RESCUED SECRETION OF MISFOLDED MUTANT PROINSULIN

by

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DEDICATION

To my beautiful wife Pamela, who lovingly tolerates my unpredictable hours in the lab, who willingly listens to multiple iterations of every talk I give, and who patiently calms me when everything is going wrong and motivates me when everything is going right. And to my adorable daughter Jayda, whose joyous "Daddy!" as I walk in the door can make the worst day fade into insignificance.

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ABSTRACT

In pancreatic beta cells, the insulin precursor proinsulin is folded in the endoplasmic reticulum (ER), forming three critical intramolecular disulfide bonds. After homo-dimerizing, native proinsulin exits the ER en route to secretory vesicles, where it forms hexamers, is endoproteolytically cleaved to mature insulin, and is stored until it is secreted in response to elevated blood glucose. In Mutant Ins-gene induced Diabetes of Youth (MIDY), misfolded mutant proinsulin is retained in the ER and acts in a dominant-negative manner to impair maturation of wild-type (wt) proinsulin, leading to decreased insulin release and eventual ER stress-induced beta cell death. Using cell culture and mouse models, I have investigated two potential mechanisms to improve secretion of misfolded mutant proinsulin. First, I found that intermolecular interactions between proinsulin molecules impact strongly on the fate of those molecules. Misfolded mutant proinsulin molecules dimerize with and impair secretion of co-expressed wt molecules. Interestingly, the opposite is also true; wt proinsulin molecules also stabilize and enhance secretion of mutant molecules. Thus, there is a dynamic bidirectional interaction between dimerization partners, which we hope to exploit pharmacologically to improve clearance of misfolded proteins from the ER and alleviate ER stress-induced cell death.

In the second half of my project, I investigated how manipulating the oxidative environment of the ER may impact proinsulin secretion and beta cell health in cells expressing mutant proinsulin. ER Oxidoreductin-1 (Ero1α), the best-studied ER oxidant, contributes to oxidative folding of secretory proteins by coupling generation of de novo disulfide bonds with reduction of molecular oxygen. Due to its generation of hydrogen peroxide as a byproduct, Ero1α hyperactivity has been speculated to contribute to cell death in stressed beta cells. Surprisingly, I found the opposite to be true. Overexpression of Ero1α rescued secretion of wt proinsulin in the presence of mutant proinsulin. Furthermore, Ero1α directly rescued a subset of MIDY mutant proinsulins by improving their oxidative folding, resulting in a decrease in mutant proinsulin-induced ER stress response. These findings improve our understanding of proinsulin maturation in beta cells, and may contribute to novel therapeutic approaches in this and other secretory protein conformational diseases.

CHAPTER 1

INTRODUCTION

Insulin Function, Synthesis, and Secretion

Insulin is the primary hormone responsible for glucose homeostasis. In response to increased blood glucose after a meal, increased blood insulin levels stimulate glucose uptake and utilization in fat and muscle and inhibit hepatic glucose production, among other effects. Insulin deficiency results in marked metabolic abnormalities and disease, as will be discussed below. A basic understanding of the insulin synthesis pathway, and the cells responsible for secreting this critical hormone, is important to understanding, preventing, and treating diseases of insulin deficiency.

Insulin is synthesized and secreted from beta cells in the pancreatic islets of Langerhans. Making up approximately 50% of the cells in the ~1 million islets in a human pancreas (1), beta cells are specialized to synthesize, process, store, and secrete large amounts of insulin. The insulin polypeptide precursor, preproinsulin, is a single chain molecule comprised of the signal peptide, insulin B-chain, C-peptide, and insulin A-chain (Figure 1.1). The N-terminal signal peptide drives newly synthesized preproinsulin to and across the endoplasmic reticulum (ER) membrane, where the signal peptide is removed by signal peptidase, forming proinsulin (2). In the oxidizing environment of the ER lumen, proinsulin rapidly folds, forming three evolutionarily conserved disulfide bonds, including two inter-chain disulfide bonds B7-A7 and B19-A20, and one intra-chain disulfide bond A6-A11 (3-6). The secondary structure of the proinsulin molecule also contains three alpha-helical domains (7); the B-chain helix including residues B9-B19 and the A-chain helix including residues A13-A19 likely facilitate formation and alignment of the B19-A20 disulfide (8), while the A-chain helix comprising residues A3-A8 influences alignment of the B7-A7 and A6-A11 disulfides (9). Properly folded proinsulin dimerizes (4, 10-12) and exits the ER for delivery to the Golgi apparatus.

In the trans-Golgi network, zinc concentration increases relative to earlier secretory pathway compartments (13), and proinsulin is thought to form homo-hexamers around central coordinating Zn^{2+} ions (4, 14, 15). In the trans-golgi network, proinsulin molecules are sorted into immature secretory vesicles in a remarkably efficient process in which >99% of newly synthesized proinsulin molecules are correctly targeted (16). The mechanism of specific protein sorting and concentration in secretory vesicles is thought to occur by three potential mechanisms: sorting by entry, in which a specific signal is required for the protein to enter the vesicles; sorting for exit, in which certain proteins are specifically removed and targeted to other pathways such as the endosome; and sorting by retention, in which some signal causes the protein to be retained in secretory vesicles (17). Proinsulin sorting remains an active area of investigation, but recognition by specific receptors (18), specific membrane interactions (17), hexamer condensation (19), and selective removal of other soluble proteins to the endosomal system (20) have been suggested to play a role in formation of immature insulin vesicles. In maturing secretory granules (21-24), prohormone convertases PC1/3 and PC2 remove C-peptide at specific dibasic residues (Arg^{31}-Arg^{32} and Arg^{64}-Lys^{65}); carboxypeptidase E then cleaves the dibasic residues (25), producing mature insulin. The entire process of insulin maturation, from proinsulin folding in the ER to C-peptide cleavage in mature vesicles, is maintained in a unique cellular compartment, separate from the cytosol, that provides the appropriate conditions for the proinsulin and insulin molecules to maintain their appropriate secondary, tertiary, and quaternary structures.

Mature insulin is stored in mature beta granules. Each cell has between 5-10,000 beta granules (26, 27), with each granule estimated to contain 0.1– 1.0 million insulin molecules (28) condensed in a multimeric state (14, 29). Several hormones, neurotransmitters, and nutrients can modulate insulin release from beta cells, including somatostatin, adrenaline, glucagon, GLP-1, GABA, and acetylcholine, as well as amino acids including leucine and arginine. The most notable insulin secretagogue of all is plasma glucose, which normally ranges from a fasting level of 4-5mM to a post-prandial level of 7mM (1). In pancreatic beta cells, plasma glucose is tightly linked to glucose metabolism, which in turn is coupled to insulin secretion. Glucose is transported in a concentration dependent manner across the beta cell plasma membrane through

non-insulin dependent glucose transporters (Glut2 in rodents, Glut1 and Glut3 in humans) (30, 31). After phosphorylation of glucose by glucokinase and subsequent glucose metabolism, cytosolic ATP rises, causing closure of the ATP-sensitive potassium K_{ATP} channels (32), and subsequent membrane depolarization increases cytosolic calcium levels. This K_{ATP} channel-dependent pathway, termed the triggering pathway, is the best-understood mechanism for glucose stimulated insulin release. Another poorly understood pathway that is K_{ATP} channel-independent is responsible for amplification of the initial signal for insulin release, and may contribute to the continued release of insulin during "second phase" secretion (described below) (33). Calcium stimulated exocytosis of insulin granules depends on the SNARE complex, including syntaxin 1 and SNAP25 on the cytosolic surface of the plasma membrane and VAMP2 on the cytosolic surface of the secretory granule (17, 34). When a vesicle is docked at the plasma membrane prior to exocytosis, complete association of the SNARE proteins (and resulting membrane fusion) is inhibited by binding of the protein complexin (35). Calcium binding to another protein in the exocytic protein complex, synaptotagmin, blocks the inhibitory function of complexin, allowing quick membrane fusion and exocytosis (36). The SNARE proteins are also found in complex with voltage gated calcium channels, allowing very tight coupling of membrane depolarization and insulin release (37).

Insulin granules are found in two distinct populations, the readily releasable pool (RRP) docked at the plasma membrane and the reserve pool. Shortly after an increase in glucose, the RRP granules quickly fuse to the plasma membrane, accounting for the "first-phase" release of insulin. After 10-15 minutes, the "second-phase" of insulin secretion begins, in which RRP granules have been depleted and reserve granules must traffic to the membrane prior to exocytosis (17, 38, 39). Under basal conditions, a normal pancreas releases <100pmol of insulin per minute. During glucose-stimulated insulin release, that value increases to ~1500pmol/min during first-phase and ~400pmol/min during second phase (1, 40). Even under stimulated conditions, a relatively small fraction (approximately 1% per hour) of granules are released (41, 42), and the insulin granule pool is actively maintained with aged granules being degraded by crinophagy or autophagy (43, 44), resulting in a granule half life of 3-5 days (28, 45).

Approximately half of daily insulin secretion accommodates basal metabolic needs, while half is secreted in response to eating (46). Thus, under non-stimulated conditions, beta cells must maintain a basal level of insulin synthesis to maintain reserve pools, and under stimulated conditions, synthesis must be upregulated to replace exocytosed granules. Similar to regulation of insulin secretion, insulin biosynthesis is regulated by multiple nutrient factors, principally glucose. High glucose regulates insulin biosynthesis by altering preproinsulin translation in the short term (<4hrs) (47, 48), transcription in the medium term (>12 hours) (49, 50), and mRNA stability in the long term (>24 hours of high glucose) (28, 51). As preproinsulin accounts for 30-50% of the protein synthesized in a beta cell (52), its synthesis and processing represents the major activity of the beta cell.

While the post-ER steps in insulin synthesis (granule packaging, hexamerization, C-peptide cleavage) are fairly well understood, the ER processes are relatively more complex and are just now being elucidated. Following is a review of the current understanding of protein processing, trafficking, and signaling associated with the ER.

Entry, Folding, Modification, Trafficking, and Degradation in the ER

Translocation of secretory proteins across the ER membrane can occur co-translationally, simultaneously with continuing protein synthesis, or post-translationally, after translation has concluded in the cytosol (53). In co-translational translocation, the signal peptide of a growing polypeptide is recognized by the signal recognition particle (SRP). The SRP-nascent chain-ribosome complex then binds to the Sec61 translocon, and translation continues as the growing polypeptide is fed through the translocon into the lumen of the ER (54). Post-translational translocation is SRP-independent, relying instead on a mechanism that involves a Sec61/Sec62/Sec63 transmembrane complex associated with the ER luminal chaperone BiP (55, 56). Early studies indicated that ER translocation of preproinsulin mainly involved the SRP-dependent co-translational pathway (57, 58), but recent findings suggest that translocation of preproinsulin and other small polypeptides may also involve the Sec62-dependent post-translational pathways (2, 59). During or after translocation, signal peptidase cleaves the signal peptide (60), releasing the newly translocated protein into the ER lumen.

Upon entry into the ER, secreted proteins must fold into the correct three-dimensional (secondary and tertiary) structure, including formation of appropriate disulfide bonds (if applicable), as well as receiving additional post-translocational modifications such as glycosylation (if applicable). Secretory proteins also tend to assemble into oligomeric complexes to form their quaternary structure. These modifications can help to stabilize the protein both inside and outside the cell, and may be required for proper trafficking of the protein from the ER to the Golgi complex, as well as for the protein's ultimate function.

Protein folding in the ER begins cotranslocationally as the polypeptide emerges from the Sec61 channel (61). Though early *in vitro* studies showed that a protein's primary sequence carries the information necessary for proper folding (62-64), in more recent years, data have suggested that optimizing kinetics and efficiency of protein folding *in vivo* requires assistance from several proteins known as molecular chaperones. These chaperones, which act to prevent aggregation of unfolded proteins thereby helping to facilitate folding and limit damage caused by mis- or unfolded proteins (65), are grouped into subfamilies based on their size: Hsp40, 60, 70, 90, and 100. In the ER of mammalian cells, the Hsp70 family member BiP/GRP78 is considered a master regulator of ER function (66), though members of the Hsp40 (Erdj1-5) (67, 68), Hsp90 (GRP94) (69), and Hsp100 (TorsinA) (70) families also contribute to protein folding in the ER. As a new polypeptide enters the ER lumen, BiP binds cyclically and preferentially to short sequences of hydrophobic amino acids (71), preventing inappropriate interactions with other hydrophobic regions. Cyclical BiP binding (linked to cycles of ATP hydrolysis) occurs with greater affinity for proteins that fold slowly or unstably (72). Burial of hydrophobic side chains is thought to be the strongest force driving protein folding in an aqueous environment, with hydrogen bonding and electrostatic interactions also contributing (61). As hydrophobic regions become more oriented towards the interior of a protein structure, the exposure of BiP binding sites on that protein decreases, and BiP binding to those sites on the protein decreases in parallel. Several of the other molecular chaperones act as co-factors for BiP function. For example, ErdJ3 (p58) binds misfolded forms of several proteins in complex with BiP (73). For some substrate proteins, GRP94 binds after BiP release, which facilitates further folding (74). In the case of proinsulin folding, some specific ER chaperones have been implicated. However, while

misfolded proinsulin is known to bind BiP (11, 75) and p58 (73), the normal folding pathway for proinsulin is unknown.

Disulfide bond formation is critical for folding and stabilization of many secreted proteins. Members of the protein disulfide isomerase (PDI) family of ER oxidoreductases participate in the formation of disulfide bonds of secretory proteins. This family, consisting of 20 known members, are defined by the presence of an N-terminal ER signal peptide and at least one thioredoxin-like domain (76). The first-identified member of the family, PDI, is known to catalyze formation (oxidation) (77), breakage (reduction) (78, 79), and reshuffling (isomerization) (80) of disulfide bonds, as well as non-redox dependent chaperone activity to assist protein folding (81). Based on structural similarity to PDI, many of the PDI family members are hypothesized to have similar functions. However, the redox potential of each member remains uncertain, and still others completely lack an active CXXC motif and thus may not be involved in redox chemistry at all (76). It is far from clear why mammalian cells express such a wide range of these related PDI family members, and the role of each specific member is the subject of active investigation worldwide.

The redox active site of a PDI protein includes at least one CXXC motif, which shuttles between the reduced dithiol and the oxidized disulfide state. This dual nature of the PDI active site allows the protein to act as both a reductase and an oxidase. Formation of a disulfide bond in a secretory protein substrate is thought to be based on exchange of a disulfide from the ER oxidoreductase to the folding secretory protein, which returns the PDI active site to the reduced dithiol. Under physiologic conditions, many of the PDI family members are partially oxidized (78, 82-84), and their redox state is maintained in part by the overall redox potential of the ER that is at least in part controlled by glutathione (85, 86). But the glutathione buffer system is not thought to be capable of driving the catalysis of disulfide bond formation with the kinetics required by biological demand, so the (re-)oxidation of PDI family members for purposes of secretory protein disulfide bond formation is thought to be catalyzed through an additional mechanism.

The best-studied source of de novo oxidizing equivalents for the ER is ER Oxidoreductin 1 (Ero1) (87, 88). Ero1 is a protein residing primarily on the luminal side of the ER membrane. The Ero1-PDI pathway of oxidation, as initially described in yeast, involves oxidation of an Ero1 active site via an $FAD^+ \rightarrow FADH + H^+$ intermediate with ultimate reduction of molecular oxygen and the generation of H_2O_2. Oxidized Ero1 then internally shuttles its newly formed disulfide bond to a different Ero1 active site disulfide pair, which in turn delivers the oxidizing equivalents to PDI. This step enables PDI to oxidize its protein substrates (89-91). See Figure 1.2 for a graphic summary of Ero1 function. Mammalian Ero1 is thought to behave similarly to the yeast enzyme (92, 93) although this does not likely tell the whole story. Notably, whereas Ero1 seems to be the only source of oxidizing equivalents in the ER of yeast (88), mammalian cells express two Ero1 homologs: Ero1α (94), which is expressed ubiquitously, and Ero1β (95), which is limited to pancreatic beta cells and a few selective other cell types (96). Ero1β deficiency in beta cell lines has been shown to cause a decrease in proinsulin maturation and insulin content (97). Moreover, mice with homozygous knockout of the Ero1β gene develop a prediabetes/diabetes phenotype triggered by insulin deficiency. Nevertheless, mice lacking both Ero1α and Ero1β still retain some ability to oxidize proinsulin and produce mature insulin (96), suggesting that other Ero1-independent pathways of proinsulin oxidative folding may also be involved.

Though their significance in ER oxidation remains unclear, at least four potential alternative ER oxidation pathways exist: peroxiredoxin-IV (PRDX4), glutathione peroxidase (GPX)7 and GPX8, quiescin sulfhydryl oxidase (QSOX), and vitamin K epoxide reductase (VKOR) (98). PRDX4 can catalyze oxidation of PDI by reduction of H_2O_2 (99), which makes it particularly interesting as a supplement to Ero1 that produces H_2O_2 as a byproduct. Since PRDX4 expression rescued the phenotype of an Ero1 mutation in yeast, and PRDX4 knockdown killed cells derived from Ero1α and Ero1β double knockout mice, PRDX4 is very promising as a significant supplement to Ero1-dependent disulfide bond formation. GPX7 and GPX8 can couple H_2O_2 reduction to oxidation of some ER oxidoreductases in vitro, and can physically associate with Ero1α in cells (100), but in vivo evidence of their function as ER oxidants is lacking. Like Ero1, QSOX couples reduction of oxygen with disulfide formation, but with broader substrate specificity. In fact, QSOX has been shown to directly oxidize many substrate proteins in vitro, independent of

ER oxidoreductases (101). QSOX is expressed in many tissues throughout the body and has recently been shown to act as an oxidase in vivo (102), but its significance in protein oxidation in the ER in vivo is unknown. VKOR catalyzes reduction of vitamin K epoxide to vitamin K hydroquinone, which is critical for maturation of blood-clotting factors. As part of its catalytic function, a CXXC motif within VKOR is oxidized (103, 104), and the resulting disulfide can then be transferred to PDI family members, with special preference for the transmembrane oxidoreductases TMX and TMX4 (105), suggesting that different substrates may be oxidized via different pathways. Though Ero1 is still thought to be a major ER oxidant in mammals, further study of these alternate pathways of disulfide bond formation is still needed for a complete understanding of oxidative protein folding in the ER of distinct cell types, including pancreatic beta cells.

Disulfide bond formation is a critical step in proinsulin maturation and function. Genetically modified proinsulin molecules that lack the interchain B19-A20 or B7-A7 disulfide bonds are retained in the ER, while molecules that lack the A6-A11 intrachain disulfide bond are secreted but lack the ability to bind and activate insulin receptor. Furthermore, any proinsulin molecule lacking a single cysteine, thus having a free thiol, is also at risk of being retained in the ER. Given the requirement for proinsulin to form proper disulfide bonds before exiting the ER, enhancement of ER oxidative machinery may offer an interesting approach to improve beta cell function, as will be discussed in chapter 3 of this thesis.

