[MOLECULAR MECHANISMS OF SECRETING VESICLE BIOGENESIS AND SECRETION IN CHRONIC DEGENERATIVE DISEASES]

by

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Submitted to the Graduate Faculty of

School of Medicine in partial fulfillment

of the requirements for the degree of

Master of Science in Cell Biology and Molecular Physiology

University of Pittsburgh

2012

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

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Molecular Mechanisms of Secreting Vesicle Biogenesis and Secretion in Chronic Degenerative Diseases

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ABSTRACT

Regulated trafficking and secretion of insulin by the β cell of the endocrine pancreas is critical to maintain our body energy homeostasis. Disruption of these processes typically leads to hyperglycemia and the complications of diabetes. Compared to methods using anti-insulin or C-peptide antibodies, the fluorescent protein labeling approaches provide many advantages in live-cell, real time format with dynamic spatial and temporal monitoring. Previous studies from our lab demonstrated that by fusing a GFP within the C peptide of mouse proinsulin (Ins-C-GFP) insulin secretory granule targeting, trafficking and exocytosis could be monitored in live cells. Confocal microscopy and western blot results showed over 85% of the Ins-C-GFP can be targeted to insulin granules, with highly efficient proteolytic processing to mature insulin and C-GFP.

Our present project aims to establish the minimum molecular determinants within human proinsulin required for its targeting to secretory granules. In order to do this, we designed a viral shuttle plasmid containing only the signal peptide, the first 5 residues of the B chain, followed by a monomeric GFP(B5), chemically synthesized with restriction sites for highly efficient and systematic chimeric and point mutagenesis. Confocal microscopy and 3-D reconstruction experiments revealed that the B5 vector was successfully expressed and nearly all of the fluorescent protein appeared within the ER(5 transfections; 72 cells), whereas the full-length hIns-C-emGFP vector efficiently targets insulin secretory granules. The results make it unlikely

that the first five residues of the B chain are sufficient for human proinsulin targeting to secretory granules. The results also suggest that the middle of the C peptide is not necessary for human proinsulin targeting. We are presently characterizing a construct with the signal peptide alone without any insulin B chain residues (B0). By systematically adding back segments from hIns-C-emGFP to B5 or B0 and the following systematic point mutagenesis, we aim to establish the minimal segments and the precise residue(s) or motif(s) of human proinsulin required for targeting to secretory granules.

Key Words*: Proinsulin, Trafficking, motif, Ins-C-GFP,insulin granule, INS-1, Beta cell.*

Word Count:	*Pages*	*22*
	Words	*5691*
	Character w/o space	*32,064*

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PREFACE

This report is my master thesis for the conclusion of my Master program at the Cell Biology and Molecular Physiology (CBMP) graduate program at the University of Pittsburgh. I really appreciated many people who helped me at the program.

I would firstly thank my supervisor at Pitt, Dr. Peter F. Drain, Associate Professor. He gave me a lot of trust and flexibility on the project. Without him, I could not have involved with such a challenging project; without him, I could not have such an great experience in enjoying the freedom of research; without him, I could not collect data and complete this project in time.

I also want to extend my gratitude to my thesis committee: Dr. Meir Aridor, Linton M. Traub Ph.D., John P. Horn, Ph.D., and Donna B. Stolz, Ph.D.. I am happy that they supervised both of my comprehensive examination and thesis. They gave me not only a lot of detailed instructions on my project but also many useful practical tips on the scientific hypothesis generation and also clear scientific communication how to implement a research project.

Moreover, lab personnel are really appreciated for their assistance on my data collection and the lab routine affairs: Yali Yang and Xuehui Geng, In addition, I really appreciated Dr. Patrizia Luppi, Peter's wife, for her contribution and help to the c-peptide project.

Last but not least, I really appreciated all my friends in Pittsburgh and my parents in China. In the period of my doing my theses, they supported me all through the process.

Today I finished my report and I will continue to challenge myself in the future with what I learned.

This is not the end but only the start.

1.0 THE IDENTIFICATION OF HUMAN PROINSULIN TRAFFICKING MODULES TARGETING INTO INSULIN GRANULE

Regulated trafficking and secretion of insulin by the β cell of the endocrine pancreas requires exquisite coupling of signal flow between stimulus and response and is critical to maintain our body energy homeostasis. Disruption of these processes typically leads to hyperglycemia and the complications of diabetes. Particularly for peptide hormones, such as insulin, the sites of regulation extend from the nucleus to the plasma membrane, with regulated resupply being as important as exocytosis of the secretory vesicle. Although the clinical significance of insulin secretion disruptions has been widely recognized, the underlying mechanisms regulating its targeting into the insulin granule are still unclear. In order to achieve this goal, identification and characterization of the multiple subcellular sites, molecular interactions and kinetics of the signal flow involved are required.

Insulin, in mammals, is synthesized in the pancreas within the β-cells of the islets of Langerhans. Insulin consists of two polypeptide chains, the A- and B- chains, linked together by disulfide bonds. It is however first synthesized as a single polypeptide called preproinsulin in pancreatic β-cells. Preproinsulin contains a 24-residue signal peptide which directs the nascent polypeptide chain to the rough endoplasmic reticulum (RER). The signal peptide is cleaved as the polypeptide is translocated into lumen of the RER, forming proinsulin. In the RER the proinsulin folds into the correct conformation and 3 disulfide bonds are formed. About 5–10 min

after its assembly in the endoplasmic reticulum, proinsulin is transported to the trans-Golgi network (TGN) where immature granules are formed. Transport to the TGN may take about 30 min(Davidson 2004).

