creating a dynamic regulatory system within the exocytosis machinery. As discussed earlier, cAMP/PKA augments insulin secretion via phosphorylation of Syt7. Phosphorylation occurs at Ser103 in the linker region of Syt7 which is highly conserved among different species (**Figure 3-1**A). Moreover, despite essential role of Syt7 in calcium sensing, its

phosphorylation modification does not appear to perturb Ca^{2+} dependence of Syt7-mediated Ca^{2+}-triggered exocytosis (B. Wu et al., 2015). This suggests that phosphorylation may result in Syt7 interaction with as yet unidentified target proteins in the beta cell exocytosis machinery. To study if phosphorylation of Syt7 results in dynamic interaction network, a proteomic screening was performed using GST fused Syt7 protein with phosphomimetic or phosphodeficient mutation (**Figure 3-1**E).

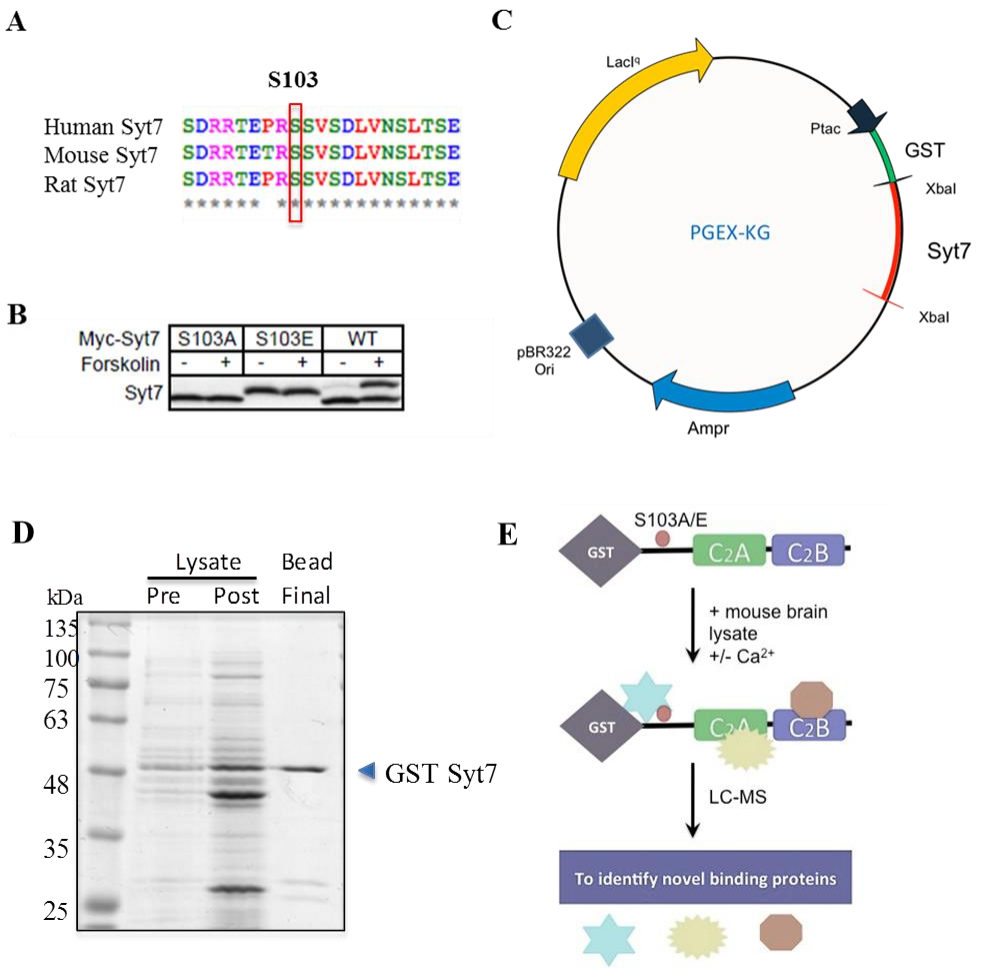

Figure 3-1 Schematic for identification of phosphorylation dependent Syt7 binding proteins

A) Sequence alignment of Syt7 residues carrying phosphorylation site from human, mouse and rat. S103 amino acid residue is conserved among all three species. B) Representative western blot for shift in band observed with treatment of HEK293T cells expressing wild type Syt7 with forskolin indicating phosphorylation of Syt7 (third lane), S103E mimics the phosphorylated state of Syt7. C) Vector map of PGEX-KG encoding GST-Syt7. C-terminal portion containing the linker region from wild type and mutant Syt7 were cloned into XbaI site of the vector to obtain GST tag at N-terminus. Protein expression is controlled by IPTG inducible tac promoter. D) Optimization of parameters for purification of GST fusion protein. First and second lane are bacterial lysates before and after IPTG induction. Final lane shows Syt7 C2A fragment expressed in GST beads. E) Successfully purified proteins were used as bait to identify interacting protein from the brain lysate in presence or absence of calcium. Bound proteins were eluted and processed by mass spectrometry to identify novel phosphorylation dependent Syt7 interacting proteins.

3.2.1 Expression and purification of GST-Syt7

Phosphomimetic (Ser103 --> Glu, S103E) and phosphodeficient (Ser103 --> Ala, S103A) mutants of Syt7 were generated by site directed mutagenesis. Mutation of Serine103 to glutamate mimics phosphorylated Syt7 due to charge and structural similarity with phosphorylated serine. On the contrary, alanine mutation mimics phosphodeficient mutant due to lack of –OH group needed for substitution with phosphate moiety. These mutants (encoding the linker and C-terminus) were then cloned into PGEX expression vector to allow N-terminal GST tag. Following successful cloning, vector was transformed into BL21 bacteria to allow expression of GST fused Syt7 protein. To avoid misfolding and aggregation of proteins during overexpression (Georgiou & Valax, 1996), following parameters were optimized prior to large-scale purification: optimal bacterial optical density $(OD)_{600}$ before induction of protein expression, concentration of IPTG required and temperature and duration of induction. The optimal condition was determined to be induction of BL21 bacterial strain expressing Syt7-PGEX plasmid at OD_{600} of 0.7 using 0.1 mM IPTG while shaking at 25 ºC for 6 h (**Figure 3-1**D**).**

3.2.2 GST pull down and mass spectrometry for identification of Syt7 interacting protein

GST beads expressing wild type and mutant Syt7 protein were incubated with precleared brain lysate from Syt7 knockout mice in presence of $CaCl_2$ or EGTA to monitor calcium dependent or independent binding. GST beads without any fusion protein were used a negative control. Bound proteins were

identified using two complementary approaches, namely SDS-PAGE gel-based ID and shotgun MS/MS based ID. One batch of eluted proteins was run on SDS-PAGE and Coomassie-stained protein bands were excised and digested by trypsin for MS analysis. Other batch of bound proteins eluted using urea buffer was subjected to in-solution trypsin digestion before being analyzed by MS. The major hits obtained using the above analyses are listed in Table 3-1. Among these proteins, those with functions that are known to be involved in exocytosis pathway were chosen as putative Syt7 interacting proteins and analyzed for cellular function associated with binding.

Table 3-1 List of proteins identified in mass spectrometry

Category	Cytoskeletal proteins	Kinase & Phosphatase	Others
S103A-Ca^{2+}	Tropomyosin Myosin-11 Drebrin Coronin	Protein phosphatase1	PIP4,5 Kinase
S103A-EGTA	ARP 2/3 complex Tropomodulin F-actin capping protein		
S103E-Ca^{2+}	Tropomyosin 14-3-3 protein Drebrin MyosinVa	PKA CamKII PKC gamma	Rabphilin3a RasGAP1 AP complex
S103E-EGTA	14-3-3 protein Actinin Beta adducin F-actin capping protein		Caldesmon Catenin alpha-2

3.3 Rph3a interacts with Syt7 in calcium and phosphorylation dependent manner

Among the proteins identified, Rph3a is of high interest for Syt7 binding, due to its known involvement in granule trafficking (Deak et al., 2006; Fukuda, 2008). In neuroendocrine cells, Rph3a binds to SNAP-25 and docks the vesicles to the membrane, thereby facilitating granule release. Conversely, in insulin secreting cells, Rph3a binds to MyoVa and regulates hormone secretion following PKA activation (Brozzi, Lajus, et al., 2012). Regardless, evidences demonstrating interactions between Rph3a and Syt7 in the regulation of insulin exocytosis are not directly assessed. To validate observations from mass spectrometry data, we sought to confirm its interactions *in vitro* and *in vivo*.

3.3.1 Rph3a is expressed in insulin secreting cells

Extensive studies have characterized the expression of Rph3a in neuronal and neuroendocrine cells. However, its expression in insulin secreting cells has been controversial (Coppola et al., 2002; Regazzi et al., 1996). To investigate whether Rph3a is indeed present in insulin secreting clonal and primary beta cells, I performed western blotting and quantitative polymerase chain reaction (qPCR) analysis of MIN6, INS1E and mouse pancreatic islets. Rodent MIN6 (mouse) and INS1E (rat) are commonly used insulin secreting clonal cell lines that mimic primary beta cells (Poitout, Olson, & Robertson, 1996). Consistent with previous observations (Inagaki, Mizuta, & Seino, 1994), Rph3a protein was abundantly expressed in brain (positive control) and was also detected in

MIN6 cells (Figure 3-2A). The antibody used for detection recognizes amino acid 671-684 of rat/mouse Rph3a sequence and has been validated before (Schluter et al., 1999). However, as reported earlier (Inagaki et al., 1994; Regazzi et al., 1996), Rph3a protein expression was undetectable in INS1E cells (Figure 3-2A) and islets, this could be due to relatively low abundance of this protein in these cells as evidenced by its low transcript level (Figure 3-2). Our finding is in agreement with the previous study that documents expression of Rph3a in pancreatic beta cells (Brozzi, Diraison, et al., 2012; Brozzi, Lajus, et al., 2012). Moreover, the presence of Rph3a in insulin secreting cell lines suggests a functional role in insulin granule exocytosis.