A majority of secreted proteins undergo glycosylation within the ER, especially N-linked glycosylation. N-linked glycosylation can play an important role in protein quality control, trafficking, and function. Because proinsulin, the subject of this thesis, is not a glycoprotein, the N-linked glycosylation process will be reviewed only briefly here. Upon entry of nascent glycoproteins into the ER, oligosaccharyl transferase attaches a preformed glycan containing three glucose, nine mannose, and two N-acetyl glucosamine residues to asparagine side chains in Asn-X-Ser/Thr consensus sites (106). These sugar residues then undergo consecutive rounds of trimming and reglucosylation by glucosidases and glucosyltransferases, providing sufficient time for proper folding to occur, assisted by cyclical association of lectin-like ER chaperones calnexin and calreticulin. Near-native molecules continue in the calnexin-calreticulin cycle until

molecules that achieve a native conformation can proceed forward to the Golgi, and terminally-misfolded molecules can be targeted for degradation (65).

A final step that many proteins undergo prior to ER exit is oligomerization, in which multiple subunits combine into a single complex. Intermolecular interactions stabilizing the complex may include intermolecular disulfide bonds or non-covalent hydrogen bonds and/or hydrophobic interactions. The nature of the oligomer and the sequence of folding and dimerization vary. Newly synthesized thyroglobulin (Tg), for example, is initially detected in high-molecular weight aggregates bound to molecular chaperones (107, 108). After ten minutes, these aggregates resolve into monomers that no longer bind chaperones. Shortly thereafter, Tg is detected as a dimer and is eventually trafficked to the Golgi. For other proteins, oligomerization can occur prior to or during monomer folding (109), as is seen with the Trp repressor in E coli (110). The immunoglobulin IgM forms more complex oligomers (111): its secreted form is made up of one J-chain and five subunits, each comprising two heavy and two light chains, stabilized by intermolecular disulfide bonds (112). Some oligomeric proteins can, alternatively, be secreted as monomers (113-115), but quite often, formation of dimers or higher order oligomers is required for export from the ER. In the case of immunoglobulins, the heavy chain is retained in the ER unless it is bound to a light chain (111). Similarly, in the T-cell receptor, addition of the ζ chain confers stability and allows ER exit of the previously assembled hetero-oligomer (116).

Protein oligomerization has also been recognized to play a role in the pathogenesis of human disease. Dominant and recessive mutations in the Cl-/HCO3- anion exchanger (AE1) have been found to cause distal renal tubular acidosis, a kidney defect, as well as southeast asian ovalocytosis, a red blood cell defect (117, 118). Normally, AE1 is expressed on the plasma membrane of alpha-intercalated cells in the kidney or red blood cells, but mutant forms of the protein are retained in the ER and fail to reach the membrane. When mutant AE1 is co-expressed with wild-type AE1, the two proteins form hetero-oligomers, which causes the wild-type AE1 to also be retained in the ER (118, 119). Interestingly, hetero-dimerization between mutant and wild-type kAE1 also rescues some of the mutant molecules, allowing them to exit the ER and reach the plasma membrane (120, 121). As part of further investigation into how protein

oligomerization in the ER can affect human disease, chapter 2 of this thesis will focus on how mutant and wild-type versions of two proteins interact to influence pathogenesis of two distinct diseases: Mutant Ins-Gene Induced Diabetes of Youth (MIDY, described below) and Congenital Hypothyroidism with deficient thyroglobulin.

In order to leave the ER for transport to the Golgi complex, proteins must pass the ER quality control (QC) system. It is thought that QC acts at two levels: primary (or general) QC applies to all proteins exiting the ER, while secondary QC applies to specific proteins (122). In primary QC, ER chaperones and other proteins recognize common characteristics of mis- or unfolded proteins, such as exposed hydrophobic regions, mobile loops, free thiols, or a general lack of compactness. Such characteristics of incompletely folded proteins trigger binding by chaperones like BiP, GRP94, and the PDIs, resulting in retention in the ER. The lectins calnexin and calreticulin are important in retention of misfolded glycoproteins, as they bind to the specific glucosylated glycans characteristic of misfolded proteins. The primary QC system is relatively stringent, since the multitude of folding enzymes and chaperones form a redundant system in which each protein recognizes unfolded substrates in a different manner (123). In secondary QC, cargo receptors recognize molecules to specifically target them for ER exit. Examples of secondary QC proteins include ERGIC-53 (124), which recognizes a subset of glycoproteins, and β-catenin (125), which binds to E-cadherin and enhances its export to the plasma membrane. Transport from the ER to the Golgi occurs via COPII coated vesicles budding from specialized exit sites of the ER. Entry of proteins into COPII coated vesicles can occur by a bulk flow mechanism (126) but receptor-mediated export can increased the kinetics and efficiency of ER export (127). In the former mechanism, properly folded cargo is incorporated into ER-exit vesicles because there is no means (such as persistent ER chaperone binding) to exclude such incorporation; whereas the latter mechanism requires binding of cargo proteins to receptors for active extraction from the main ER compartment into ER-exit vesicles. Both of these models likely contribute to differing degrees, depending on the particular substrate protein being exported (122). Upon uncoating, the COPII vesicles fuse to form the ER-Golgi intermediate compartment (128). Vesicle membrane and ER resident proteins that may have escaped can be recycled back to the ER (129) via COPI coated vesicles, with the help of ER-retrieval receptors such as the KDEL receptor (130).

Proteins that are terminally misfolded are targeted for degradation by the process known as ER-associated degradation (ERAD). After recognition by ER chaperones, the misfolded protein must be retrotranslocated from the ER to the cytosol (131-133). If disulfide bonds have been formed, it is believed that they must be broken before retrotranslocation, a process that is likely to involve the reductase function of some of the PDI family members (134-136). The process of retrotranslocation remains incompletely understood, though it does include at least in part the Sec61 complex (133, 137). Ubiquitination of ERAD substrate proteins during retrotranslocation, through the concerted action of Hrd1, gp78, RMA1, TEB4, and CHIP (138), targets the misfolded substrate to the cytosolic 26s proteasome for degradation (139).

Accumulation of misfolded proteins can be harmful to individual cells and to whole organisms. When unfolded or misfolded proteins accumulate, cells can experience "ER stress". To adapt to protein load in the ER and to prevent such accumulation, cells activate an ER stress response also known as the unfolded protein response (UPR). The ER stress response serves to balance ER protein load with folding capacity by inhibiting general protein translation while upregulating genes involved in protein folding and degradation. This conserved stress response consists of three individual signaling branches activated by the three ER membrane-associated sensors Ire1, ATF6, and PERK (140). All of these sensor activities are thought to be repressed by binding of their luminal domains to BiP. When the load of unfolded or misfolded proteins in the ER increases, BiP shifts its binding distribution towards these proteins and away from the sensors, which can allow them to activate their respective response pathways (141-143). Direct binding of misfolded proteins by the sensors may also contribute to pathway activation (144). Activated Ire1 dimerizes and auto-phosphorylates and then cleaves a 26 base pair fragment from the *Xbp1* mRNA (145), which can then be translated into an active Xbp1 transcription factor that induces expression of many genes involved in ER function (146). Activated Perk homodimerizes and ultimately phosphorylates Ser51 of eIF2α (147), which attenuates global protein translation to help relieve ER stress. Curiously, phospho-eIF2α allows for the increased translation of ATF4, a transcription factor that also induces expression of genes related to ER function, apoptosis, and oxidative stress (148). Activated ATF6 exits the ER and is delivered to the Golgi complex where it is cleaved by S1P and S2P proteases to release the transcription-activating form

pATF6α(N) (149), which acts similarly to active Xbp1 in turning on ER stress-response genes (150). Though the UPR is a protective response meant to maintain functional balance in the ER, chronic unremitting ER stress can induce apoptosis through the ATF4-induced expression of the pro-apoptotic transcription factor CHOP (151).

ER stress and Misfolded Proinsulin in Diabetes

The existence of several forms of monogenic diabetes caused by mutations to ER-related genes emphasizes the importance of the ER in pancreatic beta cell function and insulin secretion. In Wolcott-Rallison syndrome, a rare disorder characterized by neonatal insulin-dependent diabetes and other organ system manifestations, mutations in PERK (152) result in pancreatic hypoplasia and β-cell loss (153). In Wolfram syndrome, a mutation in the WFS1 gene (154), which encodes a protein that affects ER calcium homeostasis, results in early-onset diabetes associated with selective β-cell loss, among other manifestations (155). Furthermore, mutations in ATF6 (156, 157) and CHOP (158) have been identified as contributing to increased risk for type 2 diabetes.

Type 2 diabetes, which accounts for >90% of the 25.8 million cases of diabetes in the United States (159), results when defects in insulin secretion and peripheral insulin action contribute to loss of maintenance of euglycemia. Progressive insulin deficiency seems to be what drives patients from a state of "pre-diabetes" to overt diabetes and is recognized as a crucial feature in the pathogenesis of the disease (160-162). Impaired beta cell function, as evidenced by impaired first- and second-phase insulin release (163-165) and increased secretion of unprocessed proinsulin (166), and loss of beta cell mass all likely contribute to insulin deficiency in diabetes. Patients with type 2 diabetes have been shown to have fewer beta cells (167, 168) and an increase in apoptotic beta cells (169); these findings have also been replicated in multiple animal models of the disease (170-175). Though not the only factor involved in beta cell death, ER stress has been implicated as a key factor in the progression of diabetes (175-180). Notably, Laybutt et al observed increased expression of several ER stress markers in islets of diabetic *db/db* mice and in pancreatic sections of patients with type 2 diabetes. They also found that decreasing ER stress, by overexpressing BiP, attenuated lipid-induced apoptosis in mouse beta cell cultures (176). Similarly, Song et al showed that knockout of the gene encoding the ER stress-induced pro-apoptotic transcription factor, Chop, protected from beta cell death and

diabetes in multiple genetic and diet-induced models of the disease (175). Though these and other studies establish a link between ER stress-induced apoptosis in pancreatic beta cells and type 2 diabetes, the proximal mechanism responsible for ER stress signaling is less clearly established.

One potential contributor to ER stress-induced beta cell death in diabetes is islet amyloid polypeptide (IAPP) (181). This protein, which is synthesized as an 89 amino acid chain and cleaved to a 37 residue form (182), is trafficked through the insulin secretory pathway and cosecreted with insulin (183). Its physiologic function is poorly understood, but it does have a paracrine effect to inhibit insulin secretion (184). It may also inhibit insulin-stimulated glucose uptake in muscle cells (185), delay gastric emptying, and suppress appetite (186). IAPP was originally discovered due to its tendency to form extracellular amyloid deposits in islets of diabetic patients (187, 188). Transgenic expression of human IAPP gives mice and rats a greater predisiposition to diabetes (189-191), based on a deficit in beta cell mass due to increased apoptosis (192). Evidence now suggests that the toxic effects of IAPP are not due to the formation of extracellular fibrils (as was originally hypothesized) (193), but rather due to its propensity to form intracellular toxic oligomers (194-196). The mechanism by which IAPP causes cell death may involve UPR activation, since increased ER stress markers were observed in beta cell lines and islets of transgenic IAPP rodents (177, 197), but other pathways may also be involved, since p38 MAPK (198), JNK1 (199), and Fas-associated death receptor (200) expression is increased in cells exposed to IAPP. Even though IAPP has well-established potential to harm beta cells, its significance in pathogenesis of type 2 diabetes is less well-defined; clearly, other proteins and factors in the cell are involved.

Proinsulin is definitively the most abundantly synthesized islet protein, accounting for up to 20% of total mRNA (201) and up to 50% of all protein synthesized in beta cells (202). It is therefore reasonable to hypothesize that misfolded or unfolded proinsulin may contribute significantly to ER stress and apoptosis in the pathogenesis of type 2 diabetes. Indeed, a subpopulation of newly synthesized proinsulin molecules are detectably misfolded in mouse islets and beta cell lines (3) and this amount increases under conditions of high secretory demand (203, 204), as in insulin-

resistant pre-diabetic patients. These findings raise questions about the pathophysiological consequences of increased levels of misfolded proinsulin in the ER of pancreatic beta cells.

The negative effect that misfolded proinsulin can have on beta cells was first established in the *Akita* mouse (205). In animals of this lineage, one allele of the *Ins2* gene harbors a Cys to Tyr mutation at position A7, producing a misfolded proinsulin molecule that cannot form the critical B7-A7 disulfide bond (75). This subpopulation of misfolded protein molecules is retained in the ER (206) and, remarkably, this impairs secretion of co-expressed wild-type proinsulin by recruiting bystander proinsulin into aberrant disulfide-linked protein complexes (207) and triggers ER-stress induced beta cell apoptosis and diabetes in early life (208).

In the past six years, the same insulin mutation as that found in the *Akita* mouse, as well as 26 other proinsulin mutations (209-216), have been discovered to cause autosomal dominant permanent neonatal diabetes as part of a syndrome termed Mutant Ins-gene Induced Diabetes of Youth (MIDY) (7).   Like the *Akita* proinsulin, these mutants are retained in the ER, induce ER stress, and negatively impact secretion of co-expressed wild-type proinsulin (217-219). As is detailed in Table 1.1, of these 27 human Ins gene mutations, twelve either introduce or remove a cysteine: two in the B chain, eight in the A chain, and two in the C-peptide or flanking cleavage sites. As these mutations result in an unpaired reactive thiol, the mechanism by which they cause diabetes is likely similar to that of the *Akita* mutation (207). The three MIDY mutations located in the preproinsulin signal peptide appear to act by affecting ER translocation and/or signal peptide cleavage (2). The remaining twelve mutations all occur in conserved B chain residues that are believed to be important for alignment of the B and A chains leading to formation of the insulin interchain disulfide bonds (7, 220-224); indeed, these MIDY mutations are likely to impair formation of native proinsulin disulfide bonds. As a consequence, these mutants behave like those that introduce or remove a cysteine in proinsulin, being retained in the ER, with dominant-negative effects on the export of co-expressed wild-type proinsulin, to an extent greater than that caused by chemical induction of ER stress (219). Thus, the current model for this disease is that misfolded proinsulin in the ER specifically interacts with wild-type bystander molecules, augmenting the impairment of proinsulin export, and thereby causing insulin deficiency.   As insulin secretion is impaired, blood glucose increases, leading to further

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upregulation of proinsulin expression and synthesis. This positive feedback loop only exacerbates ER stress and beta cell apoptosis, leading to diabetes (see Figure 1.3).

The MIDY mutants are an interesting model of proinsulin folding in their own right, but their greater significance lies in providing insights into the basic mechanism by which proinsulin misfolding may bring about insulin deficiency in other forms of diabetes. Understanding the proinsulin folding pathway, and defects in proinsulin folding, as a fundamental link to beta cell failure, may contribute to improved understanding of common forms of type 2 diabetes. The experiments detailed in this thesis investigate two novel approaches to improve secretion of misfolded proinsulin. In chapter 2, I exploit the MIDY mutant proinsulin-G(B23)V to explore the biological significance of the relative abundance (ie., stoichiometry) of wild-type and mutant proinsulin forms. In chapter 3, I exploit Ero1 proteins to enhance the oxidative capacity of the ER, in order to examine effects on the export of mutant proinsulin molecules that are otherwise prone to misfolding. The findings detailed in both chapters shed new light on the problem of secretory protein folding in the ER, and they may contribute to novel therapeutic approaches for diabetes and other diseases.

Figures and Tables
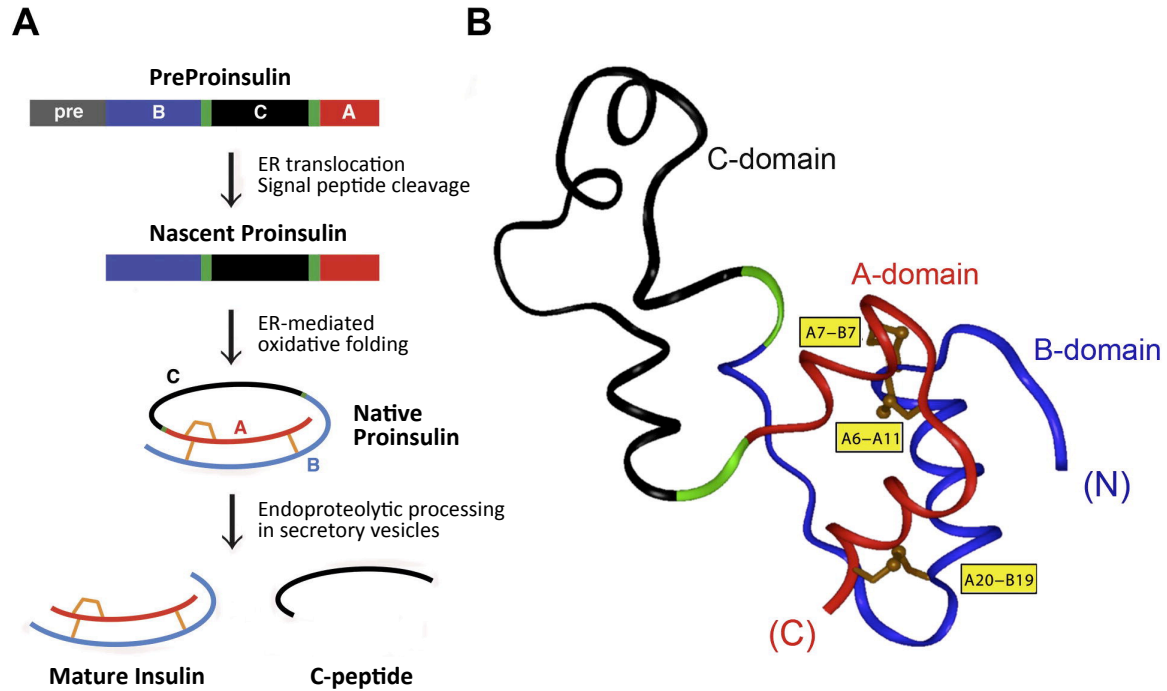
Figure 1.1 Structure and processing of proinsulin