Proinsulin undergoes maturation into active insulin through the action of cellular endopeptidases known as prohormone convertases (PC1 and PC2), as well as the exoprotease carboxypeptidase E(Steiner and Oyer 1967). The endopeptidases cleave at 2 positions, releasing a fragment called the C-peptide, and leaving 2 peptide chains, the B- and A- chains, linked by 2 disulfide bonds. The cleavage sites are each located after a pair of basic residues (lysine-64 and arginine-65, and arginine-31 and -32), and after cleavage these 2 pairs of basic residues are removed by the carboxypeptidase. The C-peptide is the central portion of proinsulin, and the primary sequence of proinsulin goes in the order "B-C-A" (the B and A chains were identified on the basis of mass and the C-peptide was discovered later)(Davidson 2004).The resulting mature insulin is packaged inside mature granules waiting for metabolic signals (such as leucine, arginine, glucose and mannose) and vagal nerve stimulation to be exocytosed from the cell into the circulation.

Regulated insulin granule trafficking and exocytosis have been typically measured by using insulin antibodies labeled by radioactivity or enzymes, by using capacitance changes, or by using amperometry(Watkins, Geng et al. 2002). By far the most popular measure of insulin vesicle trafficking and secretion in the basic research lab or clinic is by immunoassays using costly I^{125} radioisotopes or enzymes conjugated to a secondary antibody onto a relatively large-sized biopsy or perfusate tissue. Though in widespread use, these methods reveal little about the underlying molecular and cellular mechanisms at work within the cell. The fluorescent labeling approach, however, can study complex molecular and cellular mechanisms underlying regulated

secretory peptide vesicle trafficking and exocytosis in a live-cell, real-time format with dynamic spatial (subcellular, one-cell, multi-cell, islet) and temporal (millisecond, second, min, to hour) monitoring.

Previously in our lab, we combined confocal and live-cell imaging with a novel molecular strategy aimed at revealing mechanisms underlying glucose-regulated insulin vesicle secretion(Watkins, Geng et al. 2002). The 'Ins-C-GFP' reporter monitors secretory pep- tide targeting, trafficking, and exocytosis without directly tagging the mature secreted peptide. We trapped a green fluorescent protein (GFP) reporter in equimolar quantity within the secretory vesicle by fusing it within the C peptide of proinsulin which only after nascent vesicle sealing and acidification is cleaved from the mature secreted A and B chains of insulin. This construct provides us great advantage in solving the mystery of how human proinsulin is targeted in β cell.

1.1 INTRODUCTION

The "Overall Question" of this project is trying to address is to investigate how human proinsulin gets sorted from the TGN into the secretary granule in pancreatic beta cells. The major hypothesis for how secretory proteins are sorted to secretory granules postulates the existence of membrane receptors (so-called "trans"-element) that bind regulated secretory proteins at the level of the trans-Golgi network and direct them to secretory granules.

Correspondingly, targeting molecules contain a "*cis*"-element sorting determinant ensuring successful delivery. My over-arching hypothesis is the B chain and/or A chain of proinsulin contain(s) the *cis*-element(s) determining successful delivery into the secretary granules. Supporting evidence or assumptions for this model include: 1) mutants in the signal

peptide are expected to be excluded because the signal peptide is cleaved from proinsulin and does not enter the ER; 2) known ER folding mutants will not exit the ER and therefore are not testable candidates for subsequent Golgi to secretory granule sorting(Stoy, Steiner et al. 2010); 3) C-peptide is excluded for three reasons: the Ins-C-GFP mutants with emGFP inserted in proinsulin C peptide was successfully directed into the regulated secretary granules(Watkins, Geng et al. 2002); C-peptide sequence is highly varied among spices, replacement of proinsulin C-peptide with hIGF-1 C-peptide does not disrupt the insulin granule targeting(Powell, Orci et al. 1988); and various single-chain insulin mutants with a short peptide insertion can also goes into the regulated secretary pathway(Groskreutz, Sliwkowski et al. 1994; Rajpal, Liu et al. 2009). Finally, there are reports that complete deletion of the C peptide does not prevent proinsulin sorting to secretory granules(Powell, Orci et al. 1988; Liu, Ramos-Castaneda et al. 2003). To test the hypothesis, we generated mutants of human insulin and evaluating their trafficking in the secretion pathway in INS1 cell.

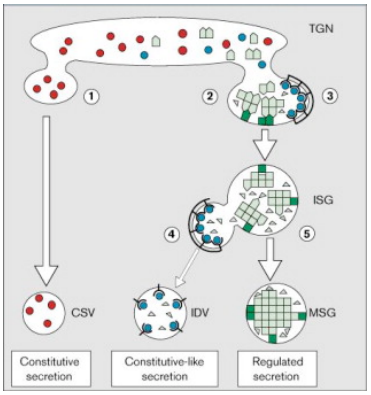

Figure 1. Secretory proteins can be sorted at either of two levels during secretory granule biogenesis – at the trans-Golgi network (TGN) or the immature secretory granule (ISG).