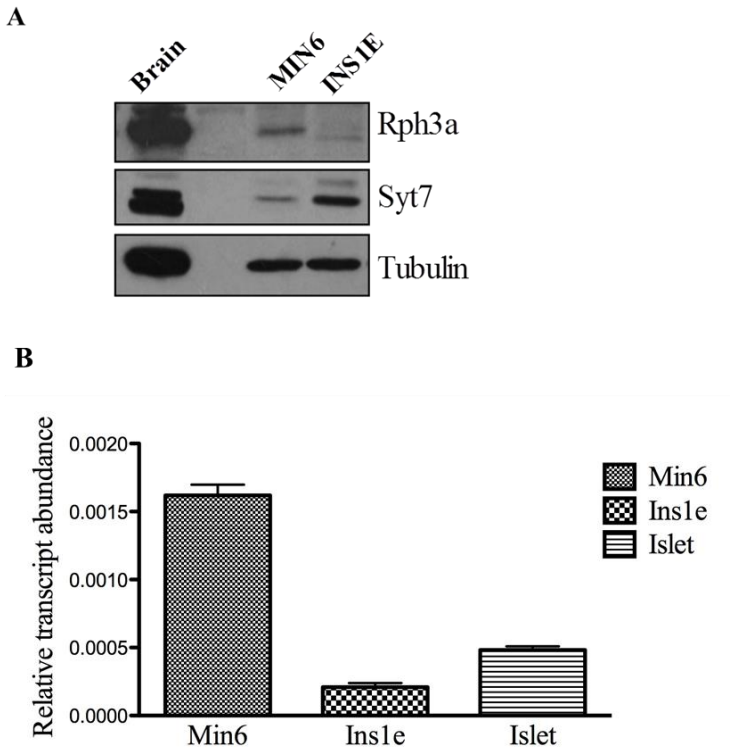

Figure 3-2 Rph3a is expressed in insulin secreting cell lines and primary beta cells.

A) Western blot analysis shows a 75kDa band corresponding to Rph3a in MIN6 cells. Brain lysate served as a positive control for Rph3a. Tubulin was used as a loading control. B) qRT-PCR analysis of Rph3a transcript abundance in insulin secreting cell lines and primary islets. The transcript abundance of Rph3a was normalized to GAPDH, a housekeeping gene. Data is presented as mean±sem, n=3.

3.3.2 Phosphorylated Syt7 exhibits enhanced binding to Rph3a

3.3.2.1 Syt7 binds to Rph3a *in vitro*

Following the confirmation of Rph3a protein expression in brain and MIN6 cells, pull down experiments in mouse brain lysates was performed using phosphomimetic and phosphomutant GST-Syt7. Bound proteins were eluted and analyzed by standard western blotting as mentioned in methods section (section 2.7). A 75 kDa band corresponding to Rph3a was observed in eluents from GST-Syt7 but not from GST only beads, indicating that the interaction between Syt7 and Rph3a is specific. Furthermore, greater binding was observed with phosphomimetic (S103E) GST-Syt7 beads compared to phosphomutant (S103A) GST-Syt7 beads (Figure 3-3A), suggesting that phosphorylation of Syt7 enhances binding to Rph3a.

To further confirm the binding interaction between phosphorylated Syt7 and Rph3a, coimmunoprecipitation was performed. Coimmunoprecipitation is another widely used method to identify stable protein-protein interactions and involves co-expression of epitope tagged proteins in cell lines of interest. Epitope tagging simplifies the immunoprecipitation experiments due to the availability of high affinity antibodies for the tags. Since they are short sequences, they usually do not interfere with protein expression and function (Terpe, 2003). Plasmids encoding Rph3a constructs were kindly provided by Dr. Zhiping Pang (UT Southwestern Medical Center). Flag tagged Rph3a was co-expressed with either wild type or phosphomutant myc tagged full length Syt7 in HEK293T cells. Monoclonal antibody against c-myc bound to

magnetic beads was used to immunoprecipitate Rph3a in calcium or EGTA containing IP buffer. As observed in GST pull down, Rph3a bound slightly more strongly to phosphomimetic Syt7 than with phosphodeficient form (Figure 3-3B). Furthermore, Rph3a bound to Syt7 both in presence and in absence of calcium, however calcium significantly enhanced both Syt7 and Rph3a interaction, suggesting that Syt7-Rph3a binding is both phosphorylation and calcium dependent.

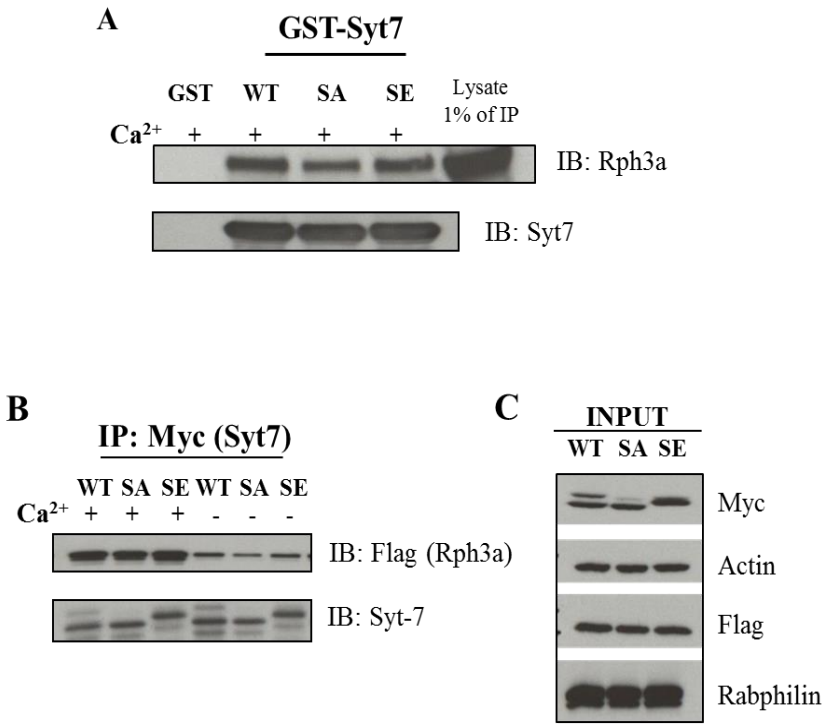

Figure 3-3 Syt7 interacts with Rph3a *in vitro*

A) Wildtype (WT), phosphodeficient (SA) and phosphomimetic (SE) GST Syt7 beads were used to pulldown brain lysate. Rph3a interacts with higher affinity to phosphorylated Syt7 (SE) compared to phosphodeficient (SA) form. B) Flag tagged Syt7 and Rph3a plasmids were coexpressed in HEK293T cells and c-myc antibody was used to pull down Rph3a in presence or absence of calcium (Ca^{2+}). Rph3a binds to Syt7 in a phosphorylation and calcium dependent manner. C) Input shows equal amount of protein being loaded. Figures are representatives of two independent experiments.

The GST-pull down and coimmunoprecipitation assay results described above confirm the interaction of Syt7 with Rph3a. Although both these methods provide evidence of *in vitro* binding, they do not necessarily reflect or recapitulate that these protein interact *in vivo* since *in vitro* studies only provide biochemical endpoints of multiple protein interactions under solubilized condition. Therefore, these interactions need to be demonstrated to occur *in vivo.*

3.3.2.2 Binding of Syt7 to Rph3a is promoted by PKA signaling *in vivo*

To determine whether the Syt7-Rph3a interaction characterized through *in vitro* experiments occurred in real time scale of insulin release in physiological settings, coimmunoprecipitation experiments were performed in MIN6 cells. MIN6 cell was chosen for this set of experiment due to the higher abundance of Rph3a expression compared to other insulin secreting cell lines as discussed earlier in section 1.3.1. MIN6 cell line was originally derived from a transgenic mouse expressing large T-antigen of SV40 in pancreatic beta cells, and is one of a few cell lines that display characteristics of pancreatic beta cells, including insulin secretion in response to glucose and other secretagogues (Ishihara et al., 1993; J.-I. Miyazaki et al., 1990)

To mimic endogenous PKA stimulation, MIN6 cells were treated with either DMSO or with 10 µM forskolin (FSK) for 30 mins. Forskolin, a potent activator of AC, rapidly increases intracellular accumulation of cAMP, which in turn activates PKA signaling (Eddlestone, Oldham, Lipson, Premdas, & Beigelman, 1985) that phosphorylates Syt7 at Ser103 position (B. Wu et al., 2015). To further confirm phosphorylation, I used P-PKA substrate antibody that recognizes phospho-serine/threonine residues that are preceded by arginine at the -3 position (RXXpS/T), comprising the PKA recognition site. Indeed, forskolin stimulation for 30 min robustly increased the overall PKA phosphorylation in MIN6 cells (Figure 3-4A). Following treatment, endogenous Rph3a was immunoprecipitated using polyclonal Syt7 antibody. Clearly, Rph3a was immunoprecipitated only when the cells were treated with

forskolin indicating phosphorylation dependent binding (Figure 3-4B). Rabbit IgG was used as control for immunoprecipitation.