A) In pancreatic beta cells, the signal peptide of preproinsulin is cleaved upon co-translational translocation to the ER, producing nascent proinsulin. After oxidative folding and formation of the three intramolecular disulfide bonds, native proinsulin exits the ER for transport to secretory vesicles, where C-peptide is cleaved, producing mature insulin. **B)** Native proinsulin is made up of the B chain (blue) with an α-helix at residues B9-B19, the unstructured C-peptide (black, with flanking dibasic residues shown in green), and the A chain (red) with α-helices at residues A3-A8 and A13-A19. Disulfide bonds are formed between C(B7)-C(A7), C(B19)-C(A20), and C(A6)-C(A11). Modified from (225).
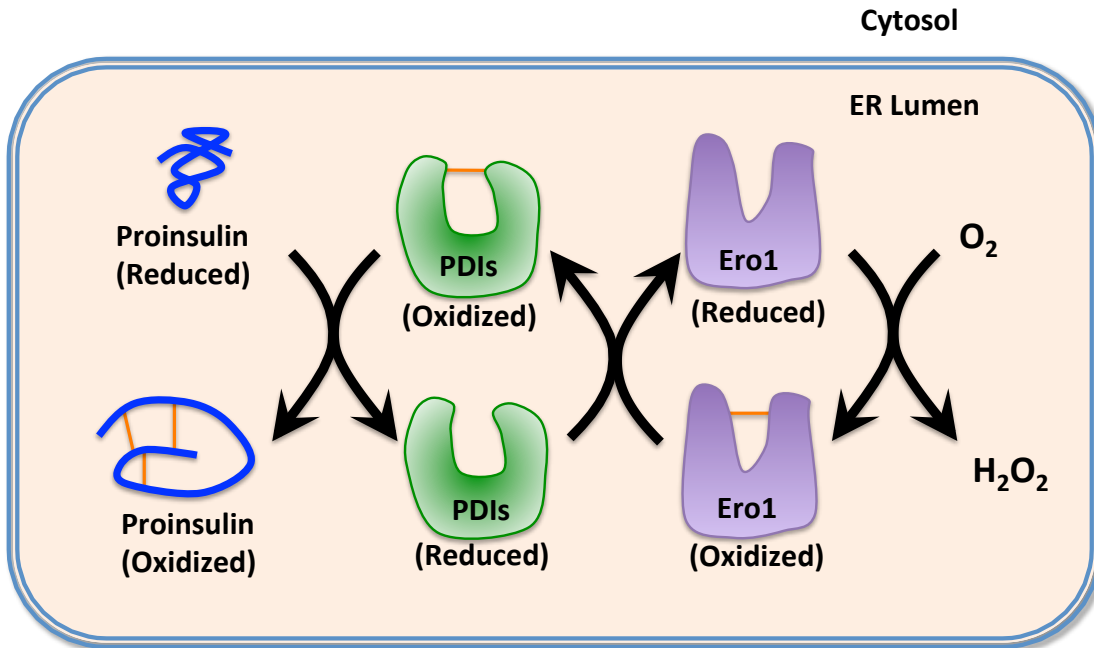
Figure 1.2. Proposed model for proinsulin disulfide bond formation. Within the ER, proinsulin must form three intramolecular disulfide bonds. Members of the PDI protein family (green) transfer disulfide bonds (orange line) to the proinsulin molecule, and then must be re-oxidized by Ero1 (purple). Ero1 in turn forms de novo disulfide bonds by reducing molecular oxygen, producing hydrogen peroxide. This cycle must repeat three times to form the required proinsulin disulfide bonds.
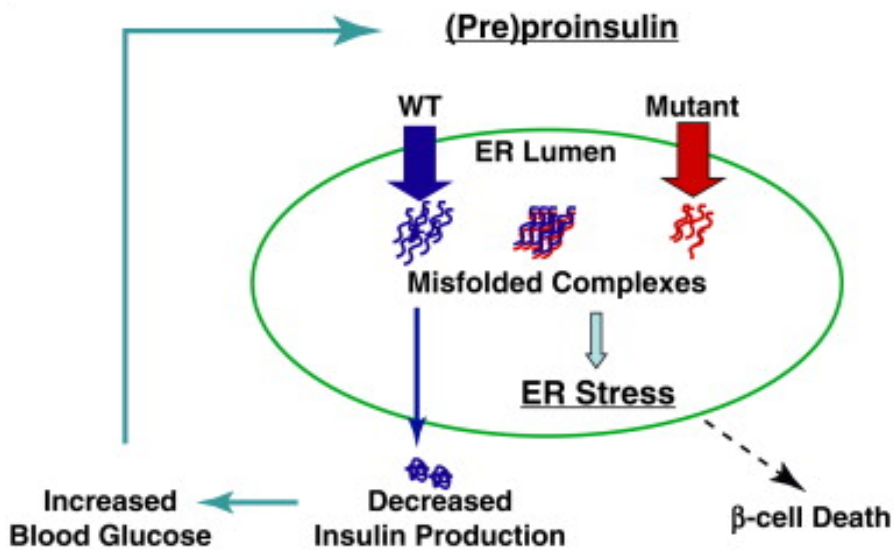
Figure 1.3 Proposed molecular mechanisms of beta cell failure caused by misfolded proinsulin. When WT (blue) and mutant (red) proinsulin are coexpressed in beta cells, the mutant proinsulin promotes WT proinsulin retention in the ER, decreasing insulin production, and forcing beta cells to use up insulin secretory granules from their progressively depleted insulin storage pool. The insufficiency of insulin increases blood glucose, and this initially stimulates the production of even more wild-type and mutant preproinsulins, exacerbating ER stress induced by misfolded proinsulin-containing protein complexes. Inexorable progression of insulin insufficiency leads to frank diabetes, further ER stress, and beta cell demise. Figure from (7).

	Chain	MIDY Mutation	Age of Onset	Original Diagnosis
Cysteine Mutations	B	C(B19)G	most <6 mo	Neonatal DM
	B	F(B24)C	<6 mo	Neonatal DM
	C-pep	R(Cpep -2)C	<6 mo or 8-37 yr	Type 1 DM or MODY
	C-pep	R(Cpep+2)C	most <6 mo	Neonatal DM
	A	G(A1)C	<6 mo	Neonatal DM
	A	C(A6)Y*	<6 mo	Neonatal DM
	A	C(A7)S	<6 mo	Neonatal DM
	A	C(A7)Y**	<6 mo	Neonatal DM
	A	S(A12)C	<6 mo	Neonatal DM
	A	Y(A14)C	<6 mo	Neonatal DM
	A	Y(A19)C	<6 mo	Neonatal DM
	A	Y(A19)Stop	<6 mo	Neonatal DM
Non-Cysteine Mutations	SigPep	R(SP6)C	15-65 yr	MODY
	SigPep	R(SP6)H	15-65 yr	MODY
	SigPep	A(SP24)D	most <6 mo	Neonatal DM
	B	H(B5)D	<6 mo	Neonatal DM
	B	L(B6)M	<6 mo or 17-36 yr	Neonatal DM or MODY
	B	L(B6) P	<6 mo or 17-36 yr	Neonatal DM or MODY
	B	L(B6)V	<6 mo or 17-36 yr	Neonatal DM or MODY
	B	G(B8)R	most <6 mo	Neonatal or Type 1 DM
	B	G(B8)S	most <6 mo	Neonatal or Type 1 DM
	B	L(B11)P	<6 mo	Neonatal DM
	B	LY(B15,16)H	<6 mo	Neonatal DM
	B	C(B19)G	most <6 mo	Neonatal DM
	B	R(B22)Q	13-35 yr	MODY
	B	G(B23)V***	<6 mo	Neonatal DM
	B	F(B24)C	<6 mo	Neonatal DM

*Analogous to Munich mouse
**Analogous to Akita mouse
***Focus of this thesis

Table 1.1 Human proinsulin mutations that cause MIDY.
Of the 27 known MIDY mutations, 12 introduce or delete a cysteine residue. Shown are the locations of the mutations (C-peptide, B Chain, A Chain, or Signal Peptide), the age at onset of diabetes, and the original diagnosis(7).

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CHAPTER 2

DOMINANT PROTEIN INTERACTIONS THAT INFLUENCE THE PATHOGENESIS OF CONFORMATIONAL DISEASES[1]

Abstract

Misfolding of exportable proteins can trigger endocrinopathies, including Mutant *INS*-gene Induced Diabetes of Youth (autosomal dominant) and Congenital Hypothyroidism with deficient thyroglobulin (autosomal recessive). Both proinsulin and thyroglobulin normally form homodimers; mutant versions of both proteins misfold in the endoplasmic reticulum (ER) triggering ER stress; and in both cases, heterozygosity creates potential for cross-dimerization between mutant and wild-type (wt) gene products. Remarkably, we find in both cases, that whereas conditions favoring an increased stoichiometry of mutant gene product dominantly inhibits export of the wt partner, increased stoichiometry of the wt gene product helps to rescue secretion of the mutant partner. Unlike approaches involving the regulation of proteostasis networks, these dramatic effects appear protein-specific. Surprisingly, the bi-directional consequences of secretory blockade and rescue occur simultaneously in the same cells. Expression level and stability of wild-type subunits — influencing the ratio of the assembly partners — may be a critical factor influencing which effect dominates the clinical phenotype, as demonstrated in two mouse models with secretory protein misfolding in the ER. The results offer new insight into dominant versus recessive inheritance of conformational diseases, and offer opportunities for the development of new therapies.

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Introduction

Several human 'conformational diseases' of the secretory pathway are caused by mutations in exportable proteins blocking their export from the endoplasmic reticulum (ER) (1). Loss of function in post-ER compartments is often observed as autosomal recessive disease. By contrast, other disorders caused by gain-of-toxic-function mutations can ultimately lead to cell death that may trigger autosomal dominant disease (2).

Given that both autosomal dominant and recessive mutations can be found in exportable proteins that form homodimers, and the much higher frequency of heterozygosity than homozygosity in the global population, it is critical to understand how cross-dimerization between wild-type (wt) and mutant gene products might influence clinical phenotypes. To study this, we have examined mutant forms of proinsulin (linked to autosomal dominant disease) and thyroglobulin (linked to autosomal recessive disease).

Proinsulin, the major protein synthesized by pancreatic beta cells, is co-translationally translocated into the ER. In the ER, proinsulin is thought to form noncovalent homodimers that proceed in the distal secretory pathway to form homohexamers that undergo endoproteolytic processing to mature insulin and C-peptide (3). Recently, 26 distinct coding sequence mutations in proinsulin have been found responsible for a gain-of-toxic-function underlying the autosomal dominant syndrome of **M**utant *INS* gene-induced **D**iabetes of **Y**outh (MIDY), in which secretion of co-expressed wild-type proinsulin is inhibited, resulting in insulin-deficient diabetes (4). When expressed recombinantly, MIDY proinsulin mutants, including proinsulin-G(B23)V, are not appreciably secreted from heterologous cells (5). Curiously, however, some MIDY mutants expressed in Min6 pancreatic beta cells, including proinsulin-G(B23)V, undergo successful anterograde transport in the secretory pathway, even to the extent of becoming endoproteolytically processed in secretory granules (6). The difference in the secretory fate of proinsulin-G(B23)V in cells lacking a wt proinsulin allele versus beta cells that express endogenous proinsulin, raises the question of whether wt proinsulin could impact on transport of the mutant proinsulin-G(B23)V.

Such a question is also interesting when considering thyroglobulin (Tg), the major protein product of the thyroid gland that serves as precursor for thyroid hormone synthesis. The large Tg primary structure comprises three disulfide-rich upstream regions ("I-II-III") followed by the cholinesterase-like (ChEL) domain (7). Similar to proinsulin, Tg forms noncovalent homodimers in the ER (8). The ChEL domain functions as an intramolecular chaperone to promote oxidative folding of I-II-III but also functions in Tg homodimerization (9, 10). In the disorder known as Congenital Hypothyroidism with Deficient Tg, ChEL is a commonly affected mutation site both in humans (11) and rodent models (12-14). Homozygous *rdw/rdw* rat dwarfs express a single ChEL point mutation [equivalent to G(2298)R of mature mouse Tg] and develop thyroid atrophy (15) from thyrocyte cell death (16). When co-expressed, mutant rdw-Tg can cross-dimerize with wt Tg (17).

Understanding the cell biological behaviors of misfolded versions of exportable proteins in the presence of their properly folded partners is of great importance for understanding potential therapeutic approaches to conformational diseases. In the current study, we have investigated the selectivity of interactions between two ER-retained mutant secretory proteins and their wt counterparts.

Materials and Methods

Materials

Lipofectamine 2000, Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, fetal bovine serum, zysorbin, penicillin, and streptomycin were from Invitrogen. Glucose, 3-isobutyl-1-methylxanthine (IBMX), tolbutamide, and Brefeldin A were from Sigma. Endoglycosidase H (EndoH) was from New England Biolabs. Complete protease inhibitor cocktail was from Roche. Citrisolv was from Fisher. Rabbit anti-myc, anti-GFP and chicken anti-myc were from Immunology Consultants; mouse mAb anti-HA was from Covance. Rabbit anti-ACTH antibody was a gift from Dr. M. Low (U. Michigan, Ann Arbor, MI). Human proinsulin specific RIA was from Millipore. Mouse proinsulin specific ELISA and mouse proinsulin-specific antibody was from Alpco. Trans^{35}S label and Na^{125}I were from Perkin Elmer.

Cell Culture and Transfection

293, 293T, and AtT20 cells were cultured in DMEM with 10% fetal bovine serum and penicillin (100 U/ml) and streptomycin (100 μg/ml). INS1 and INS1E cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 mM pyruvate, 10mM HEPES, penicillin (100 U/ml) and streptomycin (100 μg/ml), and 50 mM 2-mercaptoethanol. Proinsulin and thyroglobulin variants were expressed in pCDNA3.1 or pTarget mammalian expression vectors. The hPro-CpepSfGFP-KDEL vector was a gift from Dr. E. Snapp (AECOM, Bronx, NY). Transfections, using Lipofectamine 2000 were performed in 12-well plates. Total plasmid DNA was held constant within each experiment by addition of empty vector. Cells were harvested 24-48 h after transfection, and lysed in either boiling SDS-gel buffer (4% SDS, 20% glycerol, 120 mM Tris pH 6.8), RIPA buffer (0.1 M NaCl, 0.2 % deoxycholate, 25 mM Tris-pH 7.4, 1% Triton X-100, 0.1% SDS, 10 mM EDTA pH 8.0, and proteinase inhibitor cocktail), NP40-CoIP buffer (1% NP40, 0.1 M NaCl, 2 mM EDTA, 25 mM Tris pH 7.4) or TX-CoIP buffer (0.1% Triton X-100, 0.1 M NaCl, 5 mM EDTA, 25 mM Tris pH 7), as indicated. For glucose-stimulated secretion measurements, cells were preincubated in 2.8 mM glucose for 30 min. Fresh basal media (2.8mM glucose) was then collected for 90 min, followed by stimulation media (21 mM glucose, 1 mM tolbutamide, 1 mM IBMX) for 90 min, with cell lysis thereafter in SDS-gel buffer.

Generation of mouse lines

Mice expressing hProC(A7)Y-CpepGFP transgene driven by the *Ins1* promoter bearing with hetero/homozygous disruption of endogenous *Ins2*, were as previously described (18). A full description of *rdw-Tg3xMyc* transgenic mice is forthcoming; briefly, a transgene consisting of the bovine *Tgn* promoter (19) immediately upstream of the full-length mouse *Tgn* ORF encoding rdw-Tg plus a triple-myc epitope tag (i.e., rdw-Tg3xMyc) (10) was expressed in C57BL/6 mice. These *rdw-Tg3xMyc* transgenic mice in a $Tgn^{+/+}$ genetic background were crossed and then backcrossed with $Tgn^{cog/cog}$ mice to generate $Tgn^{+/cog}$ and then $Tgn^{cog/cog}$ ± *rdw-Tg3xMyc* mice. All animals were used in accordance with the University of Michigan's University Committee on Use and Care of Animals.

Confocal Imaging

Formaldehyde-fixed cells were permeablized with 0.4% TX100, blocked (TBS containing 3% BSA and 0.2% TX100), and then either directly mounted or stained overnight at 4°C with primary antibodies: chicken anti-myc (1:5000 dilution) and rabbit anti-ACTH (1:25000). Thereafter, slides were rinsed and incubated with secondary antibody conjugates, mounted with Prolong Gold with DAPI, and imaged by confocal epifluorescence with a 60x oil objective. GFP intensity in granule (ACTH-staining) and non-granule regions was quantified using Metamorph (Molecular Devices). For cells transfected with hPro-CpepMyc, only cells positively staining with chicken anti-myc antibodies were quantified.

For imaging of mouse islets, excised pancreata were fixed, paraffin-embedded, micro-sectioned, and deparaffinized with Citrisolv. Sections underwent antigen retrieval (RetrieveALL-1), were blocked with 3% BSA, and were immunostained overnight at 4°C with mouse anti-mouse proinsulin, guinea pig anti-insulin, and rabbit anti-calnexin antibodies. After rinsing and incubation with secondary antibody conjugates, slides were mounted with Prolong Gold with DAPI, and by confocal epifluorescence as above. For quantification of wt mouse proinsulin localization, analyzers were blinded to mouse genotype. Four to five islets per mouse were analyzed, scoring each nucleated cell expressing proinsulin as either "majority ER" (colocalizing with calnexin) or "majority Golgi" (perinuclear crescent of increased signal intensity).

Metabolic Labeling, Immunoprecipitation, EndoH digestion

24 h post-transfection, cells were trypsinized and re-plated on poly-D-lysine-coated plates. After 6 h, cells were pulse labeled with ^{35}S-amino acids for 10 min and chased as indicated, with cells lysed in RIPA buffer. For measurement of mutant proinsulin stability, lysate and media were combined, precleared with zysorbin, and immunoprecipitated with anti-insulin or anti-myc. Immunoprecipitates (normalized to TCA-precipitable counts) were analyzed by reducing 4-12% acrylamide gradient SDS-PAGE, phosphorimaging, and band quantitation (ImageQuant).

For ^{125}I labeling of secreted rdw-Tg3xMyc, thyroid glands were labeled for 30 min at 37°C in 200 μL complete medium containing 0.1 μM NaI plus 1.0 μCi/μL Na^{125}I. After labeling, samples were washed thrice with ice-cold PBS, sonicated in 200 μL of RIPA buffer, diluted to 1

mL, and immunoprecipitated with rabbit anti-myc antibody normalized to total DNA in the lysate.

EndoH digestion was performed according to manufacturer instructions; EndoH sensitive and resistant rdw-Tg3xMyc standands were from transfected 293T cells radiolabeled with ^{35}S-amino acids. EndoH digests were analyzed by SDS 5%-PAGE and phosphorimaging. Controls establish that anti-myc does not immunoprecipitate untagged endogenous thyroglobulin (*not shown*).

Co-Immunoprecipitation

After lysis in ice-cold CoIP buffer, lysates were precleared and then immunoprecipitated with the indicated antibodies, washed, and analyzed by reducing SDS-PAGE and immunoblotting.

Proinsulin Measurements and Western Blotting

At 24 h post-transfection, an overnight incubation in fresh media was initiated, and cells were lysed in RIPA buffer. Secreted and cellular proinsulin was measured by human proinsulin-specific RIA or mouse proinsulin-specific ELISA, normalized to total protein in the lysate. For western blotting, proteins (10 μg/lane) were resolved by 4-12% acrylamide gradient SDS-PAGE, electrotransferred to nitrocellulose, and immunoblotted with the indicated antibodies. Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch with proteins visualized by ECL (Millipore). α-Tubulin was measured as a loading control. Western blot bands were quantified using ImageJ and ImageQuant software. The location of relevant molecular weight markers are indicated.

Statistics

Statistical analyses were conducted using GraphPad Prism software. Data are presented as mean ± SEM, unless otherwise noted (as in Figure 2.8). Two-tailed student's t test was used to assess statistical significance, with a threshold for significance of $p<0.05$.

Study approval

The handling and euthanizing of mice was performed entirely in accordance with national guidelines and with approval from the Committee on Use and Care of Animals at the University of Michigan.

Results

Trans-dominant retention of a well-folded wt secretory protein partner

To test whether simple ER retention of one homodimerization partner can confer retention to a wt bystander, we expressed various epitope-tagged proinsulin constructs: human proinsulin (hPro) bearing or not bearing a myc- or SuperfolderGFP-tag within the C-peptide sequence (hPro-CpepMyc and hPro-CpepSfGFP, respectively), or mouse proinsulin (mPro). Epitope-tagging the C-peptide does not significantly affect the folding or ER export of wt proinsulin (20). We also expressed proinsulin(s) bearing a C-terminal KDEL sequence reported to confer proinsulin retention within the ER (21). All of these recombinant proinsulins were comparably expressed, and the expression level for each protein could be experimentally controlled (although the mutant hProG(B23)V-CpepMyc is less stable, see below) as it was proportional to the amount of plasmid DNA included in our transfections (See Appendix, Supplemental Figure S1). hPro-CpepSfGFP-KDEL was retained intracellularly in INS1 beta cells (See Appendix, Supplemental Figure S1), primarily in the same compartment as that marked by a ER-RFP, a red fluorescent protein bearing the KDEL retention signal (6).