Two levels of sorting to the regulated pathway of protein secretion can be distinguished: sorting in the trans-Golgi network during the formation of immature secretory granules (vesicular intermediates in secretory granule biogenesis), and sorting in the immature secretory granules during their maturation(Molinete, Irminger et al. 2000). Proinsulin is processed and crystalized in the mature insulin granules. Thus, the properties fitting the mutant that get into the TGN but not the SGs---Secreted but not processed or TGN accumulation (little chance because of the passive flow for the constitutive secretary pathway). Currently, there are two models in the insulin sorting proposed(Arvan and Castle 1998): the receptor-mediated sorting or the concentration-dependent sorting. They may coexist in targeting proinsulin into the insulin granule.

One of the limits of previous studies for the intracellular proinsulin trafficking are most of them are done in the neuroendocrine cell lines, most in pituitary-derived cell lines, rather than the native insulin secreting cell lines which make people question about whether they are authentic in the beta cell or insulin secreting cell line derived from them.

1.2 RESULTS AND DISCUSSION

In order to identify the Golgi targeting mutation with In-C-emGFP insertion, mutations of N-terminal half of human proinsulin (B chain + 1-17th amino acids of C peptide) and C-terminal half (18-31th amino acids of C peptide and A chain) are made to replace the wide type fragment. To achieve this purpose, Nco I - Not I enzyme site pair was used for the N-terminal half while Kas I and Hpa I sites for the C-terminal half. To rule out the potential weak dimerization between emGFP proteins, the Alanine 206 of the emGFP was substituted with Lysine.

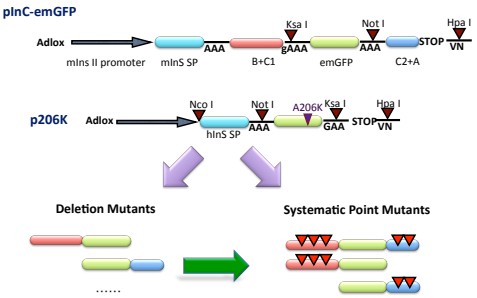

Figure 2. Mutating strategies of human proinsulin to identify the trafficking mutants.

Using this strategy, five mutants are chosen to make: the Wakayama, the LA, HisB10Asp SerB9Asp and EL-A16/17SA mutants for the following reasons:

The Wakayama and LA are clinically characterized insulin mutants causing diabetes associated with familial hyper-proinsulinemia indicating they may be mis-sorted at TGN into the constitutive secretory pathway explaining the hyper-proinsulinemia(Stoy, Steiner et al. 2010).

The HisB10Asp and SerB9Asp mutants form dimer and monomers only with no further polymerization to the hexamers formed by wildtype proinsulin(Brange, Owens et al. 1990). The HisB10Asp mutant was reported to be mis-sorted at the Immature Secretory Granules (ISGs) and secreted constitutively(Quinn, Orci et al. 1991; Irminger, Vollenweider et al. 1994). These two constructs can be used to test/exclude the condensation-dependent sorting model.

The ELA16/17SA is a mutant reported by the Loh group as constitutively secreted in the unprocessed proinsulin form, indicating it is mis-sorted at the TGN(Dhanvantari, Shen et al. 2003). The paper proposed Carboxypeptidase E(CPE) might be functioning as its potential receptor. However, this idea is challenged by subsequent studies: the transgenic CPE-deficient CPEfat/fat mouse shows normal insulin processing and secretion(Irminger, Verchere et al. 1997); How the less abundant receptor CPE can direct proinsulin for its targeting(Thiele, Gerdes et al. 1997)?

The C-terminal GFP-tagged proinsulin is also constructed as it was reported only partially sorted into the insulin secretary granules (~12%) indicating it does reach the TGN(Pouli, Kennedy et al. 1998). We would like to see whether it is processed and secreted constitutively or in a regulated (glucose stimulated) manner.

In order to address these questions, the following mutants were constructed.

Table 1. Construction of human proinsulin mutants

Construct/Mutant	Constructed	Puro Vector	Stable cell line	Sequenced
B0	Yes	Yes	Selecting clones	Yes
B5	Yes	No	-	Yes
WT	Yes	Yes	Yes	Yes
BC1	Yes	-	-	Yes
C2A	Yes	-	-	Yes
BCA	Yes	Yes	Selecting clones	Yes
Wakayama	Yes	Yes	In process	Yes
LA	Yes	No	-	Yes
HisB10Asp	Yes	-	-	In process
SerB9Asp	Yes	-	-	In process
A16/17_SerAla	In progress	-	-	

1.2.1 Intracellular Trafficking of Human Proinsulin inside INS-1 Cell.

First, we made the human proinsulin mutant construct containing peptide only the signal peptide, and the first 5 residues of the B chain followed by a monomeric GFP (B5). The first 5 residues of the B chain are included to ensure the proper processing and cleavage of the signal peptide when the protein translocates into the ER lumen. The restriction sites were chemically synthesized with for highly efficient and systematic chimeric and point mutagenesis for the strategy shown in Fig. 7 and Fig. 8. When this B5 construct was transfected into the INS-1 cell line, it shows the even phenotype across the cytoplasm except little black dot (Fig.3). Co-expressing experiment in INS-1 cells with the B5 and the pInC-mCherry shows these little block spots co-localize with pInC-mCherry, as shown in lower panel in Fig.3, suggesting they are insulin granule. Compared the

B5 with the construct expressing emGFP under the same promoter without signal peptide which expressed in the cytosol with clear nucleus, the accumulation of fluorescence inside nucleus suggests B5 was, partially at least, expressed in ER. This results shows that the first 5 amino acids are insufficient for the human proinsulin molecule to exit ER.