To avoid issues with antibody cross-reactivity to target and cellular proteins, I performed reciprocal immunoprecipitations in similar treatment condition but this time endogenous Syt7 was immunoprecipitated using monoclonal antibody against mouse Rph3a. Similar observation was made whereby binding of Syt7 to Rph3a was observed only in presence of forskolin stimulation (Figure 3-4C).

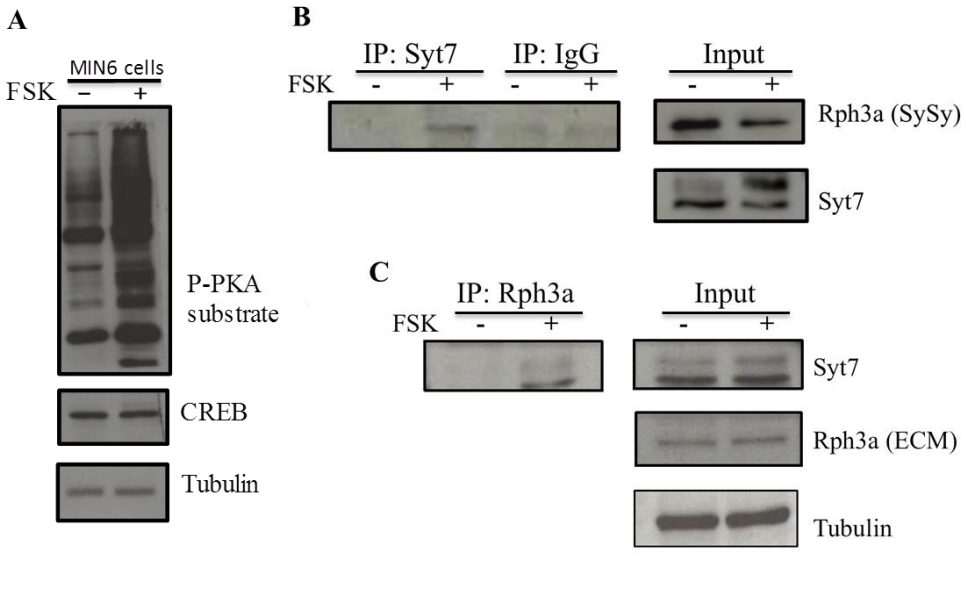

Figure 3-4 Rph3a interacts with Syt7 *in vivo*

A) MIN6 cells were treated with 10 µM forskolin (FSK) for 30 min to stimulate PKA-dependent phosphorylation. P-PKA substrate antibody was used to confirm overall increase in phopshorylation of proteins. B) Polyclonal rabbit antibody against Syt7 was used to immunoprecipitate DMSO or forskolin treated MIN6 cells. Rabbit IgG was used as negative control. Rph3a was detected only in lane with forskolin treatment, indicating phosphorylation dependent binding of Syt7 and Rph3a. No band was observed in IgG control. C) For reverse coimmunoprecipitation, monoclonal antibody against mouse Rph3a was used to immunoprecipitate Syt7 from MIN6 cell lysates treated with or without forskolin. Syt7 was only detected in lane with forskolin treatment, confirming phosphorylation dependent interaction. Input shows equal amount of proteins being loaded. Figures are representative of two to three independent experiments.

Taken together, these data confirm that Rph3a binds to Syt7 and phosphorylation of both proteins by PKA enhances their binding affinity. Notably, the amount of endogenous Rph3a protein that co-immunoprecipitated with endogenous Syt7 is relatively small. This is expected because only a small fraction of Syt7 is phosphorylated under the stimulated condition.

3.3.3 Phosphorylation of Rph3a further enhances binding to Syt7

It is well established that Rph3a can be phosphorylated by calmodulin- and cAMP-dependent protein kinases *in vitro* and by PKA in neurons (Fykse, Li, & Sudhof, 1995; Lonart & Sudhof, 1998). The major phosphorylation site is Serine 234 (S234) located in the linker region between Rab binding amino-terminus and the carboxyl-terminal C_2 domains. Phosphorylation of Rph3a is thought to be involved in PKA induced activation of long-term-potentiation in neurons although no striking synaptic phenotype was observed in Rph3a knockout mice (Lonart & Sudhof, 1998). To confirm that phosphorylation occurs in exogenously expressed Rph3a, HEK293T overexpressing wild type Rph3a was treated with forskolin. Robust phosphorylation of Rph3a was observed within 15 mins of stimulation and was detected by p-serine antibody as well as previously characterized phospho-specific Rph3a antibody (Lonart & Sudhof, 1998) (Figure 3-5A).

Since both Syt7 and Rph3a are phosphorylated by PKA, it is possible that phosphorylation of Rph3a would further enhance its binding with Syt7 to enhance PKA-dependent Syt7-mediated insulin release. To address this, I generated phosphorylation-deficient (Ser→Ala, phosphodeficient S234A) and phosphomimetic (Ser→Glu, phosphomimetic S234E) mutants of Rph3a using site-directed mutagenesis. Successful mutation was verified by sequencing using primers that span the phosphorylation region. These constructs were then overexpressed in HEK293T cells and GST-Syt7 beads were used to perform pull down assay. Notably, phosphomimetic Rph3a (S234E) readily

bound to GST Syt7 (S103E) as compared to phosphodeficient mutant (S234A) as shown in Figure 3-5B indicating phosphorylation of Rph3a further enhances binding to Syt7.

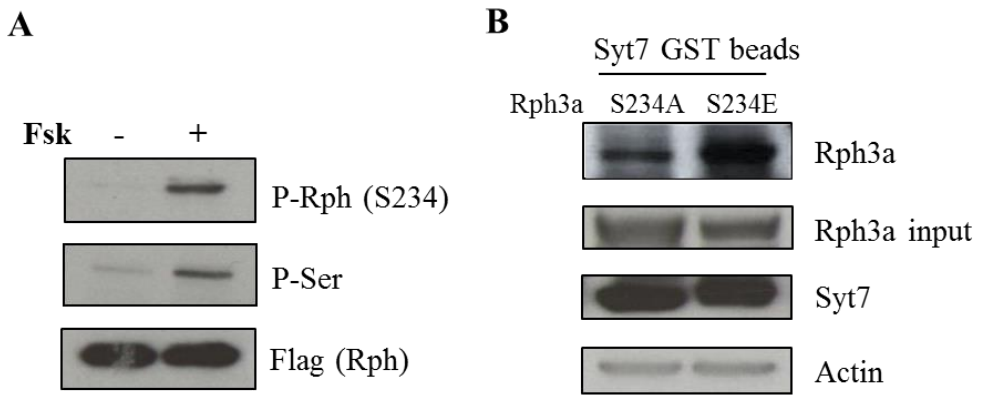

Figure 3-5 Phosphorylation of Rph3a further enhances Syt7 binding

A) Phosphorylation of Rph3a is induced by forskolin at Ser234 in the linker region. HEK293T cells overexpressing Flag Rph3a was treated with 10 uM forskolin for 15 mins. Robust phosphorylation was observed as detected by phospho-Rph3a (p-Rph) and P-Serine (P-Ser) antibody. B) Binding of Syt7 to Rph3a is further enhanced when Rph3a is also in a phosphorylated state. Ser234A and S234E are phosphodefective and phosphomimetic mutant of Rph3a respectively. Figures are representative of two independent experiments.

Collectively, these results suggest that the phosphorylation of Syt7 and Rph3a proteins by PKA signaling further promote their association, which may in turn regulates granule trafficking during PKA activation.

3.3.4 Syt7 interacts with C_2A domain of Rph3a

Rph3a contains a Rab binding domain (RBD) at the N-terminus followed by two tandem C_2 domains at the C-terminus. Having observed the interaction of Syt7 and Rph3a *in vitro* and *in vivo*, the domain(s) of Rph3a necessary for Syt7 binding was investigated by GST pull down of Rph3a truncated mutants. Full length N-terminus Flag tagged Rph3a and the different Rph3a truncations carrying only the Rab binding domain, or the C_2 domain were subcloned into PCMV5 vector (Figure 3-6A). Each of these constructs was transfected in HEK293T cells and their expressions were confirmed using antibody against Flag epitope (Figure 3-6C). Whole cell lysates expressing the truncated mutants were then incubated with GST Syt7 beads and the eluted proteins analyzed by SDS-PAGE and Western blot for anti-Flag antibody. Notably, only the fragment encompassing isolated C_2A domain of Rph3a bound to Syt7. By contrast, fragments containing RBD or C_2B domain failed to interact with Syt7 (Figure 3-6B), indicating that the C_2A domain of Rph3a is primarily responsible for binding to Syt7.