Interestingly, when co-transfected with hPro-CpepSfGFP-KDEL plasmid, the intracellular content of wt hPro-CpepMyc increased, and its secretion decreased (Figure 2.1A). Intracellular wt hPro-CpepMyc co-immunoprecipitated with hPro-CpepSfGFP-KDEL, perhaps to an even greater extent than its co-precipitation with wt hPro-CpepSfGFP (Figure 2.1B bottom row). Nevertheless, even as increasing doses of mPro-KDEL dramatically inhibited secretion of wt human proinsulin (Figure 2.1C *upper bar graph measured by human proinsulin RIA*), secretion of wt TgGFP was largely unaffected in the same cells (Figure 2.1C *bottom*). These results suggest that blockade of export of the well-folded wt secretory protein partner involves specific interactions with its ER-retained dimerization partner. Indeed as shown in Figure 2.1D, secretion

of wt hPro-CpepMyc was largely restored upon overexpression of wt mPro "competitor" even in the presence of mPro-KDEL.

Misfolded mutant secretory protein also assembles with and impairs secretion of its WT partner. We have previously suggested that in the autosomal dominant disease called MIDY, misfolded mutant proinsulin can inhibit wt proinsulin export by recruitment of the wt gene product into aberrant protein complexes within the ER (5, 20). Indeed, when wt hPro-CpepMyc was co-expressed with misfolded mutant mProG(B23)V in place of wt mPro, the wt hPro-CpepMyc secretion was impaired *in trans* (Figure 2.2A). By contrast, the mutant *rdw* allele of Tg functions as an autosomal recessive (15); nevertheless, surprisingly, we found that co-expression of rdw-Tg also could greatly decrease the secretion of wt Tg3xMyc (Figure 2.2B) with the extent of this dominant-negative inhibition dependent upon the relative abundance of mutant versus wt gene products (*see below*). Unlike secreted wt Tg3xMyc, wt Tg3xMyc retained intracellularly in the presence of co-expressed rdw-Tg was recovered in an endoglycosidase H (EndoH)-sensitive state indicating molecules that had not advanced to the Golgi complex (Figure 2.2B). The rdw-Tg has a point mutation in the ChEL domain that is involved in Tg homodimerization (10, 17). To test if the rdw ChEL domain can indeed associate with the wt Tg ChEL domain, wt ChEL-HA was co-expressed with rdw-ChEL-Myc. Similar to the situation with the mutant proinsulin hProG(B23)V-CpepMyc (Figure 2.2C *bottom*), we found that mutant rdw-ChEL could co-precipitate with its wt dimerization partner (Figure 2.2D *bottom*).

WT-mutant cross-dimerization offers secretory rescue to the mutant gene product.
In INS1 cells, wt hPro-CpepSfGFP efficiently reached secretory granules and was well secreted, whereas hProC(A7)Y-CpepSfGFP (which harbors the same mutation as that found in the *Akita* mouse) was retained in the ER (Figure 2.3A *upper*) rather than being secreted (Figure 2.3A *lower*) (22). Interestingly, however, recombinant hProG(B23)V-CpepSfGFP showed intermediate behavior, with partial ER retention and a partial secretory granule distribution, and an intermediate level of secretion — consistent with a previous report (6). This stands in contrast to findings that secretion of mutant hProG(B23)V is negligible in heterologous cells that do not express endogenous wt proinsulin (5). We therefore considered whether secretion of hProG(B23)V might be improved in cells expressing a wt proinsulin partner. To test this, we

expressed wt hPro-CpepSfGFP in the mouse pituitary cell line AtT20 (which forms secretory granules containing ACTH but does not express proinsulin). In this cell line, wt hPro-CpepSfGFP co-localized with endogenous ACTH in secretory granules that accumulate at the distal tips of cellular processes (Figure 2.3B *upper row*). When expressed by itself, hProG(B23)V-CpepSfGFP exhibited primarily an ER distribution and did not reach secretory granules (Figure 2.3B *middle row*). However, when co-expressed with wt hPro-CpepMyc, the hProG(B23)V-CpepSfGFP was partially rescued, becoming visible in secretory granules (Figure 2.3B *lower; quantification of granule GFP intensity shown at right*). These results indicate that expression of wt proinsulin enhances intracellular transport of mutant hProG(B23)V.

To determine the selectivity of this rescue, 293T cells were co-transfected with fixed equimolar amounts of hProG(B23)V-CpepMyc and rdw-TgGFP, and simultaneously co-transfected with either wt mPro or wt Tg (empty vector was included to keep constant the total DNA in each transfection). As measured by human proinsulin-specific RIA, secretory rescue of hProG(B23)V was provided selectively by wt mPro (Figure 2.4A *upper panel; confirmed by immunoblotting in Figure 2.4B*) but not by wt Tg (Figure 2.4A *upper*). Conversely, rescue of rdw-TgGFP secretion was conferred upon co-expression of wt Tg (17) but not by wt mPro (lane "M", Figure 2.4A *lower panel*). This phenotype involved authentic intracellular transport through the secretory pathway (rather than cell death) as rescue was blocked in cells treated with Brefeldin A (which blocks anterograde transport, Figure 2.4C). Thus, not only can an ER-retained dimerization partner impair secretion of its wt counterpart (Figures 2.1, 2.2), but expression of wt dimerization partner augments secretion of its misfolded counterpart (Figures 2.3, 2.4).

WT dimerization partners dose-dependently stabilize their misfolded counterparts for secretory rescue.

To examine the stability of hProG(B23)V-CpepMyc, we used metabolic pulse-labeling of cells with ^{35}S-amino acids and measured the fraction of newly-synthesized protein remaining at 20 h after synthesis. hProG(B23)V-CpepMyc stability was significantly increased by co-expression of wt mPro (Figure 2.5A). In parallel, wt Tg3xMyc increased both the intracellular as well as secreted amounts of rdw-TgGFP — indeed, these observations were dependent on the dose of wt Tg3xMyc (Figure 2.5B). Rescue of hProG(B23)V-CpepMyc also was observed with increasing

concentrations of co-expressed wt mPro (Figure 2.5C). Notably, rescue appeared more dependent upon the ratio between wt and mutant proteins than the absolute amount of wt protein expressed, because increasing rescue was also observed with decreasing expression of misfolded mutant rdw-TgGFP rather than raising the amount of wt Tg3xMyc (Figure 2.5D).

Rescue of mutant and blockade of wild-type proinsulin and thyroglobulin occur within the (patho)physiological context.

To determine if the effects of altering wt : mutant stoichiometric ratio in heterologous cells also are observed in pancreatic beta cells, we expressed two different doses of hProG(B23)V-CpepSfGFP in INS1E cells that are known to secrete endogenous proinsulin and insulin under basal conditions and at greater levels in response to elevated glucose. Unsurprisingly, INS1E cells transfected with less plasmid expressed less mutant proinsulin (Figure 2.5E *left panel*). Interestingly, INS1E cells expressing less mutant proinsulin (and thus a higher wt : mutant stoichiometric ratio) exhibited higher fractional secretion of the mutant protein under both basal and glucose-stimulated conditions, including both unprocessed hProG(B23)V-CpepSfGFP and its CpepGFP processing product (Figure 2.5E *right*).

We next wished to extend these findings to tissues of animals with diseases of protein misfolding in the ER. First we looked directly at the rescue of mutant rdw-Tg3xMyc in thyroid tissue from animals lacking or bearing wt Tg. For this, we prepared an *rdw-Tg3xMyc* transgene whose expression in the thyroid gland was driven by a thyroglobulin promoter. The rdw-Tg protein was expressed in thyroid tissue of otherwise wild-type C57BL/6 mice, or mutant *cog/cog* mice (with homozygous expression of mutant Tg-L2263P) in a C57BL/6 background. Both strains of mice expressed the rdw-Tg protein in the thyroid gland (See Appendix, Supplemental Figure S2), and both strains can iodinate secreted proteins (23). However, in thyroid tissue from the *cog/cog* background (lacking wt Tg), no rdw-Tg could become iodinated, indicating an inability of rdw-Tg to reach the iodination site (Figure 2.6A). By contrast, in thyroid tissue of C57BL/6 control mice expressing wt Tg, some mutant rdw-Tg was rescued based on the ability to become iodinated — moreover, the iodinated rdw-Tg was endoH-resistant, indicative of molecules that had undergone normal intracellular transport through Golgi/post-Golgi compartments (Figure 2.6A).

We also wished to determine if decreased wt : mutant stoichiometry can promote blockade of the wt gene product. For this, we examined mice expressing the *Akita*-like mutant hProC(A7)Y-CpepGFP transgene (18) with deletion of either one allele or homozygous knockout of endogenous *Ins2* to progressively decrease wt proinsulin expression. We then used mouse proinsulin-specific immunofluorescence to examine the intracellular distribution of the remaining endogenous (primarily *Ins1*) gene product. In wt, *Ins2*$^{+/-}$, and *Ins2*$^{-/-}$ mice lacking the mutant transgene, most pancreatic beta cells exhibit strong proinsulin immunostaining in the Golgi region, consistent with previous reports (22, 24). However, in the presence of the mutant hProC(A7)Y-CpepGFP [which itself is entrapped in the ER (18)], more cells began to appear with endogenous mouse proinsulin in an ER-like pattern (co-localizing with calnexin, Figure 2.6B upper two rows). There is published evidence that endogenous proinsulin synthesis/content is heterogeneous in the population of islet β cells (25, 26). Nevertheless, in *Ins2*$^{-/-}$ mice, as the ratio of wt : mutant proinsulin decreased, the fraction of cells exhibiting endogenous wt proinsulin in an ER-staining pattern increased — and this effect was apparent in mice matched for random blood glucose levels ≤ 250 mg/dL (Figure 2.6B, *quantitated at right*). These findings demonstrate that in tissues from actual conformational disease models, both mutant secretory protein rescue (Figure 2.6A) and wt secretory protein blockade (Figure 2.6B) are influenced by the ratio of wt : mutant secretory protein in the ER.

Can secretory rescue and secretory blockade occur simultaneously?

To examine whether both rescue (of mutant) and blockade (of wt) dimerization partners can occur simultaneously, we first transfected 293T cells with wt mPro, or hProG(B23)V, or both. We then independently measured mouse proinsulin and human proinsulin secretion by species-specific immunoassay. Secretion of wt mPro was significantly diminished by the presence of hProG(B23)V (Figure 2.7A *upper*). Remarkably, from the same cells, hProG(B23)V secretion was improved by the co-expression of wt mPro (Figure 2.7A *lower*). Similarly, when transfecting with progressively increasing ratios of wt Tg3xMyc to mutant rdw-TgGFP, rdw-TgGFP secretion improved (Figure 2.7B *upper*), whereas wt Tg3xMyc secretion was significantly diminished when the ratio favored rdw-TgGFP (Figure 2.7B *lower*). Intermediate ratios showed both rescue and blockade (Figure 2.7B). Both sets of data in Figure 2.7 indicate that these effects occur simultaneously in the same cells.

Is secretory rescue of misfolded proinsulin unique to the G(B23)V substitution?

Multiple MIDY mutants cause dominant-negative blockade of wt proinsulin export (5). Structurally, MIDY can be subdivided into those mutants that cause the gain or loss of a Cys residue to create an unpaired cysteine, and other mutants affecting conserved hydrophobic residues that perturb disulfide pairing of the natural cysteine partners (4). To see which class of mutants could be rescued by co-expression of wt proinsulin, we compared secretion of a series of co-expressed MIDY mutants. Notably, secretion of most proinsulin mutants containing an unpaired cysteine, such as C(A6)Y, C(B19)H, or the C(A7)Y mutant responsible for diabetes in the *Akita* mouse, was not improved by co-expression of wt proinsulin (Figure 2.8). By contrast, secretion of several other misfolded MIDY proinsulins [e.g., A(SP24)D, H(B5)D, G(B8)S] were rescued in addition to G(B23)V. These data suggest that this mechanism does not work in all cases; nevertheless, cross-dimerization of proinsulin (Figure 2.1B, 2C) or thyroglobulin (Figure 2.2D) exhibits plasticity, highlighting the potential for intracellular transport rescue of a variety of misfolded exportable proteins by their wt counterparts.

Discussion

There are a large number of disorders linked to misfolding, ER entrapment (27) and degradation of exportable proteins (28), and several new approaches have been proposed for the development of therapies for these diseases. Some of these proposals include pharmacological modification of the rate of protein synthesis to avoid overloading protein folding capacity (29), others involve manipulation of the intraluminal ER ionic milieu (30, 31) or modulating ER-associated degradation (ERAD) (32), and still others involve pharmacologic chaperones (33) or modulators of endogenous ER chaperone activity (34, 35) including pre-emptive induction of unfolded protein response (36). Each of these therapies are designed to manipulate the ER quality control environment, altering the ratio of protein folding to protein folding capacity.

In addition to these critical features, we also note that there has been unrecognized selection pressure for exportable proteins to evolve as oligomeric species (37). Indeed, even upon initial description of the ER hsp70 chaperone, BiP, its ability to confer ER retention was found to be linked to its selective association with unassembled subunits of exportable protein oligomers (38). Oligomerization limits chaperone re-binding and helps to relieve ER entrapment (39, 40),

often by limiting exposure of unpaired Cys residues that can frequently be associated with ER retention (41-43). Typically, monomer folding precedes oligomerization (44) but there are examples where oligomer formation may be a very early folding step (45). Either way, for many exportable proteins, achieving an oligomeric state is a critical decision point in determining anterograde transport versus ERAD (46).

It has been shown that expression of misfolded mutant proteins have the potential to cause their wt (bystander) dimerization partners to be retained in the ER (5, 20, 47-50). Although protein-specific rather than general — lowering the levels of the mutant partner (51) and raising wt levels — has been proposed as one of the more efficacious therapeutic approaches, by maintaining general protein homeostasis while allowing escape from ER entrapment of a specific disease-linked gene product (52, 53). In the present study, we emphasize that one potential consequence of raising the wt:mutant protein ratio is that an increased fraction of mutant protein may cross-dimerize with wt, allowing for novel, protein-specific enhancement of protein export. Our findings are unequivocal because we have selectively epitope-tagged the respective wt and mutant partners, and experimentally controlled the expression levels of the respective products (Figures 2.1, 2.5-2.7). In this report, we have studied proinsulin mutants causing MIDY, and thyroglobulin mutants causing congenital hypothyroidism, as two genetically-unrelated representatives of a broad class of conformational diseases of exportable proteins. The organs affected by these diseases appear quite different in terms of their ability to expand tissue mass, and they might have different intrinsic susceptibility to ER stress (a goiter can grow large whereas expansion of pancreatic beta cell mass is more limited), yet studying these molecules in parallel has allowed us to address persistent questions about the extent to which phenotypes linked to cross-dimerization are protein-specific, and the extent to which the pathogenesis of these diseases is secondary to generalized ER stress.

In the current study, we demonstrate that retention of proinsulin-KDEL, via a mechanism involving neither misfolding nor ER stress, is sufficient to induce export blockade of its wt dimerization partner (Figure 2.1A, C) in conjunction with direct physical association between the partners (Figure 2.1B), and in a manner competed by further addition of wt proinsulin (Figure 2.1D). In the same cells, wt Tg secretion continues unimpeded in the face of proinsulin

blockade, demonstrating specificity (Figure 2.1C). We have every reason to believe that the initial pathogenesis of MIDY is based on a similar underlying cell biological principle: in the case of mutant versions of exportable proteins, chaperone-mediated retention of the misfolded gene product is the rule (54). While formation of misfolded protein complexes was once thought to be nonspecific (55), we have found that expression of the proinsulin-G(B23)V mutant selectively co-precipitates its wt partner (Figure 2.2C) and blocks secretion of that partner (Figure 2.2A) while neither associating with nor blocking wt Tg in the same cells (Figure 2.1C and *data not shown*). This dominant effect on wt proinsulin appears to account for the dominant inheritance of MIDY (Figure 2.9), producing insulin deficiency from the wt allele (4). Moreover, expressing the *Akita*-like hProC(A7)Y-CpepGFP transgene in mice first with heterozygous and then homozygous loss of endogenous *Ins2* (i.e., changing the relative expression of the two protein partners) results in progressive blockade of remaining wild-type proinsulin, independent of changes in random blood glucose (Figure 2.6B).

Despite an autosomal recessive pattern of inheritance, we found surprisingly similar molecular behavior for the genetically unrelated thyroglobulin protein. The rdw-Tg, which bears a mutation in the ChEL domain and causes thyrocyte cell death only when expressed in homozygotes (16), also exhibits cross-dimerization with wt Tg in heterozygotes (17). Herein we show that the molecular mechanism involves direct interactions between the mutant and wt dimerization (ChEL) domains (Figure 2.2D). Remarkably, we found that expression of the recessive rdw-Tg also can dominantly block export of its wt Tg partner (Figure 2.2B), and this phenotype is linked to lowering the wt:mutant protein ratio (Figure 2.7B). The fact that heterozygosity does not generate hypothyroidism in vivo indicates that *rdw/+* rats express a Tg protein ratio favoring the wt gene product. Further, when expression of the wt gene product is favored, rescue phenotypes become apparent. Such rescue was directly demonstrated in thyroid tissue of mice expressing a mutant rdw-Tg3xmyc transgene in a wild-type genetic background: unlike in *cog/cog* mice, rdw-Tg3xmyc acquired endoH resistance and even became iodinated in animals making endogenous wild-type Tg (Figure 2.6A). Moreover, rescue is also observed for proinsulin mutations that are ordinarily transmitted with autosomal dominant inheritance. In pancreatic beta cells, partial rescue of proinsulin-G(B23)V to secretory granules is already in evidence (Figure 2.3A), conferring enhanced secretion under both basal and stimulated

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conditions (Figure 2.5E) (6), and such an effect can be directly attributed to co-expression of wt proinsulin (Figure 2.3B). Moreover, rescue of mutant proinsulin-G(B23)V or rdw-Tg is also protein specific: in cells co-expressing both mutants, secretory rescue of proinsulin-G(B23)V is accomplished exclusively by wt proinsulin, whereas secretory rescue of rdw-Tg is accomplished exclusively by wt Tg (Figure 2.4).

All evidence points to the idea that secretory rescue is a consequence of intracellular stabilization of the mutant gene product: this is true both for proinsulin-G(B23)V (Figure 2.5A) and for rdw-Tg (Figure 2.5B). And for both mutant proteins, the magnitude of the stabilization and secretory rescue is linked to the ratio of wt:mutant protein expression (Figure 2.5C, D). Most remarkable of all, rescue (of mutant) and blockade (of wt) secretion occurs simultaneously in the same cells (Figure 2.7). Thus for both proteins, there is a dynamic bi-directional balance between retention and anterograde transport of mutant cross-dimers (Figure 2.9).

We posit that for the many conformational diseases affecting exportable proteins that oligomerize in the ER, a dominant versus recessive pattern of inheritance is in part a reflection of the balance of these two activities (blockade versus rescue, see Figure 2.9). In the case of MIDY and other dominantly inherited diseases, the balance favors net retention of protein, whereas in the case of congenital hypothyroidism with defective Tg, the balance favors net export. The minimum plasmid ratio at which we observed secretory rescue was consistent with this hypothesis: rescue of proinsulin-B23V requires higher wt:mutant ratios than the rescue of rdw-Tg secretion (Figure 2.5).