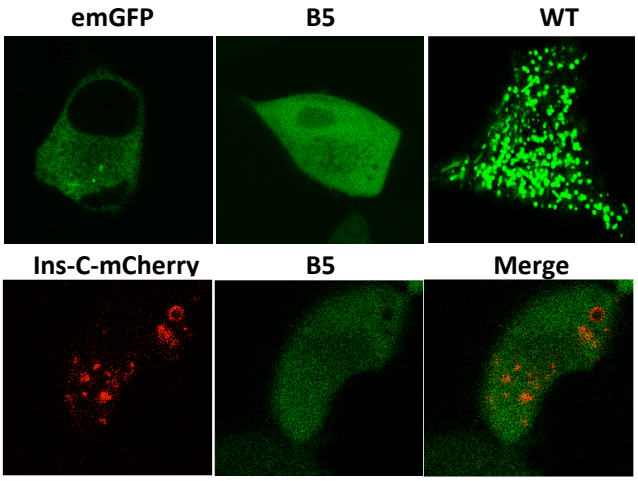

Figure 3 B5 is insufficient for the insulin granule targeting in INS-1 cell line. A. INS-1 cell was transfected with PInsII-emGFP, which is emGFP directly followed with mouse Insulin II promoter. (*n=45* from three independent transfections.) **B**. INS-1 cells was transfected with B5 plasmids.(n=52 from four independent transfections.) Representative images are shown which were taken at 24hrs post-transfection. Lower panel B5 plasmids was transfected into the INS-1 stable cell line expressing Ins-C-mCherry. Images were taken 24 hrs after transfection.

The next question is whether the 5 residues of the B chain are required for the proper signal peptide cleavage or not. In order to testify this question, we removed the 5 amino acids from the B5 construct by taking advantage of the Not I site pair at both ends of the first 5 amino acids of the B chain (B0). As shown in the Fig.4, B0 shows ER accumulation, whose phenotype is not dramatically different from the B5. This result suggests that the human proinsulin signal peptide is self-competent for properly cleavage without requirement of the involvement of any B chain residue.

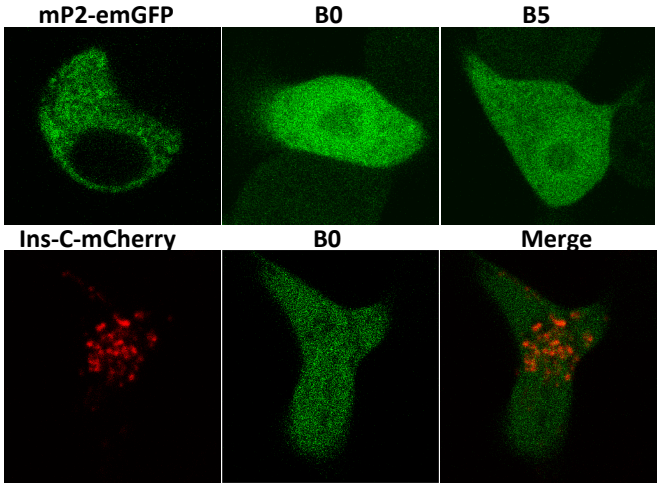

Figure 4 B5 does not influence the cleavage of signal peptide of human proinsulin in INS-1 cell line. A. INS-1 cell was transfected with mProm-emGFP, B5 and B0 constructs, respectively.(*n>30*) **B.** INS-1 cells was transfected with B5 plasmids(*n=21*). All images were taken at 24hrs after transfection.

Then, whether the intact N-terminal half and/or C-terminal half of human proinsulin are required for its the proper targeting into the insulin granule or not? To answer this question it, we made the constructs contained the B chain and the first 17 amino acids of C peptide (BC1) and the following 14 amino acids and A chain (C2A) of human proinsulin. As shown in the Fig.5, both BC1 and C2A shows ER accumulation, similar to the phenotype of B5 and B0, suggesting that both the N-terminal half and C-terminal half of human proinsulin are required its properly intracellular trafficking and β granule targeting.

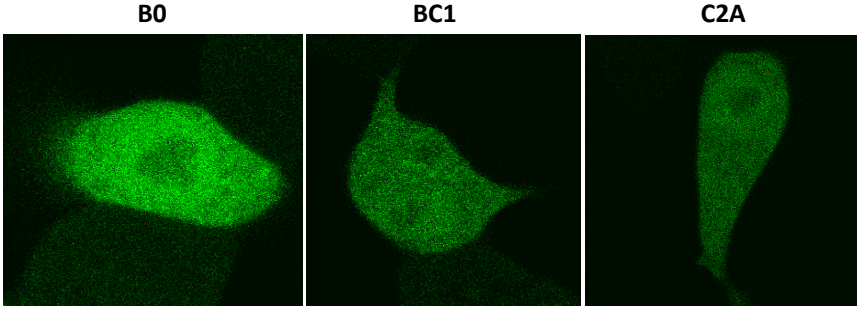

Figure 5 Human proinsulin needs both intact B chain and A chain for its proper targeting into insulin granule. INS-1 cell was transfected with B0, BC1 and C2A respectively. (*n>30*)

1.2.2 The Secretion and Procession of Human Proinsuin

Because the phenotype we are looking for is the **Secreted but not processed**. In order to detect this phenotype, we developed the protocol to concentrate the medium to test the proinsulin presence and processing status after secretion.