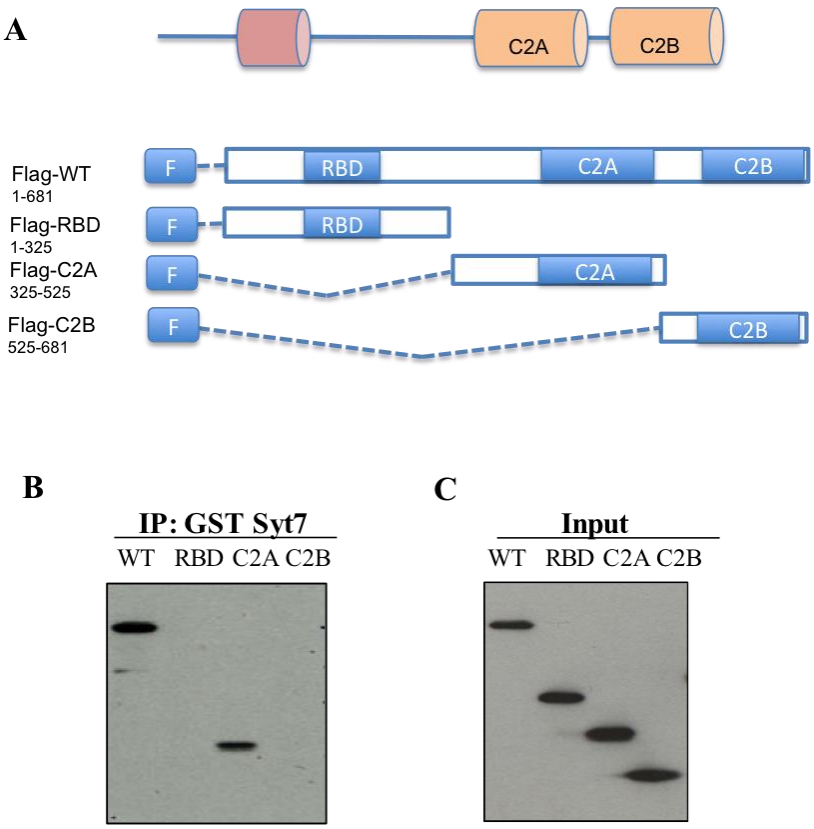

Figure 3-6 Mapping of Rph3a domain interacting with Syt7

A) Schematic of Rph3a truncation mutants. Subscript numbers are indicative of amino acid positions. All sequences were cloned into PCMV5 vector with Flag epitope tag at the N-terminus of start codon. B) C_2A domain of Rph3a binds to Syt7 but not the RBD or C_2B domain. C) Input shows comparable expression of all the mutants.

Taken together, current observations have provided strong evidence for phosphorylation dependent binding of Syt7 with Rph3a. Further understanding of the importance of Syt7-Rph3a interaction requires methods to study gain- and loss-of-function of interacting proteins. This is discussed in details in the following section.

3.4 Rph3a in regulation of GLP-1 potentiated insulin secretion

Although the significance of Rph3a in synaptic vesicle release has been largely debated due to lack of synaptic phenotype in knockout mice, *in vitro* studies in chromaffin and PC12 cells indicated its role in docking of granules (Deak et al., 2006; Fukuda, 2008; Tsuboi et al., 2007). Furthermore, Rph3a proteins are proposed to play a role in GLP-1 mediated hormone release from MIN6 cells (Brozzi, Lajus, et al., 2012). However, these observations were made in experiments involving overexpression of growth hormone, which may not reflect the ideal insulin secretion phenotype. Thus, the functional role of Rph3a in direct regulation of GLP-1 potentiated insulin release warrants further investigation. To address this, I performed knockdown of endogenous Rph3a protein from the MIN6 cells and studied its effects on insulin secretion.

3.4.1 Lentiviral mediated knockdown of Rph3a impairs insulin secretion in response to forskolin

To evaluate whether disruption of Rph3a function affects GLP-1 dependent potentiation of insulin secretion, Rph3a gene expression was silenced using lentivirus-mediated delivery of shRNA. Lentivirus is commonly used for gene delivery due to its ability to infect both dividing and non-dividing cells with minimal toxicity. Upon transfection, shRNA is transcribed via Pol III promoter and processed into active 20 to 25-nt siRNAs by Dicer. The siRNA strands get incorporated into the RNA-induced silencing complex (RISC) and bind to the target mRNA in sequence specific manner via complementary base pairing. Once bound, argonaute protein in the RISC complex cleaves the

bound mRNA. This leads to reduced levels of transcript that is available for translation by ribosomes and thus results in effective repression of target gene activity (Paddison, Caudy, Bernstein, Hannon, & Conklin, 2002). The schematic for shRNA design is illustrated in Figure 3-7.

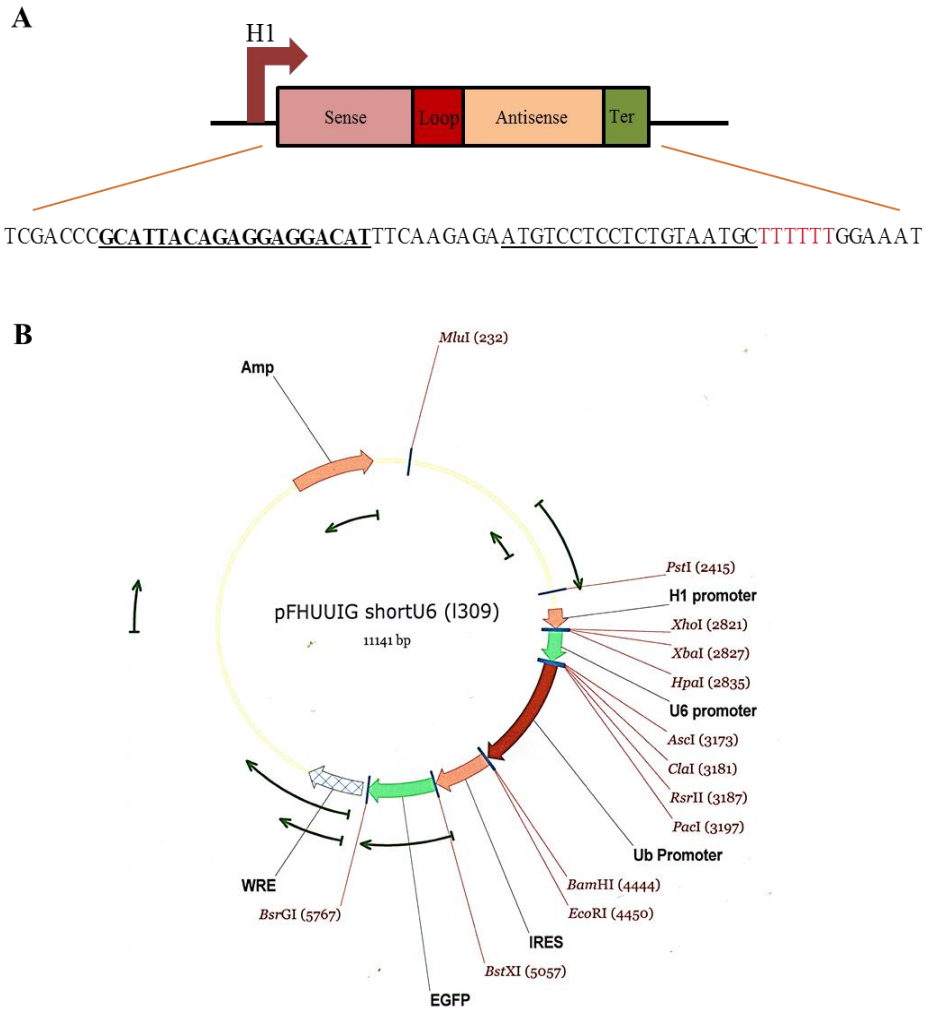

Figure 3-7 Schematic for generation of Rph3a shRNA construct

A) Schematic illustration of shRNA construct designed for Rph3a knockdown. shRNA is expressed as a hairpin that consists of sense sequence (bold and underlined), followed by 9nt spacer, an antisense sequence (underlined) and a transcription stop signal for RNA polymerase III (in red). B) Map of L309 lentiviral vector. shRNA sequence was introduced downstream of H1 promoter using XhoI/XbaI restriction sites. The H1 expression cassette provides efficient RNA polymerase III-dependent transcription of shRNA.

ShRNA construct generated against Rph3a (Sh3) significantly decreased the endogenous level of Rph3a protein expression in MIN6 cells as shown in the immunoblot from infected cells monitored 96 h after infection (Figure 3-8A). A non-targeting-scrambled (Scr) control was used to control for potential off-target effects of lentivirus infection.

After establishing effective and efficient knockdown of Rph3a, the effects of Rph3a protein loss on insulin secretion in response to high glucose and forskolin treatment were assessed. Briefly, cells were fasted for 2 h in Krebs Ringer HEPES (KRH) buffer containing 3 mM glucose. The cells were then preincubated with same buffer for 30 mins, followed by incubation for another 30 min with KRH containing 16.7 mM glucose and 16.7 mM KRH with forskolin. Amount of insulin secreted was estimated using Insulin ELISA kit (Mercodia, Sweden), the readings were normalized to total insulin content. As shown in Figure 3-8B, knockdown of endogenous Rph3a from MIN6 cells significantly impaired forskolin-induced insulin secretion. On the contrary, glucose-induced insulin release remained unchanged (Figure 3-8B).