In conclusion, the data presented herein indicate that both secretory blockade and rescue involve direct cross-dimerization between wt and mutant gene products. As cross-dimerization in the secretory pathway is certainly not limited to proinsulin and thyroglobulin (56), we expect that similar cooperativity will be observed for other exportable proteins. While the effects described in this report appear limited to protein-specific protein rescue of mutant oligomerization partners, the results imply a broader significance for understanding disease pathophysiology. Specifically, our studies have potential relevance to the finding of ER accumulation of secretory proteins even in the absence of any mutations. For example, wt proinsulin is prone to misfolding under

conditions of increased insulin demand (57, 58) and its accumulation may contribute to beta cell failure (59, 60). If oligomerization plays an important role in the retention of wt proinsulin and other wt secretory proteins, then protecting/stabilizing the interaction interface (such as with small molecule interactors) might allow ER escape of an increased fraction of exportable protein despite the presence of a misfolded subset of such molecules. Such methods might be used in combination therapy with other approaches to alter the ER environment (described above), opening remarkable new avenues for treatment of diseases of misfolding of exportable proteins.

Figures
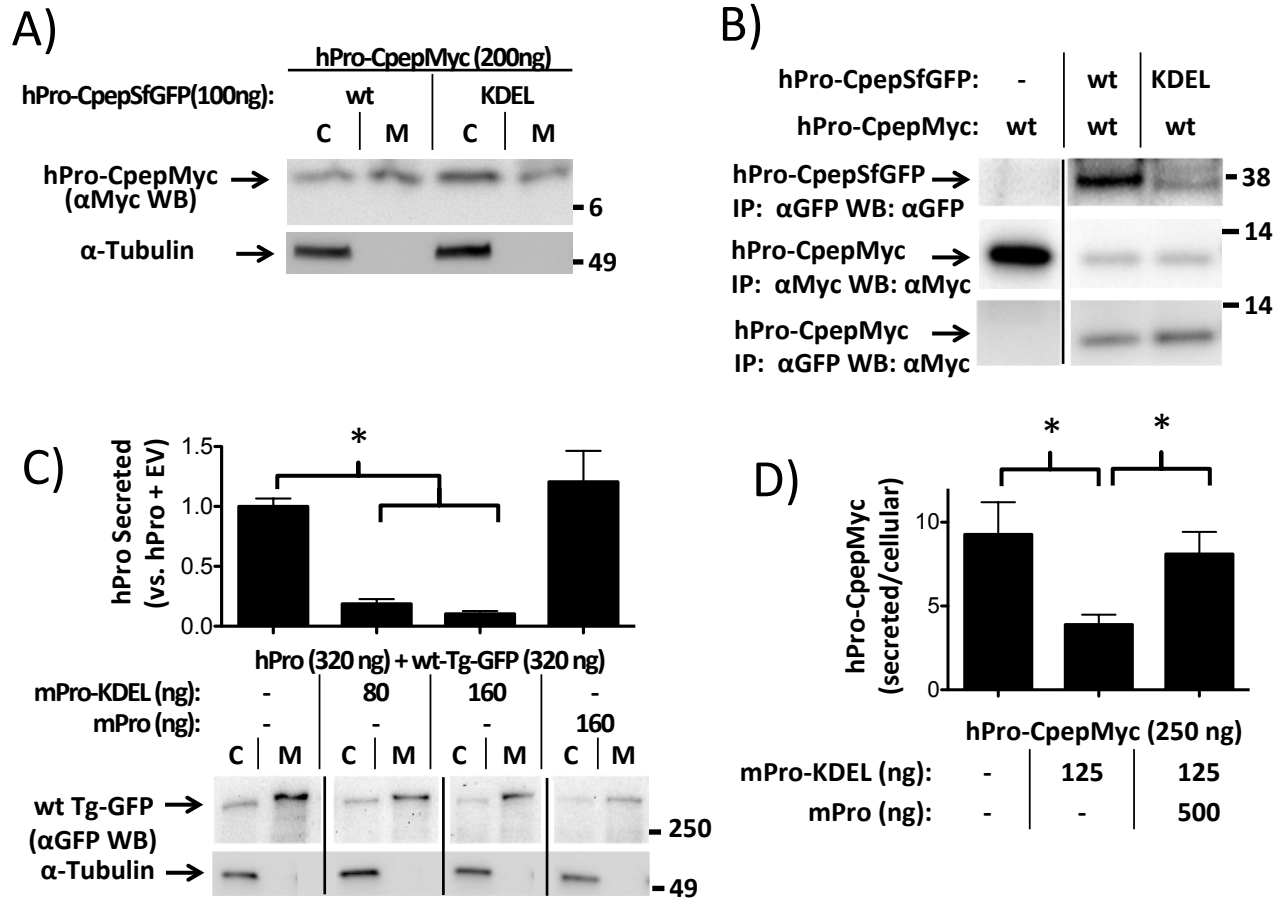
Figure 2.1. Proinsulin-KDEL interacts with and inhibits secretion of WT proinsulin. 293T cells transiently transfected with hPro-CpepMyc were co-transfected with plasmids as indicated. **A)** Cell lysates (C) and media (M) were resolved by SDS-PAGE, electrotransfer, and immunoblotting (WB) with anti-myc. The media/cell ratio of hPro-CpepMyc bands was decreased by $58.9 \pm 12.8\%$ (p=0.003, n=6) in cells co-expressing hPro-CpepSfGFP-KDEL compared to wt hPro-CpepSfGFP. **B)** Cells lysed in TX-CoIP buffer were immunoprecipitated with anti-GFP or anti-myc, resolved by SDS-PAGE, electrotransfer, and immunoblotting (WB) with anti-GFP or anti-myc as indicated. The upper two panels demonstrate expression of the indicated proteins, and the lower panel demonstrates co-immunoprecipitation. Gels are representative of three independent experiments. **C)** Cells transiently expressing wt human proinsulin plus TgGFP were co-transfected with plasmids as indicated. The media collected overnight were analyzed by human proinsulin specific RIA. TgGFP in the same cell lysates (C) and media (M) were analyzed by SDS-PAGE, electrotransfer, and immunoblotting (WB) with anti-GFP. The media/cell ratio of TgGFP bands in cells co-expressing mPro-KDEL exhibits no significant change to that from cells co-expressing wt mPro (1.9 ± 0.2 *vs.* 2.1 ± 0.8; p=0.3, n=5). In **B)** and **C)**, noncontiguous lanes from the same gel are shown. **D)** Cells transiently expressing hPro-CpepMyc were co-transfected with plasmids expressing mouse proinsulin-KDEL (mPro-KDEL) or mouse proinsulin (mPro). Media were collected overnight and cell lysates were analyzed by human proinsulin-specific RIA. The data in **C)** and **D)** represent mean ± s.e.m., each from ≥ 4 independent transfections. * = p < 0.05.
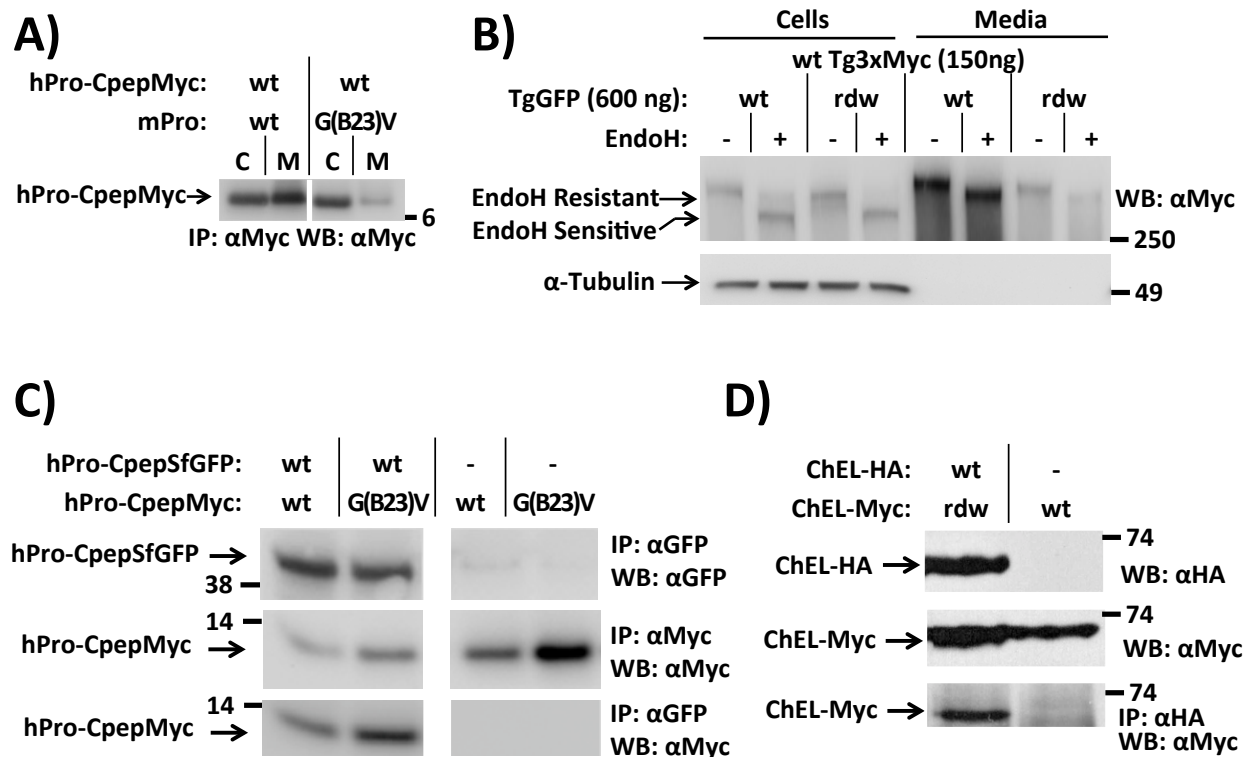
Figure 2.2. Cross-dimerization of mutant/wt proinsulin and mutant/wt thyroglobulin. 293T cells were transiently co-transfected with plasmids expressing the indicated proinsulin or Tg variants. **A)** At 48 h post-transfection, cell lysates and overnight media were collected; both were immunoprecipitated with anti-myc (to pre-purify the antigen) and then analyzed by SDS-PAGE, electrotransfer, and immunoblotting with anti-myc. The media/cell ratio of hPro-CpepMyc bands from cells co-expressing mPro-G(B23)V decreased $66.1 \pm 1.9\%$ ($p<0.001$, n=4) compared to that of wt mPro. **B)** At 48 h post-transfection, cell lysates and overnight media were collected, treated $\pm$ Endo-H, and were analyzed by immunoblotting with anti-myc. The media/cell ratio of wt Tg3xMyc bands in cells co-expressing rdw-TgGFP decreased $74.1 \pm 3.3\%$ ($p<0.001$, n=4) compared to that of wt TgGFP. **C)** At 48 h post-transfection, cells lysed in TX-CoIP buffer were immunoprecipitated with anti-GFP or anti-myc and were analyzed by Western blotting with anti-GFP or anti-myc, as indicated. **D)** Cells transiently co-expressing wt secretory ChEL-HA plus secretory mutant rdw-ChEL-Myc or secretory wt ChEL-Myc (9) were cultured in the presence of brefeldin A (5 h, 5 μg/mL) to allow intracellular co-incubation of the co-expressed constructs. Cells were then lysed in NP40-CoIP buffer and analyzed by immunoblotting with or without immunoprecipitation as indicated. For **C)** and **D)**, the upper two panels demonstrate expression of protein partners, and the lower panel demonstrates co-immunoprecipitation. Gels in **C)** and **D)** are representative of n≥3 experiments. In **A)** and **C)**, noncontiguous lanes from the same gel are shown.
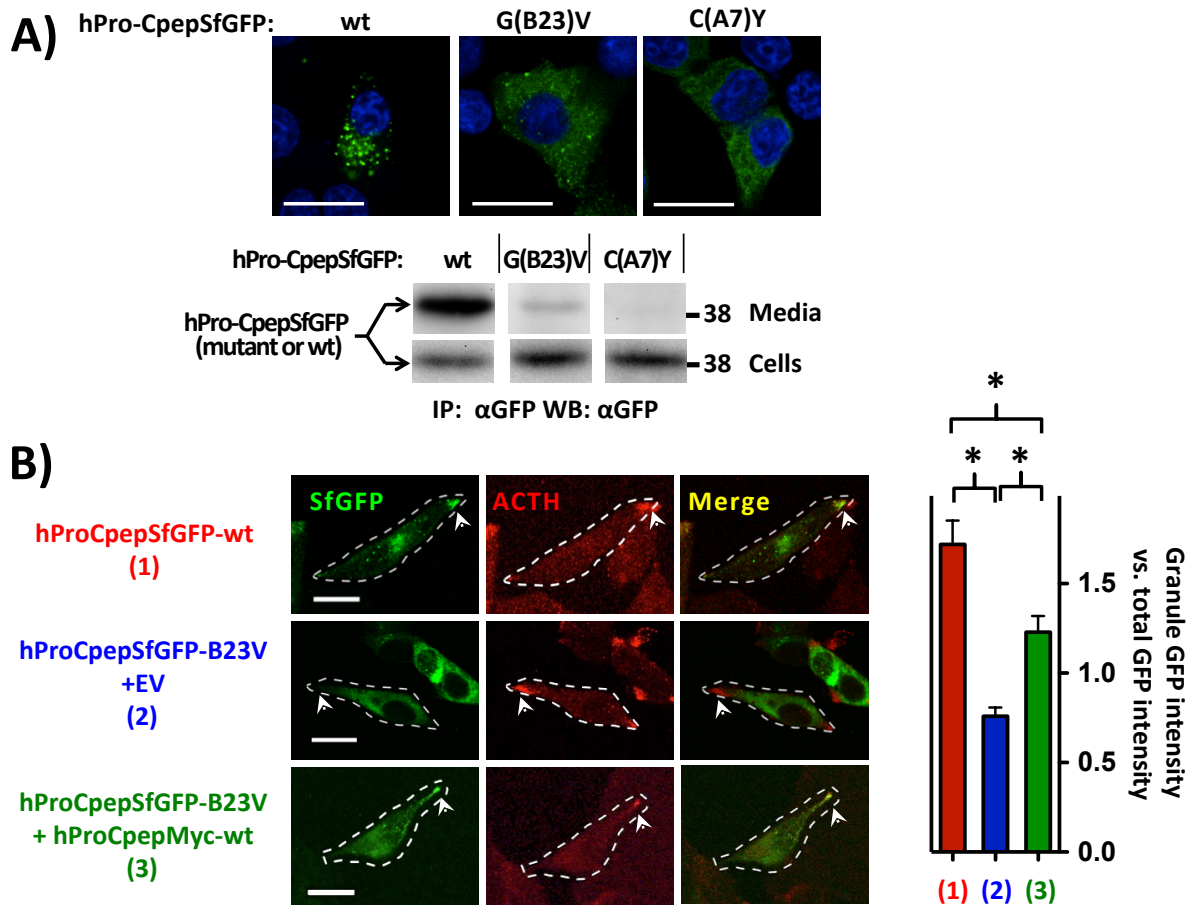
Figure 2.3. Intracellular distribution of mutant proinsulins in regulated secretory cells co-expressing or not co-expressing wt proinsulin. **A)** Cultured INS1 pancreatic beta cells (that express endogenous proinsulin) were transiently transfected to express wt or mutant hPro-CpepSfGFP, as indicated. Fixed cells (counterstained with DAPI) were examined by confocal microscopy for the distribution of SfGFP-containing peptides (*upper panels;* scale bar = 20 μm). The cell lysates and overnight bathing media were collected, immunoprecipitated with anti-GFP, and analyzed by immunoblotting with anti-GFP to examine secretion efficiency (*lower panel*). Noncontiguous lanes from the same gel are shown. The media/cell ratio for wt, G(B23)V, and C(A7)Y hPro-CpepSfGFP bands was 14.8 ± 3.8, 0.74 ± 0.04, and 0.16 ± 0.06, respectively (p < 0.05 for all groups, n=4). **B)** Cultured AtT20 pituitary corticotroph cells (that do not express endogenous proinsulin) were transiently co-transfected with one of three different plasmid combinations, as indicated. Fixed cells were examined by confocal fluorescence for the distribution of SfGFP-containing peptides (green) and immunofluorescence to localize ACTH-containing secretory granules (red) at the tips of cell (arrowheads; scale bar = 20 μm). Cell boundaries were defined from phase contrast images (*not shown*). Enrichment of average GFP intensity in the secretory granule region (*shown at right*) was compared to average GFP intensity in nongranule regions. Data represent mean ± s.e.m. from 30-38 separately imaged cells for each of the three respective transfection conditions. * = p < 0.05.
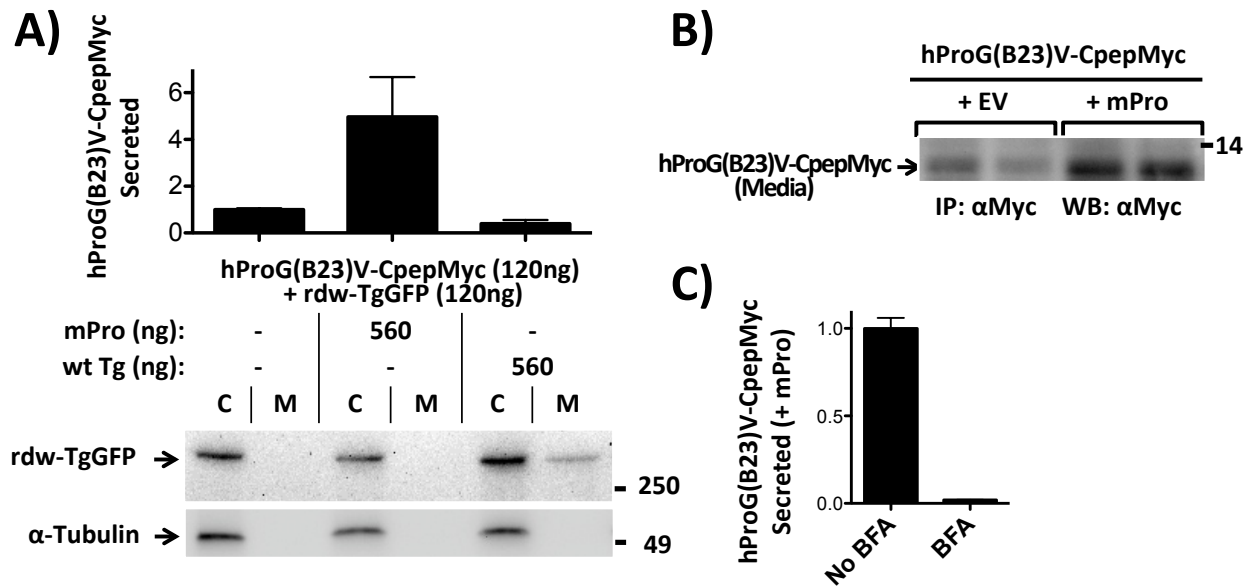