We first tested the recovery efficiency of the Chloroform/Methanol protein extraction methods in concentration the medium using BSA at different concentration. As shown in Fig.6A, about 93% of BSA will be recovered from this assay. We performed this assay to estimate the secreted fraction of insulin in the KRBB secretion buffer or medium at resting and stimulating conditions (6.5mM and 25mM glucose). We tested for 10mins, 30mins, 1hr and 2hr for stimulation and 30mins is the time we can detect the insulin in the medium. As shown in Fig6.B, GFP signal can be picked up in the medium of the INS-1 InC-emGFP WT but not those of the INS-1 and the InC-C96Y cell lines which leads to misfolding proinsulin with ER exit failure. Ins-C-C96Y is a control because it is previously established as accumulating in the ER with little if any secreted by several studies. This assay is powerful because it can also distinguish the processed and unprocessed fraction of insulin in the medium. In the lane 5 of Fig.6B or 6C, upper band is proinsulin while lower band is the C-peptide with emGFP insertion and event the partially processed insulin is also observed. When InC-emGFP stable lines were treated with KRBB secretion buffer, the secretion fraction of insulin is detectable at 30mins(Fig.6C). We have to admit that this is not controlled by the intracellular proinsulin contents. Another limit for this method is the final detection by Western Blot is not quantitative enough for quantitative purpose. We are

adapting the quantification step by with fluorescent secondary antibody or directly with ELISA.

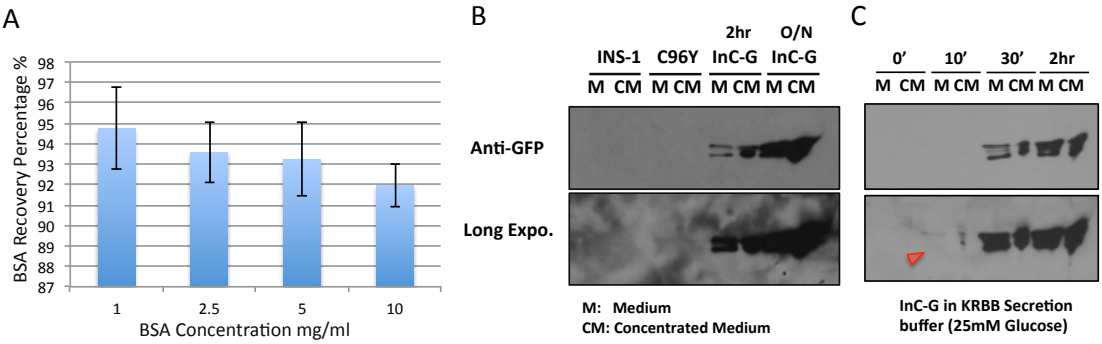

Figure. 6. The secretion assay determining the secreted fraction of insulin.

A. BSA was diluted in a serial concentration and recovered by the Chloroform/Methanol methods. Recovery rate was calculated with three for each condition(Average+/-SD). This was repeated three times. **B.** Human insulin detected with anti-GFP antibody at the in extracellular medium. Cells are treated with 6.5mM glucose and 25mM for 2hr stimulation. Medium ware collected at 30min collected for INS-1 InC-emGFP (InC-G) stale cell lines and overnight for INS-1, INS-1 InC-C96Y(C96Y) and InC-G stable cell lines was collected overnight. Experience repeated three times and reprehensive image are shown here. **C.** INS-1 InC-emGFP stable cell line is stimulated with KRBB secretion buffer containing 25mM glucose. Samples are collected at 0, 10min, 30min, and 2hrs. This experiment was repeated three times.

In order to test for the condensation model or the precise localization of the proinsulin molecules, ultrastructural studies using electronic microscopy and subcellular fragmentation assays are needed. In order to perform robust secretion assay, over 90% of cell should express the mutants. After considering other options, establishing stable cell lines might be our best choice. First we find our cell line is resistant to neomycine but not to puromycine. First, we engineered our puromycine vector to remove the two Kas I site that will cause problem for our future cloning for other fluorescent markers. Then, we cloned our constructs into the puromycine vector. As shown in the Tab. 1,

1. For B0, BCA and Wakayama mutants, we get the construct; we are establishing the stable cell line or selecting clones.
2. For WT, LA mutant, we are still working on the puromycine constructs
3. For the HisB10Asp, SerB9Asp mutants, we get the colons that are positive at double digestion in pAdlox-vector. The sub-cloning into the puro vector will be the first step.
4. For ELA16/17SA mutant is stuck at the stage of construction.

1.3 FUTURE DIRECTIONS

In order to test the requirement and the sufficiency of the molecular modules inside human proinsulin, more detailed truncation or deletion mutants of the BC1 and C2A will be the next step to construct. As shown in Fig.7, the distinction between different constructs indicates the requirement or sufficiency role of the human proinsulin fragment in its intracellular trafficking. The pInC-em206K, the positive control, targets into the insulin granule as shown in Fig.3 while B0, accumulates inside ER. Taking the C-peptide deletion mutant for example. If it shows similar phenotype with positive control pInC-em206K, it will suggest that C peptide is not required but B chain and A chain are required its proper targeting. If it shows similar phenotype with negative control, it will suggest the C peptide is required for the insulin granule targeting. It also indicates that C peptide may be sufficient for the proper trafficking inside β cells although it requires further evidence by add C peptide into a peptide that does not goes into insulin granule.