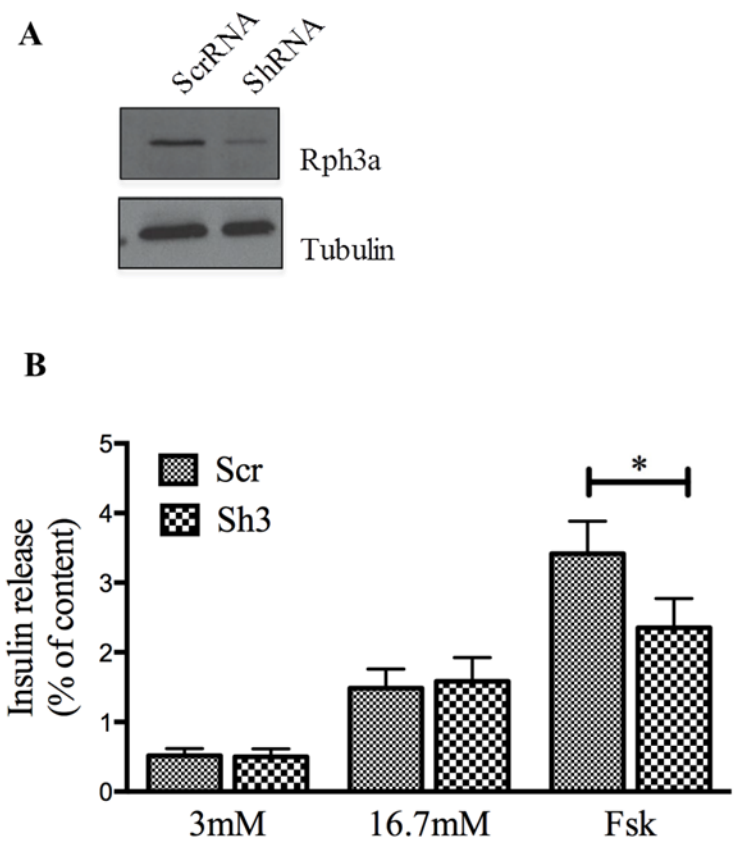

Figure 3-8 Knockdown of Rph3a diminishes insulin secretion in MIN6 cells

A) RNA interference mediated silencing of endogenous Rph3a in MIN6 cells. MIN6 cells were transduced with lentivirus expressing shRNA with non-specific (Scr) or Rph3a specific (Sh3) sequences. Figure shows representative immunoblot of MIN6 extracts confirming reduction of Rph3a protein abundance 72 h after viral transduction. Tubulin remains unchanged. B) Insulin secretion in static culture conditions. Scr or shRNA infected MIN6 cells were starved for 2 h in low glucose (3 mM) KRH and the amount of insulin secreted following high glucose (16.7 mM) and forskolin treatment were measured by ELISA. Silencing of Rph3a significantly reduced forskolin stimulated insulin secretion but had no effect in high glucose induced insulin release. Data are expressed as mean±sem, n=3. *$p<0.05$.

3.4.2 Adenoviral overexpression of phosphomimetic Rph3a in islets enhances GLP-1 potentiated insulin secretion.

Since forskolin-induced insulin secretion in Rph3a knockdown cells is defective, it is conceivable that phosphomimetic Rph3a might enhance insulin secretion. To determine if Rph3a phosphorylation could play a positive role in driving GLP-1 mediate insulin secretion, adenoviral constructs encoding wild type and phosphomutant Rph3a were overexpressed in islets and insulin secretion in response to GLP-1 analogue-exendin-4 was evaluated.

Modulation of gene expression in islet is particularly daunting due to their three dimensional structure. Pancreatic islets are organized into an organoid cluster with beta cells forming the core and alpha cells at the periphery. Most vectors used for gene delivery only infects the periphery of islets, however adenoviral infection has been demonstrated to infect both the periphery and core of islets thus making it the most potent and suitable gene transfer tool for primary islets (Mukai et al., 2007). The schematic of adenovirus generation is shown below (Figure 3-12). Briefly, recombinant adenovirus is produced by double recombination event between shuttle vector carrying gene of interest (Rph3a) and adenoviral backbone plasmid vector, pAdEasy-1. Viral plaques are then obtained from mammalian packaging cell line expressing linearized recombinant plasmid. Adenoviral particles are further amplified and directly used for infection of islets. The infection efficiency can be monitored by readily through green fluorescence.

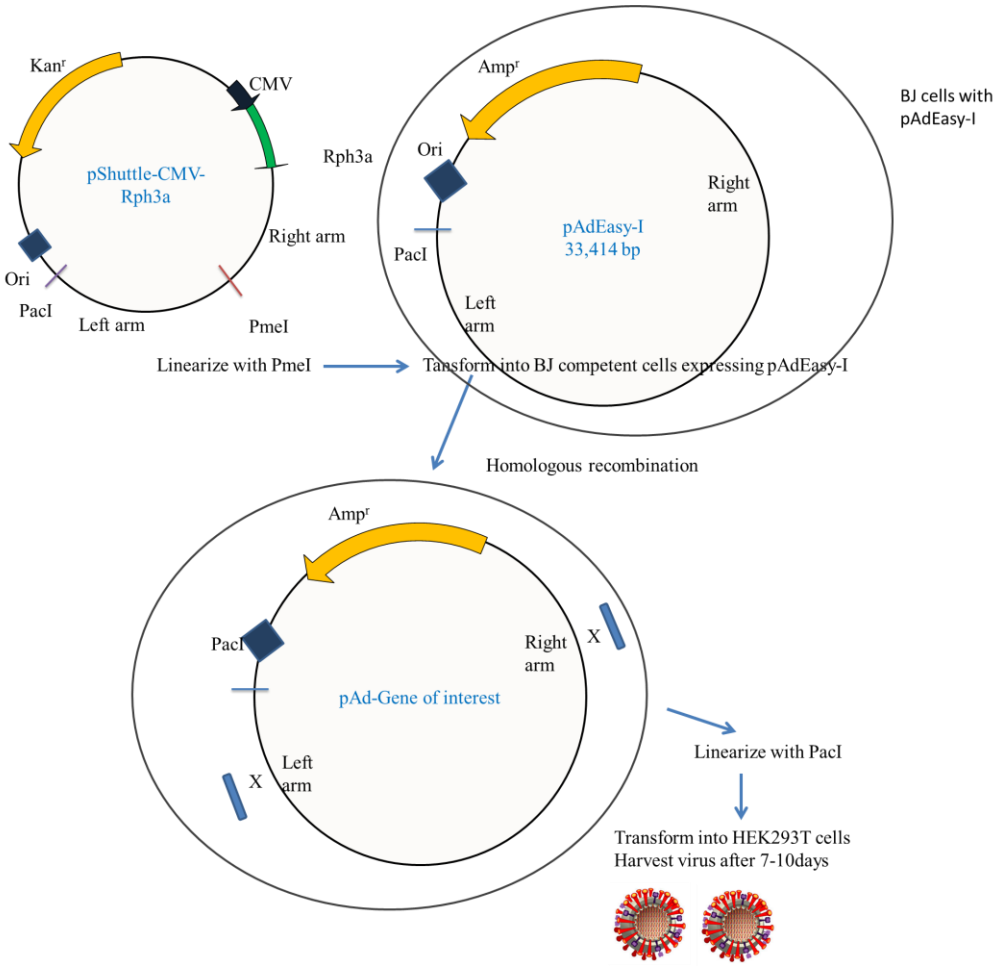

Figure 3-9 Illustration of cloning strategy for adenovirus overexpressing Rph3a

WT/SA/SE Rph3a was first cloned into a shuttle vector, pAdTrack-CMV. The resultant plasmid was linearized by digesting with restriction endonuclease PmeI and subsequently transformed into BJ competent cells containing the adenoviral backbone plasmid pAdEasy-1. The recombinant adenovirus plasmids were digested with PacI to liberate both inverted terminal repeats and transfected into HEK-293 cells. HEK-293T cells express E1 early gene that allowing virus production from backbone pAdEasy vector devoid of E1 gene. Recombinant adenoviruses are typically harvested after 7-10 days by lysis of HEK293T cells. Adenovirus particles are further amplified in HEK293T to obtain concentrated pool of virus.

Islets were infected with the concentrated pool of adenovirus containing wild type and phosphomutant Rph3a. Protein expression was monitored 48 h after infection to monitor the efficacy of the virus. Western blot analysis with Rph3a antibody revealed a single 75 kDa band corresponding to Rph3a which was absent in non-infected cells (Figure 3-10A) thus confirming the functionality of the virus. Note that Rph3a is not detectable in uninfected cells, presumably due to low amount of endogenous Rph3a protein expressed in islets.

For insulin secretion, isolated islets from Syt7 heterozygous mice was first infected with adenovirus overnight and allowed to recover for one more day. The overexpression was confirmed by visualization of GFP signal as well as by western blotting. On the day of analysis, islets were fasted in low glucose KRH for 1 h, and then stimulated with low glucose or exendin-4 for 30 mins. Supernatant was collected after each treatment and secreted insulin measured by ELISA. While overexpression of wild type Rph3a (WT) and phosphomimetic Rph3a (S234E) showed significant increase in insulin secretion with exendin-4 treatment, overexpression of phosphodeficient Rph3a (S234A) had no further enhancement in insulin release in response to exendin-4 (Figure 3-10B). These data strongly suggest that phosphorylation of Rph3a enhances GLP-1 mediated insulin release from pancreatic islets.

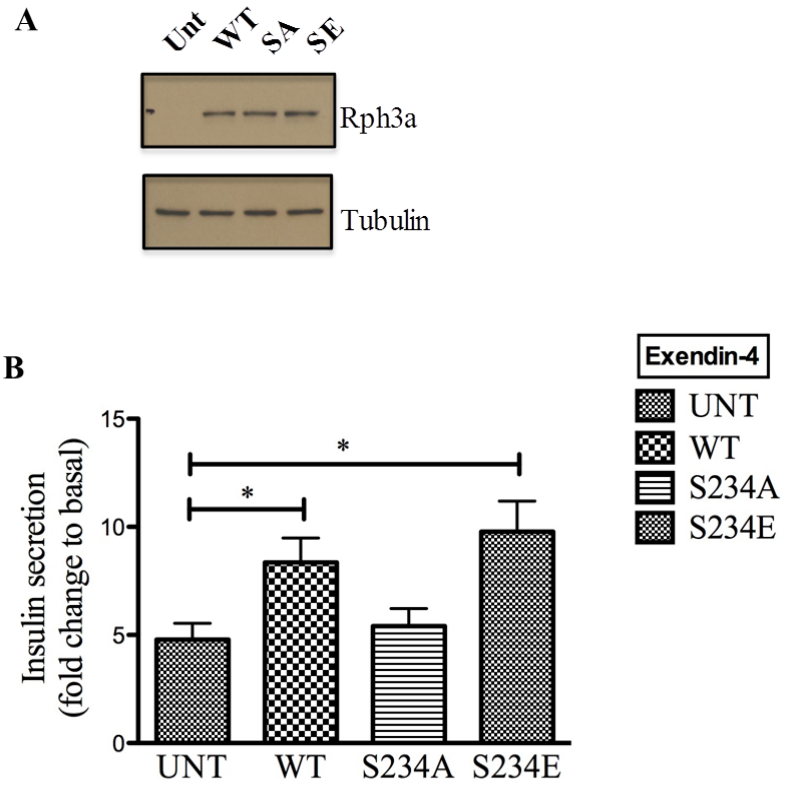

Figure 3-10 Phosphorylation of Rph3a enhances exendin-4 potentiated GSIS

A) Overexpression of wildtype (WT), phosphomutant (S234A) and phosphomimetic (S234E) Rph3a in islets for 48 h. Islets were lysed and lysates were analyzed by immunoblotting with indicated antibody. B) Islets overexpressing Rph3a were starved for 1 h in 3 mM KRH and the amount of insulin secreted following exendin-4 treatment was measured by ELISA. Overexpression of WT Rph3a and S234E mutant in islets exhibited significantly higher exendin-4 mediated insulin secretion. S234A did not potentiate secretion further. Data expressed as mean±sem, n=4. *$p<0.05$.