Figure 2.4. Cross-dimerization as a basis for secretory rescue of mutant proinsulin or thyroglobulin is specific to their respective wild-type partners. **A)** 293T cells transiently expressing both mutant proinsulin and mutant Tg were co-transfected with either wt mouse proinsulin or wt Tg. The media were collected overnight and cells lysed; proinsulin secretion was quantified by human proinsulin-specific RIA (*upper panel*). Data represent mean ± s.e.m. relative to cells lacking mPro or wt Tg ($p = 0.07$, $n = 3$). From the same cell lysates (C) and media (M), secretion of rdw-TgGFP was analyzed by SDS-PAGE, electrotransfer, and immunoblotting with anti-GFP (*lower panel*). **B)** At 48 h post co-transfection as indicated, overnight secretion of mutant hProG(B23)V-CpepMyc (in duplicate) was measured by immunoprecipitation and immunoblotting with anti-myc. The results shown in panels A and B are representative of three separate experiments. EV=empty vector. **C)** At 48 h post transfection, cells co-transfected as indicated were either untreated or treated with 5 μg/ml brefeldin A (BFA). After 5 h, the media were collected and analyzed by human proinsulin-specific RIA. The data shown are mean values ± range from two independent measurements.
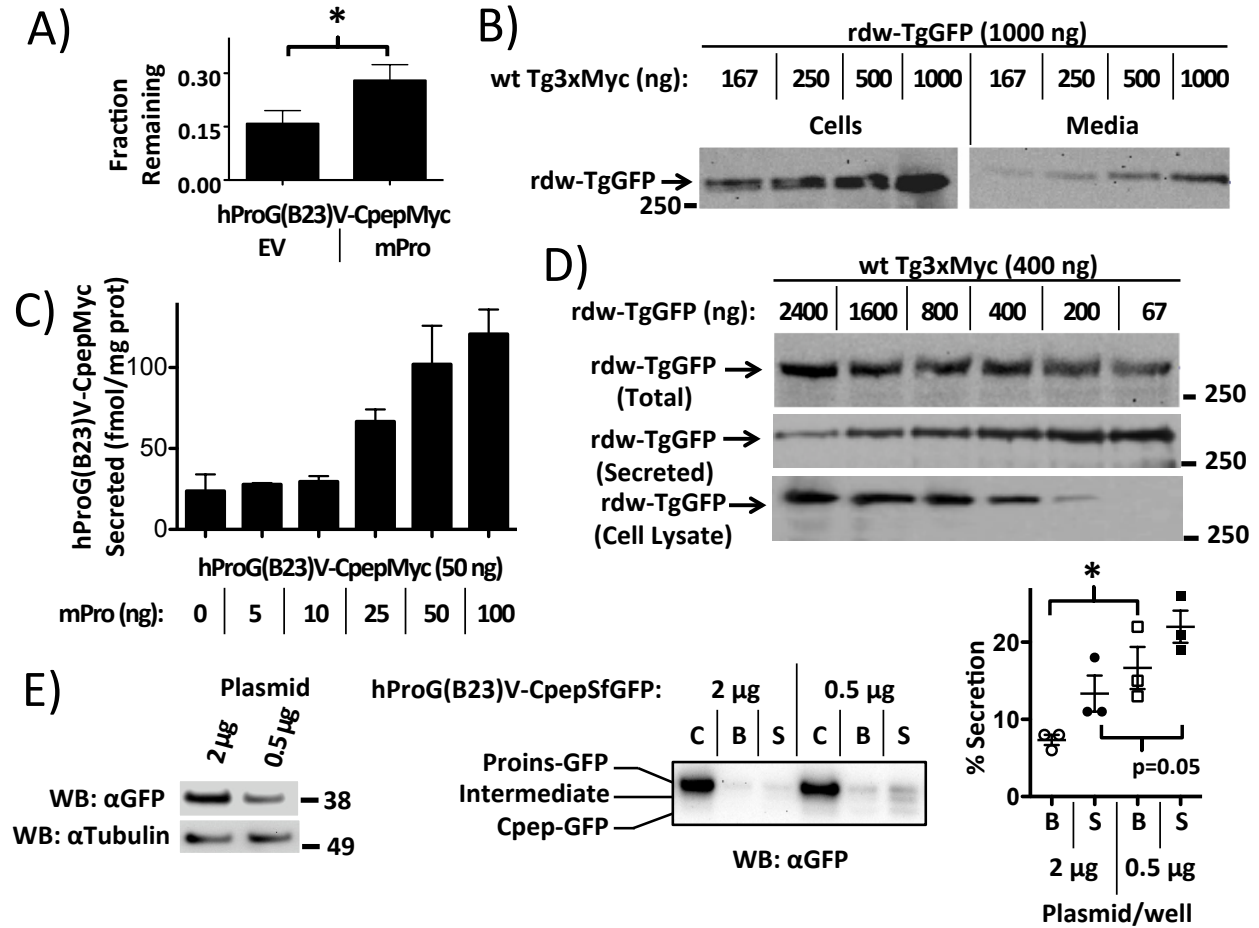
Figure 2.5. Secretory rescue and stabilization of mutant proinsulin or thyroglobulin by their wt counterparts is linked to the wt : mutant expression ratio. 293T cells were transiently co-transfected with the indicated plasmid combinations, with empty vector added to keep total DNA/well constant (**A-D**). **A)** Total mutant proinsulin recovery at 20-hour post-synthesis was measured by pulse-chase (*see Methods*). Protein stability was quantified by band recovery at 20 h chase to that at time zero; mean ± s.e.m., n=4, *=p<0.05. **B)** Overnight media and cell lysates were analyzed by immunoblotting with anti-GFP, normalized to total cellular protein. Representative blots from three experiments are shown. **C)** Overnight media were collected, and human proinsulin secretion (normalized to total cellular protein) was measured by RIA. Data represent mean ± range from two independent experiments. **D)** Overnight media and cell lysates (lower two panels) or combined lysate and media (upper panel) were analyzed by immunoblotting with anti-GFP, normalized to total cellular protein. Representative blots from three experiments are shown. **E)** INS1E cells were transfected with 2 or 0.5 μg plasmid expressing mutant proinsulin; the cellular levels of hProG(B23)V-CpepSfGFP are shown at *left*. Cell lysates (C) and basal secretion (B) and glucose-stimulated secretion (S), normalized for hProG(B23)V-CpepSfGFP protein expression (1% of total for 2 μg transfection; 3% of total for 0.5 μg transfection) were analyzed by immunoblotting with anti-GFP (*middle panel*). Percent secretion was quantified as total GFP signal in media over total in cells (*right*). The data represent mean ± s.e.m., from 3 independent experiments.
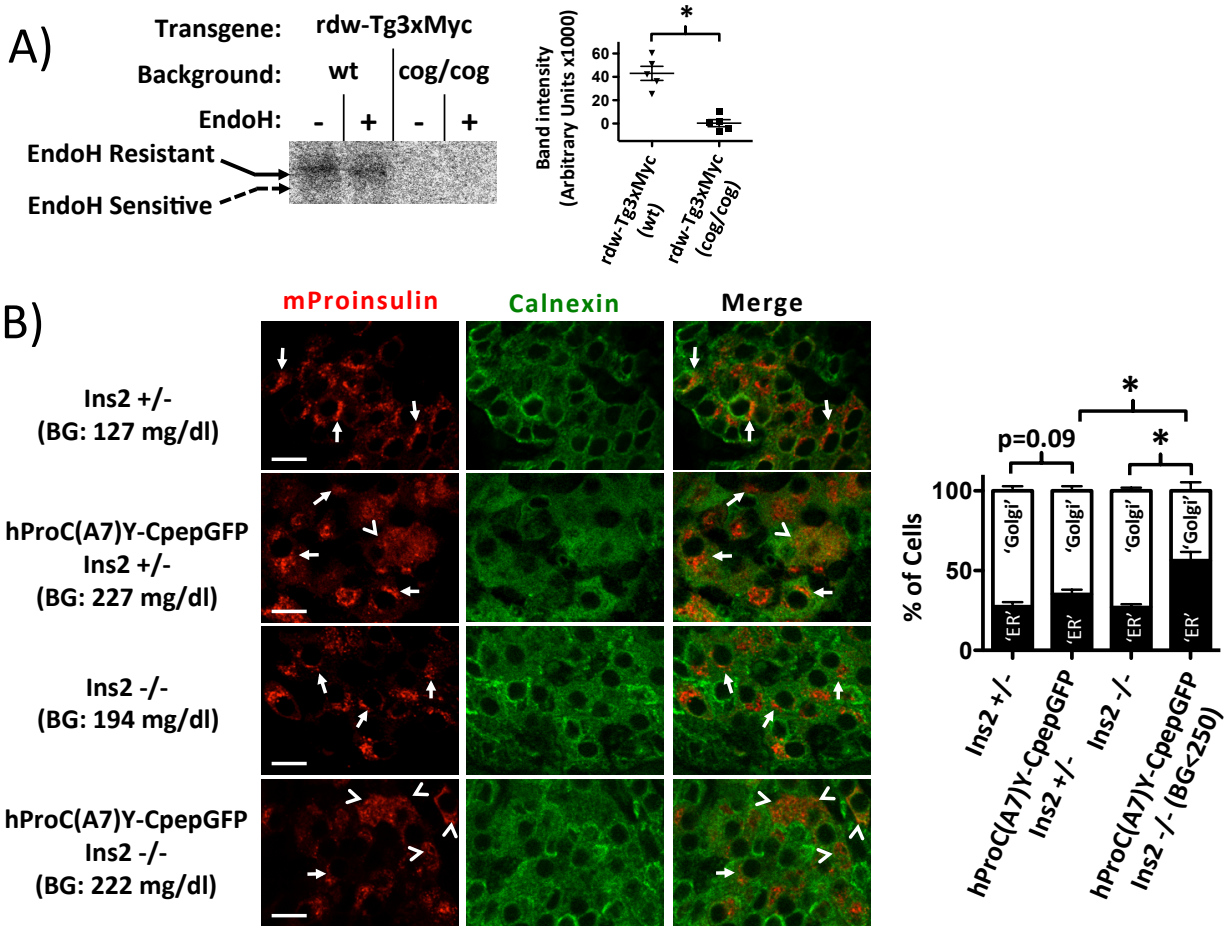
Figure 2.6. Rescue of mutant thyroglobulin and blockade of wild-type proinsulin in primary tissue from animal models of disease. **A)** Lobules of thyroid glands were freshly prepared from mice of the indicated genotypes. Secretory proteins delivered for post-translational iodination were labeled by incubation of thyroid lobules with 1.0 μCi/μL Na^{125}I for 30 min as described in *Methods*. The thyroid lobules were then lysed and immunoprecipitated with anti-myc. The immunoprecipitates were either mock-digested or digested with Endo-H as in Fig. 2B, and then analyzed by SDS-PAGE and autoradiography. **B)** Pancreata from 6 week-old mice with the genotypes indicated (*at left*) were fixed in paraffin, sectioned, de-paraffinized, and immunostained with antibodies specific to mouse proinsulin (red) and calnexin to mark the ER (green). From confocal microscope images (scale bar = 10 μm), a blinded reader scored the localization of wt mouse proinsulin in each beta cell as either a predominant juxtanuclear crescent of increased intensity ['Golgi', consistent with previous reports (22, 24), e.g., *see arrows*] or mainly co-localized with calnexin ('ER' e.g., *see arrowheads*). Quantitation of these data (*at right*) are shown as mean ± s.e.m from n=5 mice with 5 islets per mouse.
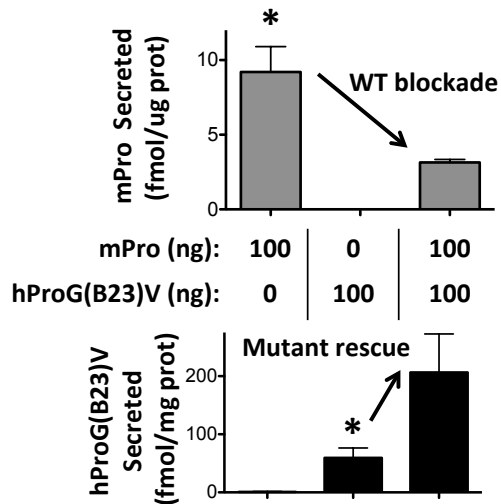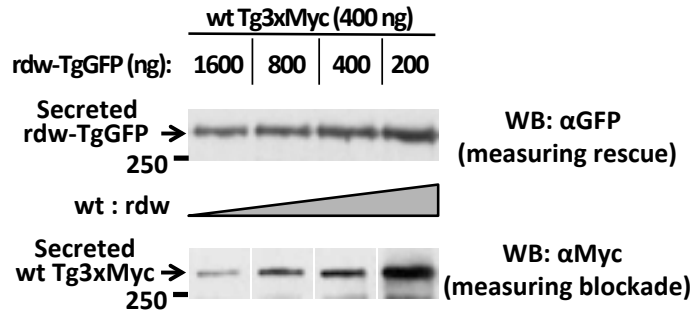
Figure 2.7. Bidirectional consequences of interactions between mutant and wt cross-dimerization partners. A) The overnight bathing media from cells co-transfected with mutant human proinsulin-G(B23)V and wt mouse proinsulin were selectively probed for simultaneous secretion of mouse proinsulin (by ELISA, *upper panel*) and human proinsulin (by RIA, *lower panel*); both assays (normalized to total cell protein) are entirely species-specific. The data represent mean ± s.e.m. from a minimum of three independent transfections, * = p < 0.05. **B)** The overnight bathing media from cells co-transfected with rdw-TgGFP and wt Tg3xMyc were resolved by SDS-PAGE and selectively probed for simultaneous secretion of mutant Tg (by specific immunoblotting with anti-GFP, *upper panel*) and wt Tg (by specific immunoblotting with anti-myc, *lower panel*), normalized to total cellular protein. For the lower panel, noncontiguous lanes from the same gel are shown. Note that whereas in single transfections rdw-Tg is not secreted and wt Tg is well secreted, in co-transfection, rdw-Tg secretion becomes enhanced while wt Tg secretion becomes inhibited. The data shown are representative of three independent experiments.
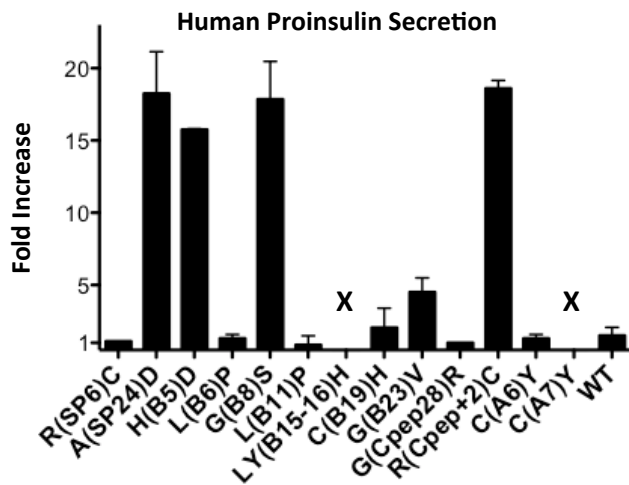
Figure 2.8. Secretory rescue by wt proinsulin is restricted to a subset of MIDY mutants. 293T cells transiently co-transfected with plasmids expressing the indicated human proinsulin mutants and either empty vector or wt mouse proinsulin, were incubated overnight in growth medium beginning at 24 h post-transfection. Media were collected and human proinsulin secretion was measured by RIA. The data shown (fold increase in mutant proinsulin secretion as a consequence of expressing wt mouse proinsulin over empty vector) are mean values ± range from two independent experiments. x = undetectable.
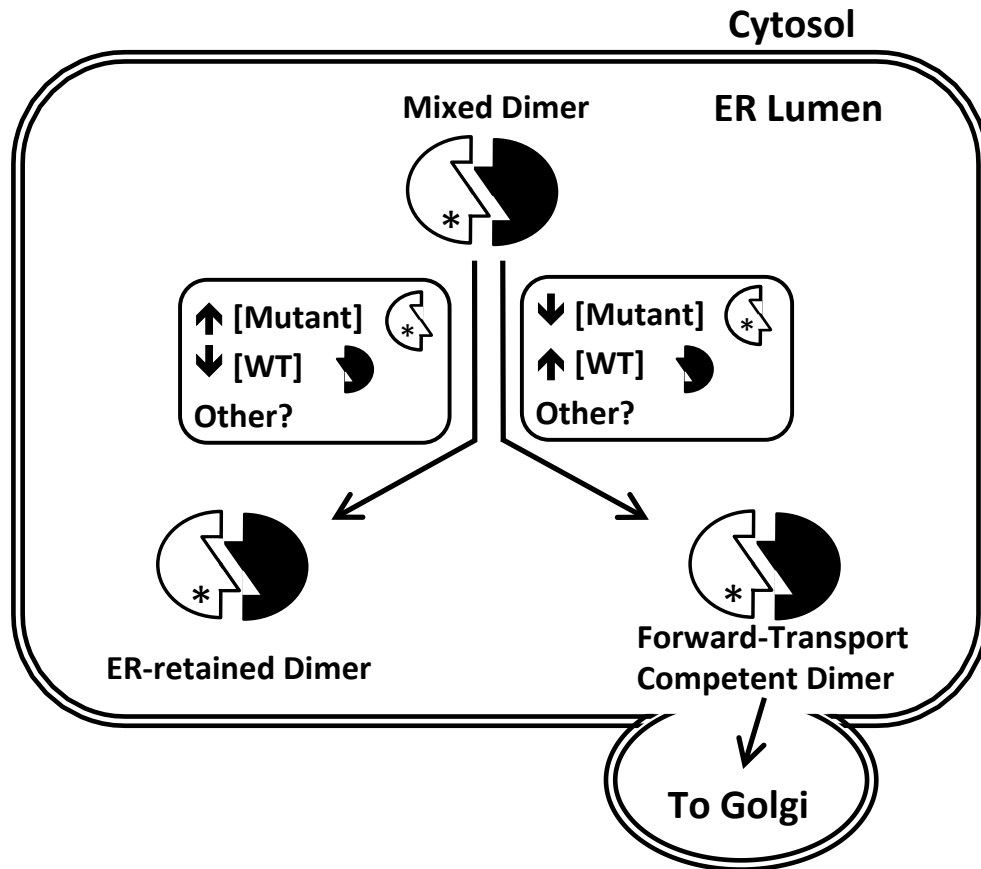
Figure 2.9. Model of bidirectional intermolecular interactions of misfolded and native proteins. Within the ER, many secretory proteins, including proinsulin and thyroglobulin, form homodimers. When mutant and wt alleles from the same gene cross-dimerize, there may be several outcomes, two of which are summarized in the figure. The wt gene product can assist the mutant partner to exit the ER, or the mutant protein can block anterograde transport of the wt protein. Among other protein-specific and general factors involved, the relative concentrations (i.e., stoichiometric ratio) of the two dimerization partners also contribute to the outcome, with lower wt : mutant ratios resulting in greater ER retention and higher wt : mutant ratios resulting in enhanced forward transport.