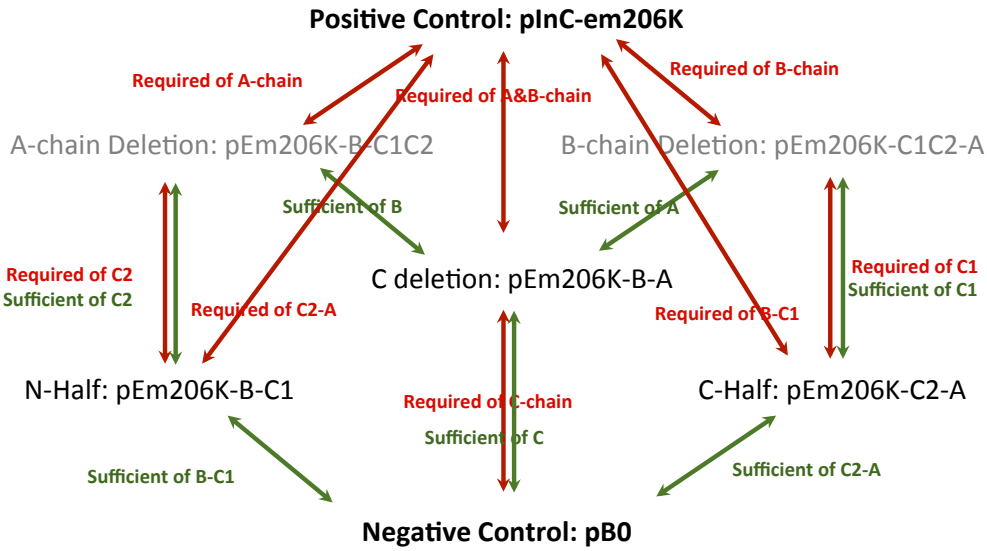

Figure 7 Strategy for identifying the requirement and sufficiency of molecular modules in human proinsulin for its targeting into the insulin granule. Red line indicates requirement while green line indicates sufficiency.

In order to identify the precise residue(s) or motif(s) responsible the ISG targeting, a systematic mutation strategy can be utilized to identify the trafficking mutant events, especially after the first round of truncation/deletion mutant screening. First, using the random mutagenesis to generate a pool of mutations in the potential fragment of human proinsulin such as B chain or A chain to replace that fragment in the wide type construct to generate a pool of mutant constructs. Then, when then construct has a puromycine selection marker, it can be used to generate a stable cell line pool to observe the phenotypic variants under a microscope. When certain mis-trafficking phenomenon, such as Golgoi accumulation, is selected by standards of live-cell microscopy or the secretion/processing system, the cell line can be sent for genomic sequencing with primers starting from the em206K to identify the site mutated to identify the mutated residue(s).s

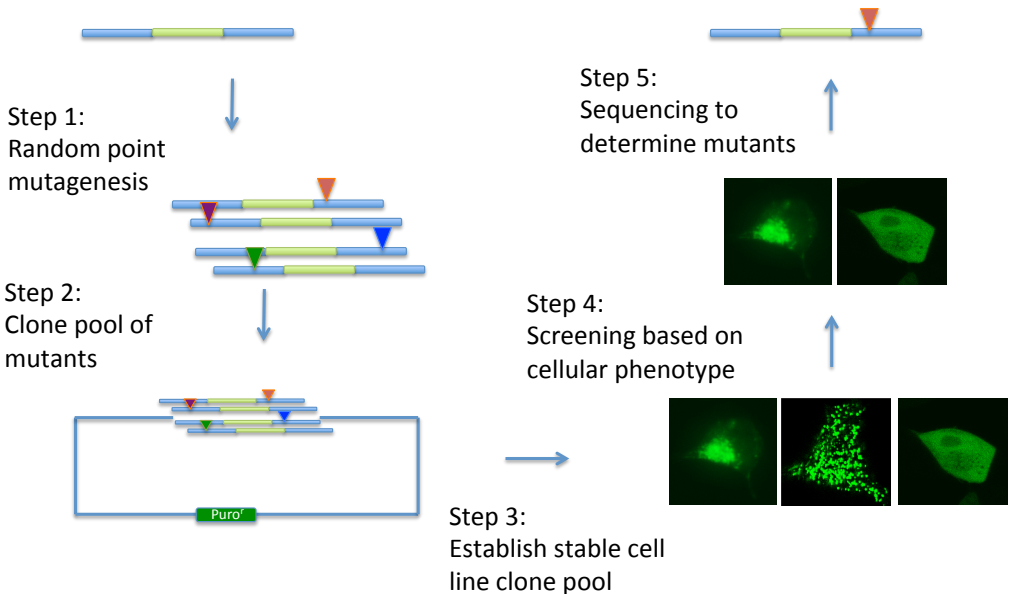

Figure 8 Strategy for systematically identification of intracellular trafficking mutants of human proinsulin.

2.0 SIDE PROJECTS

Aside, I also worked on a project on the role of C-peptide relieving the ROS responses induced by prolonged high glucose treatment in beta cell.