3.5 Investigating the potential molecular mechanism of Syt7-Rph3a binding in regulating PKA-mediated insulin secretion potentiation

The observations obtained by far suggest that phosphorylation induced enhanced binding of Syt7 and Rph3a is important for GLP-1 mediated insulin secretion. How exactly this enhanced binding promotes secretion remains undetermined. The role of Rph3a in docking of vesicles has been well characterized in neuroendocrine cells by its ability to interact with SNAP-25 in the membrane (Deak et al., 2006). In beta cells however, it is thought to interact with MyoVa, a motor protein to enhance granule translocation to plasma membrane (Brozzi, Lajus, et al., 2012). Our proteomic data indicates that MyoVa is one of the common proteins that bind to both Syt7 and Rph3a. Moreover, MyoVa was eluted alongside phosphomimetic Syt7 (**Table 3-1**). Given the evidence of interaction of Rph3a with MyoVa in presence of PKA signaling, it was anticipated that Syt7, Rph3a and MyoVa could function as a molecular machinery to mediate granule trafficking following PKA stimulation. To test this hypothesis, I determine if MyoVa can interact with Syt7 and Rph3a upon forskolin treatment.

3.5.1 MyoVa coimmunoprecipitates with Rph3a and Syt7

Min6 cells were treated with DMSO or forskolin for 30 mins to examine if MyoVa coimmunoprecipitates with Syt7 upon PKA activation. Treatment with forskolin enhanced PKA phosphorylation. Endogenous MyoVa was immunoprecipitated from the untreated and treated cell lysates using polyclonal Syt7 antibody. IgA beads were used as control for

immunoprecipitation. Interestingly, MyoVa band was only observed under forskolin treated condition suggesting phosphorylation dependent binding of Syt7 to MyoVa (Figure 3-11). Input shows equivalent amount of protein loaded.

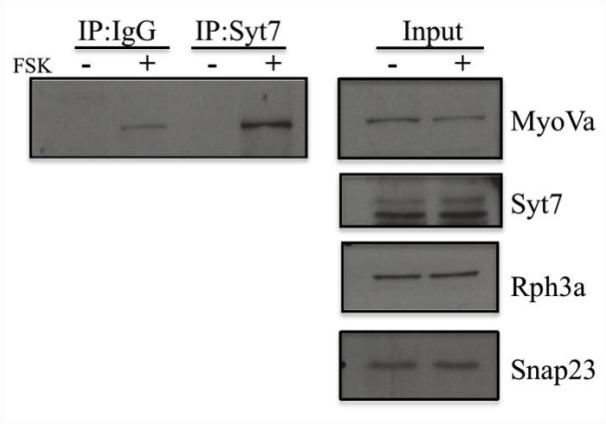

Figure 3-11 MyoVa is coimmunoprecipitated by Syt7 upon treatment with forskolin.

Coimmunoprecipitation assay of endogenously expressing Syt7 and MyoVa proteins from MIN6 lysates. Cells were treated with or without 10 μM forskolin treatment for 30 mins. Syt7 antibody was used to pull down MyoVa from cell lysates followed by immunoblotting with antibodies as indicated. Rabbit IgA was used as IP control. MyoVa coimmunorecipitated with Syt7 upon treatment with forskolin. Input shows equal amount of protein loaded. Figure is representative of two independent experiments.

3.5.2 Knockdown of MyoVa affects insulin secretion

Since Syt7 bound to MyoVa in a phosphorylation dependent manner, functional role of MyoVa in GLP-1 dependent insulin secretion was assessed by lentiviral knockdown of endogenous MyoVa. ShRNA targeting the coiled coil (CC) domain of the MyoVa protein was expressed in PLKO.1 vector. Lentivirus expressing the shRNA was then used to silence MyoVa expression in MIN6 cells.

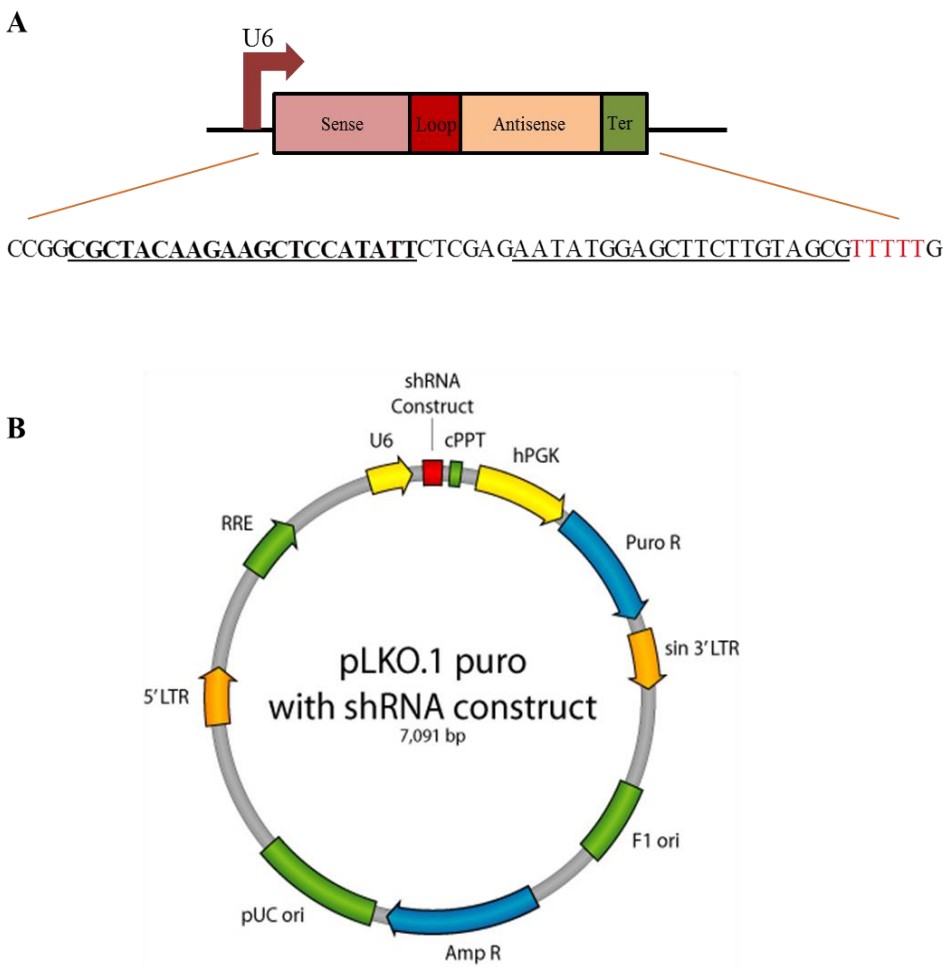

Figure 3-12 Schematic for generation of MyoVa shRNA construct

A) Schematic illustration of shRNA construct designed for MyoVa knockdown. shRNA is expressed as a hairpin that consists of sense sequence (bold and underlined), followed by 9nt spacer, an antisense sequence (underlined) and a transcription stop signal for RNA polymerase III (in red). B) Map of pLKO.1 lentiviral vector. shRNA sequence was introduced downstream of U6 promoter using AgeI/EcoRI restriction sites. The U6 expression cassette provides efficient RNA polymerase III-dependent transcription of shRNA. Infection efficiency can be readily monitored by visualization green flurescence.

Knockdown of MyoVa following infection with lentivirus expressing shRNA was verified by real time qPCR which showed approximately 40% decrease in mRNA level 48 h after infection Figure 3-13A) and confirmed by western

blotting 72 h after infection (Figure 3-13B). Following knockdown, the role of MyoVa in insulin secretion was assessed by method as described above in section 1.4.1. Insulin secretion in response to forskolin treatment was found to be significantly lower in MyoVa knockdown cells (Figure 3-13C), with no effect in glucose dependent insulin release, indicating the role of MyoVa in PKA-mediated insulin granule exocytosis.