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CHAPTER 3

ERO1A IMPROVES FOLDING AND SECRETION OF MUTANT PROINSULIN AND ATTENUATES MUTANT PROINSULIN-INDUCED ER STRESS

Abstract

Upon chronically upregulated proinsulin synthesis, misfolded proinsulin can accumulate in the endoplasmic reticulum (ER) of pancreatic beta cells, which may culminate in the development of type 2 diabetes. In Mutant Ins-gene Induced Diabetes of Youth (MIDY), misfolded mutant proinsulin impairs ER exit of co-expressed wild-type (wt) proinsulin, limiting insulin production, increasing ER stress and leading to eventual beta cell death. In this study, we have investigated the hypothesis that increased expression of ER oxidoreductin 1-alpha (Ero1α) — despite an established role in the generation of H_2O_2 — might nevertheless be beneficial in limiting proinsulin misfolding and its adverse downstream consequences. Increased Ero1α expression is effective in preventing inhibition of wt proinsulin secretion from cells co-expressing misfolded mutant proinsulin. In addition, we find that upon increased Ero1α expression, some of the MIDY mutants themselves are directly rescued from ER retention. Secretory rescue of proinsulin-G(B23)V is correlated with improved oxidative folding of mutant proinsulin. Indeed, using three different variants of Ero1α, we find that expression of either wild-type or a hyperoxidizing mutant Ero1α construct can rescue mutant proinsulin-G(B23)V, in parallel with its ability to provide an oxidizing environment in the ER lumen — whereas beneficial effects were less apparent for a redox-inactive form of Ero1α. Increased expression of protein disulfide isomerase (PDI) antagonizes the rescue provided by oxidatively active Ero1α. Importantly, the ER stress response induced by misfolded proinsulin was diminished upon increased expression of Ero1α, suggesting that enhancing the oxidative folding of proinsulin is a viable therapeutic strategy in the treatment of type 2 diabetes.

Introduction

The insulin precursor, proinsulin, is the principal protein synthesized in pancreatic beta cells and consists sequentially of the so-called B-chain, C-peptide, and A-chain. Newly-synthesized proinsulin must fold properly, including the formation of three intramolecular disulfide bonds, in order to be eligible for exit from the endoplasmic reticulum (ER) *en route* to secretory granules where it is eventually converted to mature insulin for secretion (1). Nevertheless, the specific enzymes involved in the oxidative folding of proinsulin remain poorly understood (2). Under conditions of increased synthesis, proinsulin is susceptible to increased misfolding involving the mispairing of cysteines to form non-native disulfide bonds (3, 4). Chronic accumulation of misfolded proteins in the ER—termed ER stress—leads to activation of unfolded protein response pathways, culminating in apoptosis (5). ER stress-induced apoptosis is thought to contribute to loss of pancreatic beta cells in type 2 diabetes (6). Indeed, misfolded proinsulin is an established cause of autosomal dominant diabetes in the syndrome of Mutant Ins-gene Induced Diabetes of Youth (MIDY) (1). The mutant proinsulin molecules are retained in the ER due to a defect in their folding (7); moreover, misfolded mutant proinsulin molecules exert a dominant-negative effect on the transport of co-expressed wild-type (wt) molecules through direct association, leading to ER stress and eventual beta cell death (7-9).

In recent years, increasing attention has been paid to the role of ER Oxidoreductin-1 (Ero1) in promoting the oxidative folding of proinsulin in the ER (10, 11). Mammals express two isoforms of Ero1 (α and β); both are ER luminal flavoproteins that couple reduction of molecular oxygen with the oxidation of ER oxidoreductases such as protein disulfide isomerase (PDI) (12). In turn, the ER oxidoreductases can shuttle disulfide bonds to substrates to catalyze the folding of newly-synthesized secretory proteins such as proinsulin (12). Among other pathways, Ero1 is the best-known source of disulfide bonds in the ER lumen (13). Since Ero1 deficiency impairs proinsulin maturation and causes insulin-deficient diabetes (11), it occurred to us that improved proinsulin oxidative folding may provide a novel approach to ameliorating insulin production. Indeed, both Ero1 isoforms improved proinsulin secretion from heterologous cells, though Ero1α had a markedly stronger effect than did Ero1β (14). Propelled by the hypothesis that improvement of proinsulin folding kinetics may prevent ER retention of both mutant and wt molecules, we have examined the effect(s) of increased Ero1α expression on misfolded proinsulin in the ER. We

found that Ero1α overexpression rescued secretion of mutant proinsulin, associated with improved proinsulin oxidative folding and decreased mutant proinsulin-induced ER stress.

Materials and Methods

Cell Culture and Transfection

HEK293T cells (called 293T) cells were cultured in DMEM with 10% fetal bovine serum and penicillin (100 U/ml) and streptomycin (100 μg/ml). INS1E cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 mM pyruvate, 10mM HEPES, penicillin (100 U/ml) and streptomycin (100 μg/ml), and 50 mM 2-mercaptoethanol. Flp-In T-Rex 293 cells stably transfected with empty vector (EV), Ero1α-WT, or Ero1α-Active, as previously described (18), were maintained in MEM (Sigma, M4526) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1x GlutaMAX (Gibco), 7.5 μg/ml blasticidin, and 50 μg/ml hygromycin. For doxycycline induction, they were incubated in complete media containing 1 ug/ml doxycycline for 24 hours. All cells were cultured at 37°C in a 5% CO_2 incubator. Proinsulin variants were expressed in pcDNA3.1 (Invitrogen) or pTarget (Promega), Ero1α variants were expressed in pcDNA5/FRT/TO (Invitrogen), Grx1-roGFP-iE was expressed in pcDNA3.1, and PDIflag was expressed in pcDNA3.1/V5-His TOPO TA. All plasmids have been used in prior publications, with the exception of Ero1α-Hex and proinsulin-KeepB19/A20, which were generated using the Quikchange Site-Directed Mutagenesis Kit (Agilent). Plasmids were transfected using Lipofectamine 2000 (Invitrogen) for 293T cells or Metafectene Pro (Biontex) for Ins1E and Flp-In T-Rex 293 cells. Total plasmid DNA amount was held constant within each experiment by addition of empty vector.

Proinsulin Measurements and Western Blotting

For secretion experiments, 24-48 hours post-transfection, culture media was changed and collected overnight. Cells were lysed in RIPA buffer (0.1 M NaCl, 0.2 % deoxycholate, 25 mM Tris-pH 7.4, 1% Triton X-100, 0.1% SDS, 10 mM EDTA pH 8.0, and proteinase inhibitor cocktail).   Proinsulin was measured by rat insulin or human proinsulin specific radioimmunoassay (RIA) and normalized to total protein (measured by BCA assay) in the lysate. For western blotting, proteins (10 μg/lane) were resolved by SDS-PAGE on 4-12% acrylamide

gradient gels ('NuPAGE'), electrotransferred to nitrocellulose, and immunoblotted with the indicated antibodies: anti-Ero1α (Santa Cruz), anti-Myc (Immunology Consultant Laboratories), or anti-αTubulin (Sigma). Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch with proteins visualized by ECL (Millipore). α-Tubulin was measured as a loading control. The location of relevant molecular weiht markers is indicated in the figures. Western blot bands were quantified using ImageQuant software.

Metabolic Labeling

48 hours post-transfection, cells were starved in DMEM lacking cysteine and methionine for 30 minutes. After pulse-labeling with S35 labeled cysteine and methionine (Perkin Elmer) for the times indicated in the figure legends, cells were washed in ice cold PBS containing 20mM N-ethyl-methionine (NEM, Sigma), lysed in RIPA buffer containing 2mM NEM, and immunoprecipitated using anti-Myc antibodies and protein A agarose. Immunoprecipitates were separated by reducing or non-reducing tris-tricine-urea-SDS-PAGE(Figure 3.3b) as previously described (7) or by NuPage (Figure 3.8) and analyzed by autoradiography. Gel bands were quantified using Imagequant software.

ER Oxidation

Oxidation of the ER in Flp-In TRex 293 cells, using the $HyPer_{ER}$ sensor, and in Ins1E cells, using Grx-roGFP1-iE was measured as previously described (20, 21). Briefly, Flp-In TRex 293 cells were stably transfected with plasmid expressing the $HyPer_{ER}$ sensor (33). After induction of the Ero1α variants with 1μg/ml doxycycline for 24 hours, fluorescence spectra are obtained at steady state or in the presence of 10mM DTT or 100μM H_2O_2, with excitation ranging from 410-510nm and emission at 535. Percent oxidation is calculated as previously described (20). For Ins1E, cells were washed in PBS containing 20mM NEM and lysed on ice for 1 hour in 100 mM $NaPO_4$, pH 8, containing 1% Triton-X-100 and 200 μM phenylmethylsulfonylfluoride. After removal of insoluble cell debris by centrifugation, the supernatant was immunoprecipitated using NHS-activated agarose (Thermo Scientific) decorated with anti-GFP. Samples were separated by SDS-PAGE and immunoblotted using anti-HA antibodies. Blots were analyzed densitometrically to determine the oxidized/reduced ratio (21).

BiP-Luciferase Measurement

24 hours after transfection with appropriate proinsulin, Ero1α, and BiP promoter driven luciferase plasmids, cells were split into 2 separate wells. One well was lysed in Passive Lysis Buffer (Promega) and luciferase was measured using the Dual Luciferase Reporter Assay Kit (Promega) according to manufacturer instructions. The other well was lysed in Buffer RLT (Qiagen) and homogenized using QIAShredder columns (Qiagen). RNA was isolated using the RNeasy kit (Qiagen) according to manufacturer instructions. cDNA was generated using the Superscript III First Strand Synthesis Kit (Invitrogen) according to manufacturer instructions. RT-qPCR was performed using Power SYBR Green (Invitrogen) on a StepOnePlus qPCR machine (Applied Biosystems) with human proinsulin specific primers: 5'-CGCAGCCTTTGTGAACCAAC-3' (Forward) and 5'- TGGGTGTGTAGAAGAAGCCTC-3' (Reverse). Luciferase signal was normalized to human proinsulin mRNA to account for differences in transfection efficiency.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism software. Data are presented as mean ± SEM, unless otherwise noted, as in Figure 3.2 and Figure 3.8C. Two-tailed Student's t test was used to assess statistical significance, with a threshold for significance of $P<0.05$.

Results

Ero1α rescues wildtype proinsulin in the presence of MIDY mutants

MIDY mutations cause proinsulin to act as a dominant-negative mutant that inhibits wt proinsulin transport through the secretory pathway (7-9). To test the effect of increased Ero1α expression on this dominant-negative behavior (14), we co-transfected INS1E beta cells with wild-type human proinsulin tagged with a myc-epitope (hPro-CpepMyc) plus either wt or mutant mouse proinsulin (mPro). As previously reported (7), mouse mutant proinsulins C(A7)Y or G(B23)V each impaired secretion of co-expressed human wt proinsulin, as measured by human proinsulin specific radioimmunoassay (Figure 3.1). Remarkably, co-transfection of Ero1α in beta cells rescued wt proinsulin in the presence of mutant proinsulins C(A7)Y or G(B23)V (Figure 3.1).

Ero1α directly rescues MIDY mutants

We wished to determine if prevention of dominant-negative behavior involves primarily Ero1 activity on wt proinsulin, or if it might in part be attributed to direct effects on mutant proinsulin molecules. With this in mind, we examined the effect of increased Ero1α expression on the secretion of a variety of MIDY mutant proinsulins expressed in 293T cells in which wt proinsulin is not co-expressed. Remarkably, several proinsulin mutants including G(B8)S and G(B23)V were rescued upon increased expression of Ero1α, while others including L(B11P) and C(A7)Y could not be rescued (Figure 3.2 lanes 6 and 10 versus lanes 7 and 14). These results indicate that increased expression of Ero1α can directly rescue the secretion of a subset of the misfolded proinsulin MIDY mutants.

Rescue of mutant proinsulin by Ero1α and by wt proinsulin are distinct

It is already known that wt proinsulin can dimerize with mutant proinsulin (15) to bring about a partial rescue of a subset of misfolded MIDY mutants (16). It was therefore of interest whether Ero1α-mediated rescue phenocopied the effect of wt proinsulin or whether it was quantitatively and/or mechanistically distinct. Hence, we quantified the extent of proinsulin secretion upon either treatment and asked if combined treatment showed cooperative amelioration. In the presence of wt proinsulin, we observed an approximately three-fold increase in secretion of the mutant proinsulin-G(B23)V (Figure 3.3A, second bar). Independently, we observed an 11-fold increase in proinsulin-G(B23)V secretion by increasing Ero1α expression (Figure 3.3A, third bar). When the two treatments were combined, the resulting increase in proinsulin-G(B23)V secretion was additive (Figure 3.3A, P=0.08 vs. cells expressing only Ero1α), suggesting that the two methods are mechanistically distinct.

Ero1α enhances folding and secretion of the proinsulin-G(B23)V mutant

To examine how increased expression of Ero1α impacts on proinsulin-G(B23)V folding, we co-transfected 293T cells with proinsulin-G(B23)V and Ero1α or empty vector, metabolically labeled newly-synthesized proteins with ^{35}S-amino acids, immunoprecipitated proinsulin, and analyzed the immunoprecipitates by non-reducing Tris-Tricine-Urea-SDS-PAGE. Wt proinsulin can be resolved into four differentially-migrating bands of which the fastest represents native proinsulin, the slowest represents fully reduced proinsulin, and the intermediate bands are

thought to represent incompletely or improperly folded proinsulin disulfide isomers (7). Under reducing conditions, all oxidized/partially-oxidized proinsulin bands shift up to collapse into a single reduced band (Figure 3.3B, lower panel). Under nonreducing conditions, the fraction of proinsulin-G(B23)V that migrated as the native disulfide-bonded form was less than 10% of all molecules (Figure 3.3B lane 2), whereas more than 50% of wt proinsulin had acquired the native state. Importantly, when Ero1α was co-transfected, the fraction of proinsulin-G(B23)V achieving a native-like mobility was increased (Figure 3.3B lane 3, 3'), although it remained less than that seen for wt proinsulin (lane 1). In 293T cells with co-transfection of Ero1α plasmid, the synthesis of labeled proinsulin-G(B23)V appeared to be diminished. This effect was less evident in beta cells and was not correlated with increased ER stress (*see below*) — but may reflect competition between CMV promoters on different plasmids or other mechanisms. However, the decreased synthesis of proinsulin-G(B23)V in 293T cells (Figure 3.3B lane 3) cannot readily explain its increased secretion from the same cells (Figure 3.2 lane 10). Rather, secretory rescue by Ero1α appears to be correlated with increased oxidation of proinsulin-G(B23)V.

Proinsulin-G(B23)V mutant rescue activity by Ero1α mutants ± PDI

Oxidase activity of Ero1α, involving two communicating C394-C397 and C94-C99 active site disulfides, are negatively regulated by oxidation of Cys residues at positions 104 and 131 (17, 18). We utilized a plasmid expressing Ero1α (bearing a C-terminal Myc and 6-His tag, called Ero1αMyc6His) with the regulatory Cys residues mutated to Ala; this construct (herein called Ero1α-Active) has previously been shown to have increased activity compared to Ero1α-WT (17, 19). We also mutated the four active site plus the two regulatory Cys residues in a construct called Ero1α-Hex (Figure 3.4A), which lacks the ability to generate disulfide bonds. Unlike wt proinsulin (Figure 3.4B lane 1), a negligible amount of proinsulin-G(B23)V could be secreted from cells to medium (Figure 3.4B lane 2). However, co-expression of wt Ero1α or Ero1α-Active markedly increased proinsulin-G(B23)V secretion (Figure 3.4B lanes 3 and 5). By contrast, enhanced proinsulin-G(B23)V secretion was not observed upon co-expression of the Ero1α-Hex mutant (Figure 3.4B lane 4) — although when considered as a fraction of total (cell + medium) even Ero1α-Hex promoted a statistically significant increase in fractional secretion of mutant proinsulin (Figure 3.4C).

To determine if rescue of mutant proinsulin by Ero1α correlated with increased oxidation in the ER lumen, we measured proinsulin secretion and ER redox state in two cell lines: Flp-In T-Rex 293 cells with inducible expression of wt Ero1α (18) or Ero1α-Active (17), or in transiently transfected INS1E cells. In Flp-In T-Rex 293 cells, we measured ER hyper-oxidation using the HyPer$_{ER}$ sensor (20), and in Ins1E cells, we used an ER-targeted redox sensitive green fluorescent protein, Grx1-roGFP1-iE (21). By nonreducing SDS-PAGE, Grx1-roGFP1-iE migrates farther when oxidized than when reduced, and the ratio of the two forms allow an estimation of ER redox state. Indeed, under rescue conditions for proinsulin-G(B23)V (Figure 3.5A left), there was enhanced oxidation of HyPer in the ER (Figure 3.5A right). Furthermore, in INS1E cells, wt Ero1α demonstrated a tendency towards increased ER oxidation, and Ero1α-Active produced significant oxidation (Figure 3.5B right) in parallel with increased proinsulin-G(B23)V secretion (Figure 3.5B left).

As part of the canonical ER oxidation pathway (22), Ero1 is thought to transfer disulfide bonds to ER oxidoreductases such as PDI, which can then oxidize substrate proteins. However, Rajpal et al. recently found that in pancreatic beta cells (and 293-derived cells), PDI acts as an "unfoldase" or ER retention factor for proinsulin rather than as an oxidant of proinsulin (2, 23). To determine if PDI enhances or attenuates Ero1α-mediated rescue of mutant proinsulin, we co-expressed flag-tagged PDI with hProG(B23)V-CpepMyc and Ero1αMyc6His. Remarkably, co-expression of PDI significantly impaired rescue of mutant proinsulin secretion in cells with increased expression of wt Ero1α or Ero1α-Active (Figure 3.6 lanes 3-4 and 7-8), indicating that Ero1α -facilitated proinsulin secretion was not due to enhanced PDI-catalyzed oxidation. However, co-expression of PDI had no effect in cells expressing the inactive Ero1α-Hex (Figure 3.6 lanes 5-6). Thus, the activity of PDI to antagonize mutant proinsulin export depends on the presence of active-site cysteines in Ero1α.

Ero1α decreases ER stress activated by Proinsulin-G(B23)V
Expression of hyperactive Ero1α triggers the ER stress response (17), which is likely a result of increased generation of H_2O_2 (24). It was therefore of interest to know if increased oxidation of proinsulin-G(B23)V upon co-expression of Ero1α triggers increased ER stress. To test this, we

utilized a BiP-promoter driven luciferase (BiP-luciferase) reporter (as a readout of ER stress signaling) and normalized luciferase luminescence directly to the mRNA level of proinsulin-G(B23)V that drives the ER stress response (7). Consistent with previous data (7), the G(B23)V mutation triggered a ~2.4 fold increase in BiP-luciferase compared to cells expressing wt proinsulin. However, increased expression of wt Ero1α resulted in a significant diminution of the G(B23)V-mediated increase in BiP-luciferase (Figure 3.7 lane 3); expression of Ero1α-Active also tended to inhibit the G(B23)V-mediated increase in BiP-luciferase (Figure 3.7 lane 5). By contrast, Ero1α-Hex did not decrease the ER stress response induced by proinsulin-G(B23)V (Figure 3.7 lane 4). We conclude that co-expression of active Ero1α does not further compromise ER homeostasis but rather antagonizes proinsulin-G(B23)V-mediated ER stress.