2.1.1 C-peptide relieving the high glucose treatment induces ROS response in beta cells.

Pancreatic beta cells, is among the most ROS-sensitive cells in our body(Rashidi, Kirkwood et al. 2009). Briefly, prolonged high glucose treatment stresses beta cells and induces responses in ROS generation and beta cell proliferation and eventually death(Bhatt, Lim et al. 2012). C-peptide, secreted in parallel with insulin has been shown to alleviate the diabetes-associated complexities in kidney, eye, brain and blood vessels. This is partially achieved by the decreasing ROS generation and its downstream effects in endothelial cells and neural cells. So **our question** is whether C-peptide could play a role in the beta cells by counteracting the effects of high glucose-induced ROS responses. Here are our brief results:

1. The prolonged high glucose treatment in INS-1 cell induces the translocation of NF-κB from the cytosol into the nuclear. The chromatin-associated form is also slight increased. (Fig. 9A)
2. The treatment also inhibits MAPK pathway as indicated by the lost of pErk1/2 signal in the cell lysates. (Fig. 9A)

3. The NF-κB nuclear translocation is reversed when C peptide was added but not the MAPK pathway. (Fig. 9B)

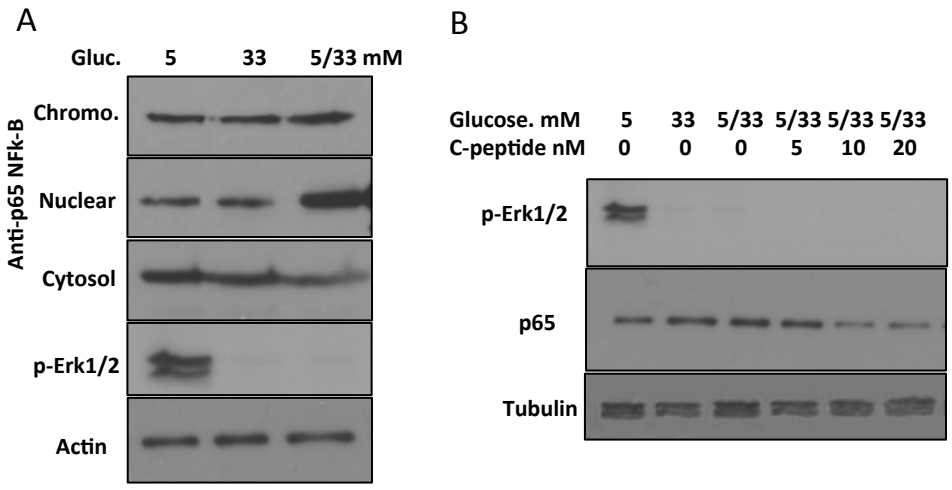

Figure.9 High glucose induces NFκB nuclear translocation and inhibits MAPK pathway.

A. INS-1 cell is treated for 72hrs with 5mM, 33mM glucose or alternating between these two concentrations every 12hrs. Cell lysates was fragmented into cytosolic, nuclear and chromatic fractions. This experiment was repeated four times. **B.** INS-1 cell is treated as in A with or without a serious concentration of C peptide. For testing nuclear NFκB, nuclear fraction of cell lysate was used. This experiment is repeated for two times.

2.1.2 Interaction of Kir6.2 and a-Synuclein at Dopaminergic neurons.

α-Synuclein has been shown to interact with K_{ATP} channel on insulin secretary granule and inhibits insulin secretion(Geng, Lou et al. 2011). However, whether it also functions in other secretary granule is unknown. Here, I used cell lines derived from the dopaminergic neurons to address this question. Here are our brief results: As shown in Fig.10A-C', α-Synuclein colocalizes with K_{ATP} channel on the dopamine secretary granule in the dopaminergic neurons. Meanwhile, co-immunoprecipitation assay shows it also physically interacts with K_{ATP} channel (Fig.10D). This interaction can be disturbed by the addition of glibenclimide, a K_{ATP} channel

opener. These results indicate that α-Synuclein interacts with K_{ATP} channel and inhibits its functions as in the INS-1 cells.

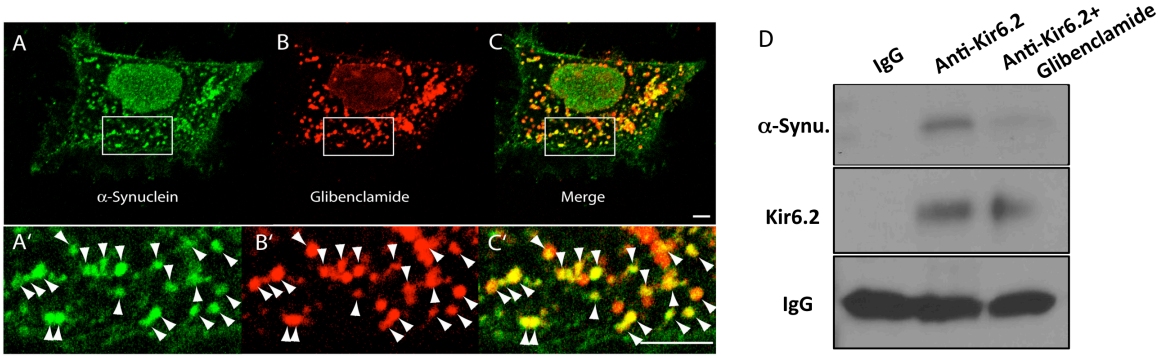

Figure.10 Interaction between KATP channel and a-synuclein on secretary granule in dopaminergic neurons. **A-C.** MND9 cells was treated with 40nM fluorescent Bodipy®TR-glibenclamide (labeling the K_{ATP} channel) and then stained with anti-α-Synuclein and images taken under confocal microscopy. **A'-C'** The subcellular section was enlarged to show their localization on the secretary granule. *n=40* cells. **D.** N27 cells lysate was treated with or without glibenclamide 250nM and then precipitated with anti-Kir6.2 antibody. This experiment has been repeated twice.