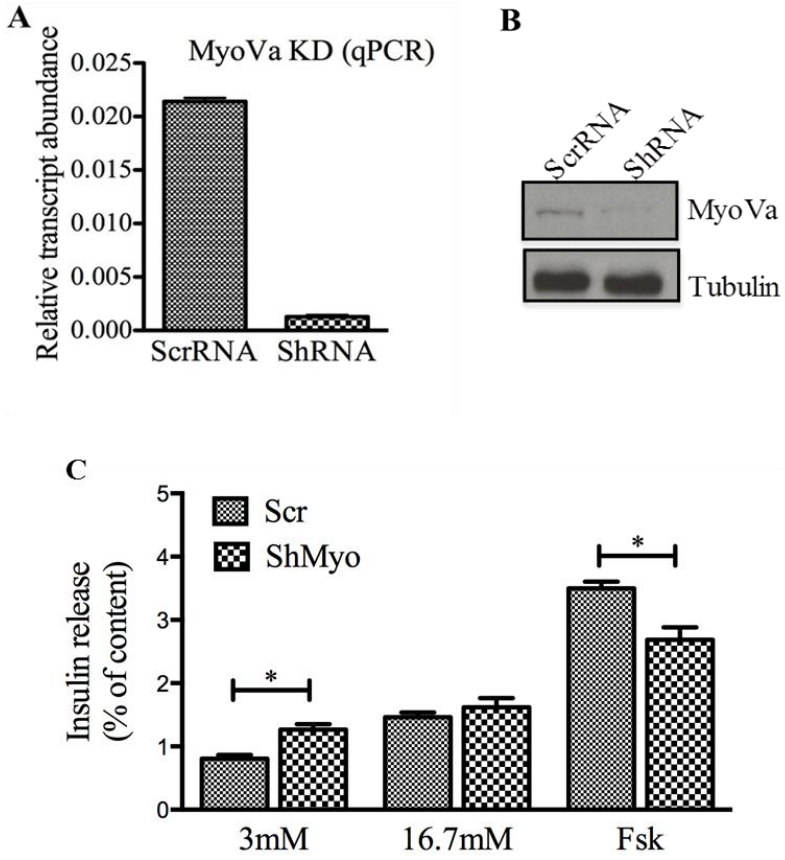

Figure 3-13 Functional characterization of MyoVa knockdown

A) qRT-PCR to confirm reduced transcript abundance of MyoVa with ShRNA to Scr control. B) Western blot confirming decrease in protein expression following ShRNA knockdown. C) Scr or shRNA infected cells were starved for 2 h in 3 mM KRH and the amount of insulin secreted following forskolin treatment was measured by ELISA. Silencing of MyoVa enhanced basal release but significantly reduced forskolin stimulated insulin secretion with no effect in high glucose induced insulin release. Data expressed as mean±sem, n=3. *$p<0.05$.

MyoVa is a crucial protein in beta cell exocytosis machinery with established roles in granule trafficking. Our current finding demonstrating Syt7 binding to MyoVa in a phosphorylation dependent manner together with decreased insulin secretion following MyoVa knockdown supports enhanced granule mobilization following phosphorylation of Syt7. Nevertheless, since Rph3a also binds to MyoVa, it is likely that these proteins together form a machinery to mobilize granules from the reserved pool to facilitate increased granule exocytosis following stimulation by PKA.

Chapter 4 DISCUSSION AND CONCLUSIONS

4.1 Protein-protein interaction plays key role in PKA-mediated phosphorylation and GLP-1 mediated insulin release

Glucose mediated insulin secretion (GSIS) serves as a primary mechanism to maintain normal blood glucose homeostasis, which is severely perturbed in diabetes. GLP-1 is a potent incretin hormone that potentiates insulin secretion in glucose dependent manner and normalizes insulin exocytotic defects in diabetic individuals. Previous studies have demonstrated that GLP-1, via activation of PKA, can directly phosphorylate proteins in exocytosis machinery to enhance granule exocytosis (Brozzi, Lajus, et al., 2012; Song et al., 2011; Sugawara et al., 2009; B. Wu et al., 2015). Several PKA substrates have been identified in pancreatic beta cells that are phosphorylated during activation of GLP-1. For example, Snapin, a SNAP-25 binding protein was highly phosphorylated in mouse model with constitutive PKA activation (Song et al., 2011). Syt7, a calcium sensor in beta cell was shown to be phosphorylated in primary islets and insulin secreting cell lines in response to GLP-1 and PKA agonists (B. Wu et al., 2015). Similarly, Myosin- and Rab-interacting protein (MyRIP) was phosphorylated in insulin secreting cell lines that in turn enhanced its interaction with MyoVa and its receptor Rph3a (Brozzi, Lajus, et al., 2012). Relating the importance of protein phosphorylation to impaired insulin secretion, previous study has demonstrated that phosphorylated snapin level is decreased in diabetic rodent islets with impaired GSIS and overexpression of phosphomimetic Snapin can rescue the insulin secretion defect in these mice (Song et al., 2011). Likewise,

phosphomimetic Syt7 can rescue the defect in GLP-1 mediated insulin secretion observed in Syt7 knockout islet (B. Wu et al., 2015), underscoring the importance of posttranslational modification in granule exocytosis.

Although the mechanisms underlying potentiation of insulin secretion by GLP-1 mediated substrate phosphorylation is not completely understood, some studies indicate regulation of protein-protein interaction of vesicle- and membrane-associated proteins within the exocytosis machinery following phosphorylation. In accordance with this hypothesis, phosphorylation of Snapin by PKA at Ser50 promotes interaction and assembly of SNAP-25, Collectrin and EPAC2 thereby converging both PKA-dependent and PKA-independent pathway (Song et al., 2011). Similarly, phosphorylation of MyRIP brings together MyoVa and Rab27 to form a complex that ultimately enhances granule release by allowing phosphorylation of Rph3a (Brozzi, Lajus, et al., 2012). These findings relate to a dynamic regulation of protein interaction within the exocytosis machinery following PKA mediated phosphorylation, thereby acting as a crucial node of regulation for successful granule exocytosis.

4.2 Significance of current study

Current study highlights the importance of protein-protein interaction as a critical node of molecular machinery underlying GLP-1 action consistent with the finding that Syt7 interacts with Rph3a upon activation of PKA signaling. Syt7 was shown to be phosphorylated by GLP-1 analogue exendin-4 in pancreatic beta cells and insulin secreting cell lines at Ser103. The site is positioned within the linker region connecting the calcium binding-C_2 domains with transmembrane domain. As the linker region is not well characterized, the significance for this phosphorylation in terms of molecular mechanism to regulate insulin secretion is not clear. In the current study, we identified Rph3a as a potential phosphorylation dependent interacting protein for Syt7 using mass spectrometry approach. Our hypothesis that Syt7 interacts with Rph3a is strengthened by several observations. Firstly, Rph3a is targeted to secretory vesicles (C. Li et al., 1994; McKiernan, Stabila, & Macara, 1996). Secondly, it binds to GTP bound Rab proteins that regulates vesicle recruitment to plasma membrane (C. Li et al., 1994). Lastly, it enhances stimulus dependent secretion in endocrine (Arribas, Regazzi, Garcia, Wollheim, & De Camilli, 1997) and neuroendocrine cells (Tsuboi & Fukuda, 2005). Consistent with previous observations (Brozzi, Lajus, et al., 2012), the expression of Rph3a was confirmed in insulin secreting cells. In addition, using multiple approaches, I demonstrated that Syt7 binds to Rph3a in calcium- and phosphorylation-dependent manner both *in vitro* and *in vivo*.

Interestingly, Rph3a can also be phosphorylated by PKA at Ser234 site in the linker region located between the Rab binding domain and C_2 domains. Phosphorylation has been suggested to regulate neuronal activity during development in a synapse-specific manner (Foletti, Blitzer, & Scheller, 2001; Fykse et al., 1995). Using *in vitro* pull down assay, I demonstrate that Syt7 binds strongly to phosphomimetic Rph3a compared to its phosphodeficient form. However, the truncation mutation analysis indicates that Syt7 binds to C_2A domain of Rph3a. Despite the C_2A fragment of Rph3a used in the experiment does not include phosphorylation site, the fact that this fragment of Rph3a still binds to Syt7 might be indicative that phosphorylation of Rph3a *in vivo* affects Rph3a localization to allow proximity with Syt7 for binding. This hypothesis is supported by an earlier finding by Foletti et al. that indicates reduced affinity of phosphorylated Rph3a to membrane (Foletti et al., 2001). Given that Rph3a rapidly shuttles between membrane and cytosol, phosphorylation then might be important for its role in vesicle mobilization for subsequent rounds of exocytosis.

In the current study, using gain and loss of function assays, I demonstrate the importance of Rph3a in insulin granule exocytosis. Lentivirus mediated knockdown of endogenous Rph3a in MIN6 cells resulted in attenuation of forskolin mediated insulin secretion without affecting the glucose dependent insulin release, highlighting its importance in PKA signaling. Furthermore, adenoviral overexpression of wild type and phosphomimetic Rph3a in primary mouse islets exhibited enhanced exendin-4 mediated insulin release, whereas

phosphodeficient mutant did not potentiate secretion. In line with this, overexpression of phosphomimetic Rph3a in Min6 cells was shown to enhance growth hormone release following forskolin treatment (Brozzi et al., 2012) although it should be noted that, insulin secretion was not assessed alongside in the same study. Accordingly, current observations further complement the observation that overexpression of phosphomimetic Rph3a facilitate GLP-1 potentiated granule exocytosis, thereby supporting the positive role of Rph3a in GLP-1 potentiation of insulin hormone secretion.

Rph3a was originally identified in synaptic vesicles as an effector for Rab3A (Shirataki et al., 1993) and Rab3c (C. Li et al., 1994). However, Rph3a knockout mice did not exhibit defects in synaptic phenotype (Schluter et al., 1999), suggesting that Rph3a does not directly regulate synaptic vesicle release in neuronal cells. In this context, Rph3a knockdown in MIN6 cells did not affect glucose-dependent insulin release. However, Rph3a deletion in Min6 cells significantly decreased GLP-1 dependent insulin secretion potentiation. This suggests that actions of Rph3a following PKA phosphorylation may preferentially regulate GLP-1 mediated insulin secretion. Given that Rph3a is targeted to Rab27 on the newly synthesized dense core granules (Tsuboi et al., 2010), and that sustained exocytosis response require continuous recruitment of newcomer granules (Gaisano, 2014), the role of Rph3a in selectively potentiating GLP-1 dependent insulin secretion is well justified. Previous studies suggest that Rph3a may be involved in vesicle transloction in both chromaffin and neuroendocrine cells (Arribas et al., 1997;

Chung, Takai, & Holz, 1995; Tsuboi & Fukuda, 2005). Moreover, these cells are shown to coexpress Rab27 and Rab3a (Fukuda, 2003). Consequently, it is speculated that Rph3a may act via Rab27 to regulate insulin granule exocytosis. Regardless, measures showing the direct interaction between Rph3a and Rab27 in facilitating insulin granule exocytosis are limited. Thus, further studies are required to validate this hypothesis.

Previous studies have shown that Rph3a interacts with several proteins within the exocytosis machinery (M. Miyazaki et al., 1994; Tsuboi & Fukuda, 2005; Willshaw et al., 2004). Of particular importance is its interaction with MyoVa in MIN6 cells (Brozzi, Lajus, et al., 2012) that allows translocation of secretory granules from the reserved pool to the plasma membrane. Here, using pull down experiments, I demonstrate that MyoVa also immunoprecipitate with Syt7 upon activation of PKA. Consistent with the finding that MyoVa recruits large dense core vesicles to the plasma membrane (Brozzi, Diraison, et al., 2012), knockdown of endogenous MyoVa in MIN6 significantly decreased forskolin mediated insulin release, suggesting its role in insulin vesicle trafficking.

Together these results support the notion that phosphorylation modification represents an important regulatory mechanism that enhances insulin granule exocytosis by bringing together proteins essential for recruiting the granules to the plasma membrane such that more vesicles are made available for release following sustained stimuli by PKA.

How does phosphorylated Syt7 binding to Rph3a and MyoVa explain GLP-1/PKA function?

Various models have been suggested for probable action of PKA. One model suggests that PKA renders granules within the readily releasable pool to be more sensitive to the stimulatory action of calcium thereby increasing release probability (Skelin & Rupnik, 2011). However, electrophysiology studies also demonstrate increased refilling of granules to the releasable pool upon activation of cAMP (Renstrom, Eliasson, & Rorsman, 1997). In line with this, stimulation of secretion following activation of PKA seems to be independent of calcium influx since the peak and integrated calcium currents were largely unaffected by PKA activators in beta cells (Ammala et al., 1994). Our findings indicate that phosphorylated Syt7 binds to Rph3a as well as MyoVa, both proteins being involved in vesicle trafficking. This supports the latter hypothesis in which PKA mediated phosphorylation events could enhance granule mobilization. Moreover, although Syt7 is a calcium sensing protein, the phosphomimetic Syt7 exhibits similar calcium binding affinity as WT Syt7 (B. Wu et al., 2015), thus suggesting calcium-independent mechanisms for GLP-1 potentiation of insulin release. Together, these findings echo acceleration of granule mobilization in response to PKA activation as the potential mechanism for enhanced secretion by GLP-1 signaling.

4.3 Future work

Within the scope of this thesis, it is evident that GLP-1 mediated exocytosis involves dynamic regulation of protein interactions that in turn enhance insulin release. However, further investigations are needed to address the potentiation of GLP-1-mediated insulin release in beta cells. Most importantly, GLP-1 also activates Epac signaling pathway that acts independent of PKA signaling. Moreover, Syt7 phosphorylation at Serine-103 is selective to PKA as Epac activator 8-CPT-2'-O-Me-cAMP failed to induce phosphorylation of Syt7 in insulin secreting cells (B. Wu et al., 2015), indicating that Syt7 may not be a target for PKA-independent pathways. Snapin, a PKA substrate is found to interact with Epac to coordinate both PKA-dependent and PKA-independent pathways. Whether Syt7 acts upstream of these events remains to be elucidated.

Current study mainly applied biochemical and cell biology approach to examine the mechanism of phosphorylated Syt7-dependent insulin release. To further delineate how Syt7-Rph3a affects regulated exocytosis of insulin granules, exocytosis response may be measured by high-resolution membrane capacitance studies in Rph3a knockdown cells including islets. In addition, the use of TIRFM to monitor real time dynamics of insulin granules exocytosis in cells with disrupted Syt7-Rph3a interaction would decipher if GLP-1 actions enhance refilling of the releasable pool as a function of substrate phosphorylation.

Equally intriguing would be the understanding of phosphorylated Syt7 regulation in diabetic condition. This can provide impetus for designing therapies that directly target Syt7 to selectively enhance insulin release therefore bypassing the off target effects of GLP-1. With proper understanding of mechanisms underlying stimulus induced insulin signaling, this may offer intervention strategies to achieve glycemic control.

4.4 Conclusion

Based on current work, a working model is proposed to explain the molecular mechanism of GLP-1 dependent insulin granule exocytosis, which is at least in part mediated by PKA-dependent pathway (Figure 4-1). Initiation of PKA signaling by GLP-1 binding to its cognate receptors in beta cell membrane leads to phosphorylation of Syt7. Phosphorylated Syt7 interacts with vesicle associated Rph3a and MyoVa upon activation of PKA signaling. Knockdown of either interacting proteins result in diminished PKA-mediated insulin secretion. Given the role of both interacting proteins in granule mobilization, PKA activation by GLP-1 likely enhances granule exocytosis by promoting granule translocation to the plasma membrane. While the current study highlights the importance of granule mobilization in GLP-1 induced insulin secretion, it also extends the role of Syt7 in beta cells from conventional calcium-sensing function to granule trafficking events prior to fusion upon phosphorylation.

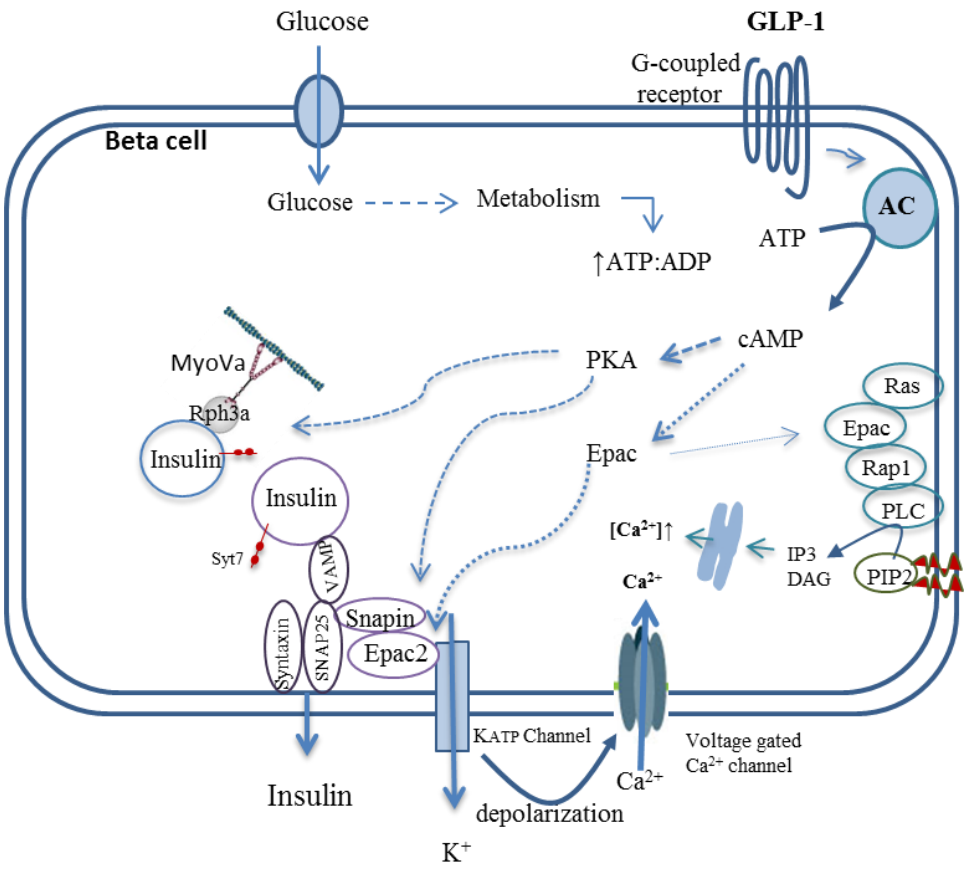

Figure 4-1 Schematic illustration depicting molecular players in GLP-1 potentiation of insulin secretion in beta cells

GLP-1 acts on G-protein coupled receptor in the beta cell membrane and activates adenylyl cyclase. Activated adenylyl cyclase converts ATP to cAMP which activates PKA-dependent and Epac-dependent pathway. PKA phosphorylates various proteins in the exocytosis machinery to enhance insulin granule exocytosis. Most importantly, phosphorylation of Syt7 by PKA enhances its interaction with Rph3a and MyoVa which ultimately enhances insulin granule exocytosis possibly by increasing granule mobilization. Epac enhances exocytosis by drectly binding to snapin and to K_{ATP} channel. Epac also binds to Rap proteins to activate phopsholipase C that converts PIP2 to IP3 and diacylglycerol (DAG) which enhance insulin release by increasing calcium release from the endoplasmic reticulum.

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