APPENDIX A

MATERIALS AND METHODS

Antibodies and Reagents.

The following antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and used according to provided instructions. Rabbit polyclonal anti-α-synuclein antibody [sc-7011 (C20)], goat polyclonal anti-α/β-synuclein [sc-7012 (N19)], mouse monoclonal anti-C-peptide [sc-57046 (5D3)], goat polyclonal anti-SUR-1 [sc-5789 (C16)], goat polyclonal anti-Kir6.2 [sc-11226 (N18)], and goat polyclonal anti-Kir6.2 [sc-20809 (H55)]. Alexa fluor 488 goat anti-rabbit IgG (H+L), and red fluorescent Bodipy®TR-glibenclamide were from Molecular Probes (Invitrogen, Carlsbad, CA).

Cell Culture and Treatment

Wide type INS1-832/13 cells or INS-1 stably expressing the live-cell fluorescent insulin reporter pAd.Ins-C-emGFPwere cultured in d-glucose-free RPMI 1640 medium (GIBCO-BRL) supplemented with 10% heat-inactivated fetal calf serum (Bio-Whittaker), 7.5 mM glucose, 10 mM HEPES, 2 mM l-glutamine, 1 mM sodium pyruvate, 0.05 mM β-mercaptoethanol, 100 μg/ml streptomycin, and 100 U/ml penicillin in a humidified 5% CO2 incubator at 37°C. MIND9

and HT22 cells were cultured in DMEM medium containing 4.5 mg/L glucose, FBS 10% and PS (100 U/ml) and incubated at 37°C, 5% CO2. N27 cells in were cultured in RPMI 1640 medium containing 4.5 mg/L glucose, FBS 10% and PS (100 U/ml) and incubated at 37°C, 5% CO2. Transfections were done with the lipotransfectamine 2000 transfection kit (Invitrogen, Carlsbad, CA) according to the manufacture's manual.

Western Blot and Immunoprecipitation

Cells were harvested and lysed in radio-immune precipitation assay lysis buffer (Upstate Biotechnology) containing protease inhibitor mixture (Sigma). Particulates were cleared by centrifugation (10 min, 12,000 *g*) and the protein concentration of supernatant was determined using Bio-Rad protein assay reagent. For immunoprecipitation assay, cell lysate was incubated with 1:30, anti-α-synuclein antibody, 610787; BD Transduction Labs; 1:75 anti-Kir6.2 antibody, sc-20809 (H55), Santa Cruz;], overnight at 4 °C on a rotator, followed by the addition of protein A/G agarose (Pierce) to the reaction containing specific primary antibody for 2-4 hrs at 4 °C. Supernatants were removed and beads were washed 3 times in co-immunoprecipitation buffer supplemented with protease inhibitor mixture, complexes were released from the protein A/G agarose by boiling for 5 min in 2× SDS-PAGE loading buffer. Western blotting was used to detect presence of target proteins in anti-Kir6.2 anti-body [1:1,000; sc-11228 (G16), Santa Cruz] or anti-α-synuclein antibody [1:1,000, sc-7011-R (C20), Santa Cruz] in 5% non-fat milk-PBS for 1 h at room temperature. Blots were washed 4× in PBS containing 0.1% Tween (PBS-T) followed by 1 h of incubation, protected from light exposure, in infrared secondary antibodies for the appropriate species (Molecular Probes). Signals were detected with ECL reagents (Amersham Biosciences).

Immunohistochemistry.

INS1-832/13 cells were fixed with 2% paraformaldehyde in PBS for 20 min, washed in PBS three times, and blocked by incubation in 2% BSA in PBS (pH 7.5) overnight at 4°C. The cells were then incubated with primary antibodies (as indicated, each at 2 μg/ml) in blocking buffer overnight at 4°C. The cells were then washed three times with PBS, incubated with labeled secondary antibodies in blocking buffer for 2 hrs at room temperature, and then washed three times with PBS. Confocal microscopy was used to detect the immunofluorescence as described below.

Confocal fluorescence microscopy.

Cells were placed into an optical recording chamber (Harvard Apparatus, Holliston, MA) at 37°C. Single-photon confocal microscopy was performed using an Nikon A10 Confocal live cell imaging. Colocalization experiments using two different fluorophores were done by sequential excitation and detection of the two channels. Control experiments showed no cross-talk detectable under the conditions of the dual-imaging experiments by comparing images from sequential acquisition of controls using single fluorophore labeling. Images were recorded beginning from the central plane of a cell. Morphometric analysis with NIH imageJ was used to localize the fluorescent granules in merged fluorescent images.

Statistical analysis.

Student's *t*-test and ANOVA with Bonferroni pairwise posttests were used accordingly to evaluate differences. $P < 0.05$ was considered significant.

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