Ero1α directly enhances formation of the proinsulin C(B19)-C(A20) disulfide bond, even in the presence of the G(B23)V mutation

Finally, to pin down where in the proinsulin folding pathway can Ero1α promote proinsulin oxidation, we engineered a proinsulin variant that can form only the B19-A20 disulfide bond. This C(B7)S,C(A6)S,C(A7)S,C(A11)S mutant that we call "KeepB19/A20" also has a myc tag and two Met residues added to the C-peptide to enhance labeling efficiency with ^{35}S-amino acids (Figure 3.8A). When transiently transfected, newly synthesized KeepB19/A20 runs by nonreducing SDS-PAGE as two distinct bands: the slower-migrating reduced band and the faster-migrating oxidized band. Because only two Cys residues remain in the protein, we can be certain that the oxidized band represents formation of the proinsulin B19-A20 disulfide bond. Overexpressing wt Ero1α or Ero1α-Active significantly increased B19-A20 disulfide bond formation (Figure 3.8B nonreduced gel, lanes 2 and 4, quantified below the gel) whereas Ero1α-Hex was without effect (Figure 3.8B, lane 3). Previous reports have suggested that the G(B23)V mutation may impair the kinetics of B19-A20 disulfide pairing (7, 25); we therefore investigated the ability of Ero1α to assist in B19-A20 disulfide bond formation in the presence of the G(B23)V point mutation. Once again, overexpression of wt Ero1α and Ero1α-Active, but not Ero1α-Hex, directly enhanced formation of this critical proinsulin disulfide bond. These data provide potential insight into the mechanism of rescue of MIDY mutants by increased activity of Ero1.

Discussion

The MIDY mutants provide an interesting model to study the effects of proinsulin misfolding on beta cell dysfunction. These mutants are known to operate in a dominant-negative mode, associating with and inhibiting the intracellular transport of co-expressed wildtype proinsulin (7-9, 16) and resulting in eventual ER stress-induced beta cell death (1). A preliminary report has indicated that increased expression of ER oxidoreductin-1 can ameliorate this dominant-negative effect (14). In the current study, we have further explored the benefits of Ero1 expression on proinsulin transport leading to elevated insulin production. Curiously, since oxidative stress has been linked to beta cell dysfunction in diabetes (6, 26), and because Ero1 activity includes H_2O_2 generation that is presumed to be detrimental to beta cells (5), published studies have attempted to improve beta cell health by decreasing Ero1 levels (11, 27). While scavenging cellular reactive oxygen species might improve beta cell insulin secretion under conditions of stress (27), in fact inhibition of Ero1β was found to have the opposite effect(10, 11). Specifically, Min6 cells with disrupted Ero1β expression have decreased insulin content and increased susceptibility to ER stress-induced apoptosis (10). Furthermore, mice with homozygous Ero1β knockout develop diabetes with insulin insufficiency, and mice expressing the MIDY mutant proinsulin-C(A7)Y develop earlier and more severe insulin-deficient diabetes when one Ero1β allele is lacking (11). Thus deficiency in the oxidative capacity of the ER may be more detrimental to beta cell insulin production than potential damage caused by reactive oxygen species generated as a byproduct of Ero1 activity.

In the current study, we have examined the consequences of increased Ero1 levels in cells expressing misfolded proinsulin, using proinsulin-G(B23)V as a model. Our work started out to further understand the improved secretion of wt proinsulin in the presence of misfolded MIDY mutants. We confirmed that increased expression of Ero1α significantly attenuates the defect in endogenous insulin production/secretion in INS1E beta cells exogenously expressing mutant proinsulin-C(A7)Y or G(B23)V (Figure 3.1). Indeed, Ero1α does not at all improve the secretion of proinsulin-C(A7)Y itself (Figure 3.2 lane 14); thus in this instance, beneficial effects of Ero1α are likely explained by actions on the co-expressed wt proinsulin partner. However, to our surprise, we found that increased expression of Ero1α markedly improved secretion of a subset of MIDY mutants including proinsulin-G(B23)V, even in the absence of any co-expressed wt

proinsulin (Figure 3.2 lane 10). These data suggest a more direct effect of Ero1α activity on the mutant proinsulin itself. We have recently found that secretion of proinsulin-G(B23)V is increased in cells co-expressing wt proinsulin (16). Interestingly, many of the same MIDY mutants whose secretion can be enhanced by co-expression with wt proinsulin (16) are among those whose secretion can also be enhanced by increased expression of Ero1α. However, Ero1α exerts a much stronger effect on proinsulin-G(B23)V secretion than does co-expression of wt proinsulin, and in fact, the two treatments together have an additive effect suggesting that each treatment acts to rescue a misfolded proinsulin molecules by different mechanisms. While rescue by wt proinsulin is likely a consequence of cross-dimerization between misfolded and wt partners (16), the mechanism of rescue by Ero1α is unknown, and in this study, we have endeavored to pursue some of the most obvious initial possibilities.

Proinsulin-G(B23)V is a useful model of misfolded proinsulin, because previous work has established that upon reaching a native disulfide bonding pattern, the insulin moiety of this MIDY mutant achieves thermodynamic stability equal to that of wild-type insulin — indeed the defect for proinsulin-G(B23)V is a kinetic barrier in achieving the native disulfide-bonded state (7). In this report, we find that increased expression of Ero1α enhances the fraction of newly-synthesized proinsulin-G(B23)V achieving a disulfide bonding pattern comparable to that of natively folded wt proinsulin (Figure 3.3B). Moreover, in two additional cell culture models, beta cells and 293 cells with inducible Ero1α expression, rescue of misfolded proinsulin by Ero1α was accompanied by increased oxidation of the ER lumen (Figure 3.5). By contrast, Ero1α-Hex, which lacks the ability to promote disulfide bonds via the canonical mechanism, had a markedly decreased ability to rescue misfolded proinsulin secretion (Figure 3.4B, C).

Our data suggest that rescue of proinsulin misfolding by Ero1α can be attributed to improvement of the proinsulin oxidation pathway. However, this oxidation pathway remains poorly understood. The best-studied Ero1 substrate is PDI, and until recently it was assumed that PDI shuttles disulfide bonds to nascent proinsulin. However, new evidence has suggested that PDI actually retards insulin production in beta cells and limits proinsulin intracellular transport (2, 23). Herein we find that co-transfection of PDI attenuates the rescue of misfolded proinsulin provided by wt Ero1α or Ero1α-Active, whereas it had no effect in cells expressing the

catalytically inactive Ero1α-Hex (Figure 3.6). These data suggest that PDI either inhibits the enzymatic activity of Ero1 (which we view as unlikely) or competes with other ER oxidoreductases that are involved in proinsulin folding, or acts directly on proinsulin in a manner that actually limits proinsulin folding and transport. Indeed, a direct physical interaction between PDI and proinsulin in the ER was demonstrated (2). Altogether, the current data clearly support the conclusion that oxidative folding of proinsulin, promoted by Ero1, is not mediated by PDI. Nevertheless, our results suggest the possibility — counter to current thinking (27) — that elevation of ER oxidative capacity (and of Ero1 activity in particular) in beta cells may be a reasonable therapeutic strategy in treatment of proinsulin misfolding. Importantly, as wt proinsulin is prone to misfolding especially when its synthesis is upregulated (3, 4), such agents could be of particular therapeutic value in type 2 diabetes where high-level proinsulin synthesis and ER-stress induced beta cell death prevail. Accordinlgy, we examined ER stress response in INS1E cells expressing proinsulin-G(B23)V and found that both wt Ero1α and Ero1α-Active decreased BiP-luciferase reporter activity, whereas Ero1α-Hex was without effect (Figure 3.7). These data provide further support for the notion that Ero1-mediated oxidation of the ER lumen may actually be beneficial to stressed beta cells (28), especially in light of recent findings that H_2O_2 generated by Ero1 may be readily consumed by concurrent activity of peroxiredoxin-4 to further enhance protein disulfide bond formation (29, 30).

While rescue of oxidative folding of proinsulin molecules that are prone to misfolding is a key contribution of Ero1α, we also note that cells expressing Ero1α-Hex also showed a modest fractional enhancement of proinsulin secretion (Figure 3.4C). Since this mutant lacks the ability to promote substrate disulfide bond formation, it is possible that Ero1α has additional activities and protein partners that influence folding and secretion of proinsulin. Further investigation is needed to explore the magnitude of these effects on secretory protein substrates and to understand the underlying mechanism and significance of Ero1α oxidative and non-oxidative activities.

Finally, we note that not only do the oxidoreductases involved in proinsulin folding remain mysterious, but even whether formation of each of proinsulin's three disulfide bonds is catalyzed remains unknown. To break this problem down to its simplest components, we generated the

KeepB19/A20 construct to study formation of the B19-A20 disulfide bond in the absence of other possible pairings. Unequivocally, both wt Ero1α and Ero1α-Active promote oxidative assembly of the B19-A20 disulfide bond (once again, Ero1α-Hex had no effect) (Figure 3.8B). More importantly, wt Ero1α and Ero1α-Active promote formation of this bond even in the presence of the proinsulin-G(B23)V mutation (Figure 3.8C). These data strongly support a mechanism for how Ero1α assists proinsulin to overcome misfolding: by facilitating formation of the critical stabilizing B19-A20 disulfide bond (31, 32).
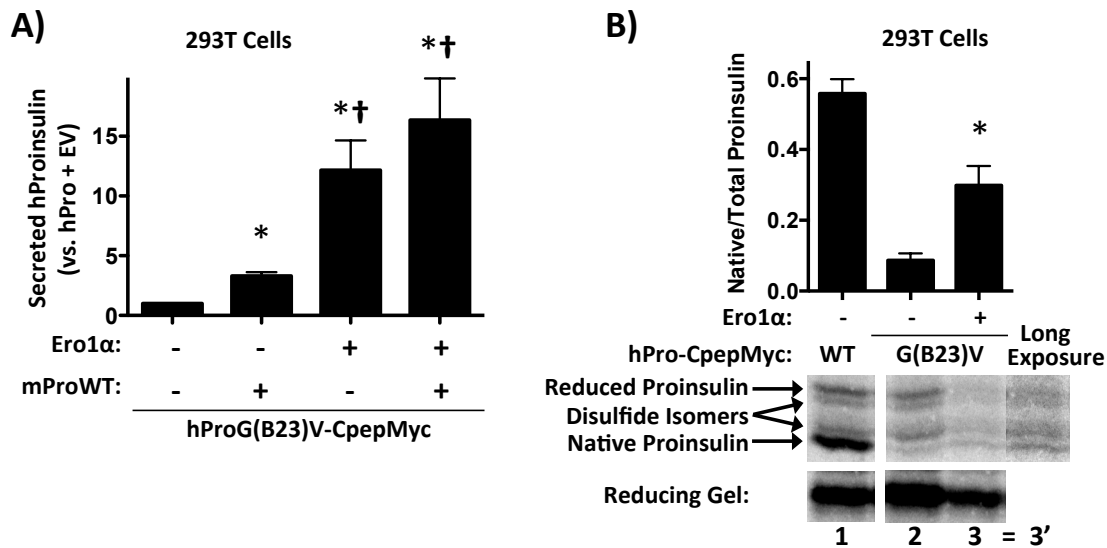
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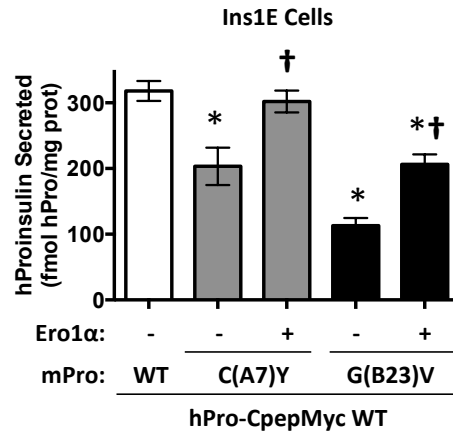

Figure 3.1. Ero1α rescues wildtype proinsulin in the presence of MIDY mutants. INS1E cells were triply transfected with plasmids expressing wt hPro-CpepMyc, the indicated mouse proinsulin mutants, and Ero1α or empty vector. 48 h after transfection, media was changed for overnight collection. Cells were lysed in RIPA buffer, and human proinsulin secretion was measured by human proinsulin specific RIA, normalized to total cellular protein content. Data represent mean ± SEM of three independent transfections. *P<0.05 vs. cells co-expressing wt mPro. †P<0.05 vs. cells untransfected with Ero1α.

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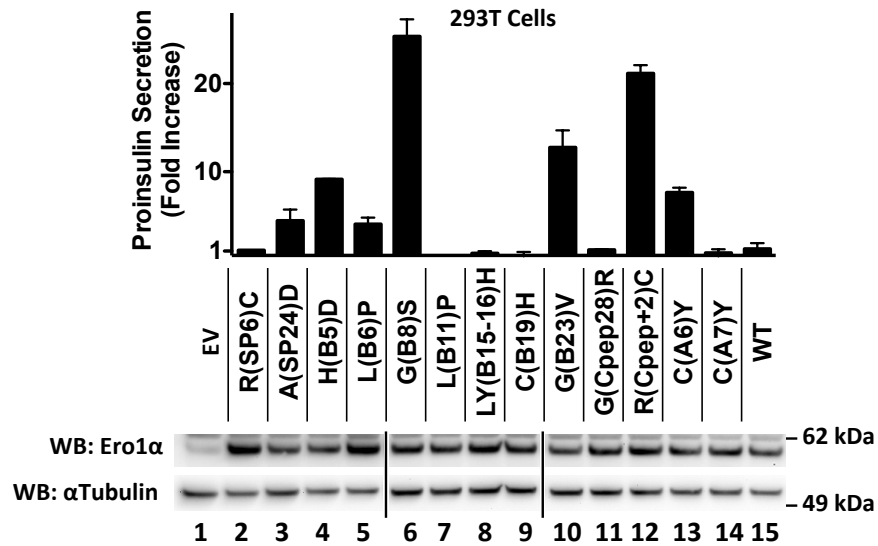
Figure 3.2. Ero1α directly rescues MIDY mutants. 293T cells were transiently co-transfected with plasmids expressing the indicated human proinsulin mutants and Ero1α or empty vector. 24 hours after transfection, media was collected overnight and cells were lysed in RIPA. Proinsulin secretion was measured by human proinsulin specific RIA and normalized to total cellular protein content. Data represent mean ± range of two independent transfections and is expressed as fold increase of secretion from cells co-transfected with Ero1α over secretion from cells co-transfected with empty vector (EV). Immunoblots of cell lysates (bottom panels) using anti-Ero1α antibodies show the level of total Ero1α expression, with α-tubulin used as a loading control. Lane 1 represents cells transfected only with empty vector, and lanes 2-15 represent cells expressing the indicated human proinsulin mutant + Ero1α. Noncontiguous lanes from the same gel were spliced together where indicated.

Figure 3.3. Ero1α enhances folding and secretion of the proinsulin-G(B23)V mutant. (A) 293T cells were triply transfected with hProG(B23)V-CpepMyc ± mProWT ± Ero1α, as indicated. 24 hours after transfection, cell culture media was collected overnight, and cells were lysed in RIPA buffer. hPro secretion was measured by human proinsulin specific RIA, normalized to total cellular protein content. Data represents mean ± SEM of three independent transfections, expressed as fold increase in secretion compared to cells transfected with hProG(B23)V-CpepMyc alone. *$P<0.05$ vs. a value of 1, †$P<0.05$ vs. bar 2. Between bars 3 and 4, $P=0.08$. **(B)** 293T cells co-transfected with wt or G(B23)V hPro-CpepMyc ± Ero1α, as indicated, were pulse-labeled with ^{35}S cysteine and methionine for 30 minutes. After washing cells in ice cold PBS containing 20mM NEM for 5 minutes, cells were lysed in RIPA containing 2 mM NEM. After immunoprecipitation with anti-Myc antibodies, samples were separated by reducing or non-reducing tris-tricine-urea-SDS-PAGE and analyzed by autoradiography. Lane 3' is a longer exposure of lane 3, to allow direct comparison of the band intensity against lane 2. Data represent mean ± SEM of densitometric quantification of native proinsulin band intensity over total proinsulin intensity. n=4, *$P<0.05$ vs. cells untransfected with Ero1α. Noncontiguous lanes from the same gel were spliced together.
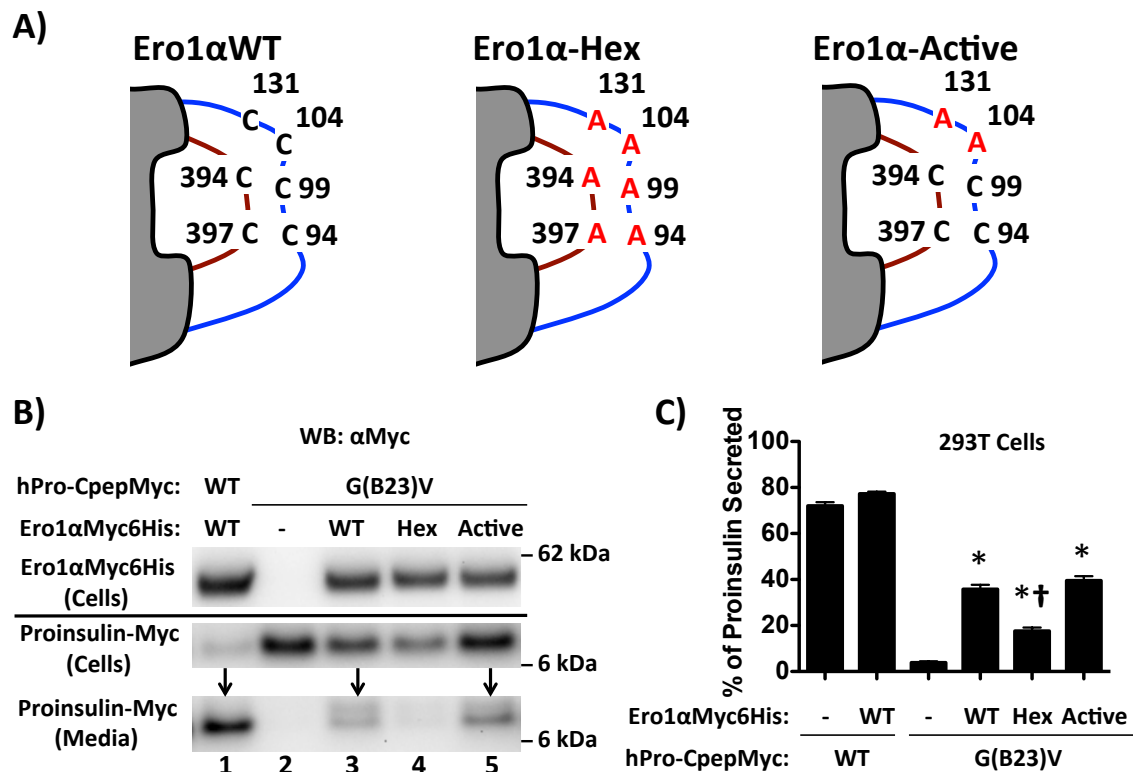
Figure 3.4. Ero1α mutants vary in their ability to rescue proinsulin-G(B23)V. **(A)** Schematic representation of the Ero1α active sites (C94-C99, and C394-C397) and regulatory cysteines (C104-C131) in wt Ero1α, Ero1α-Hex, and Ero1α-Active. **(B)** 293T cells were co-transfected with the indicated hPro-CpepMyc and Ero1αMyc6His plasmids. 24 hours after transfection, cell culture media was collected overnight, and cells were lysed in RIPA buffer. Secretion of hPro-CpepMyc and cellular content of hPro-CpepMyc and Ero1αMyc6His were measured by Western blot analysis using anti-Myc antibodies. Gel loading was normalized to total cellular protein content. **(C)** Data represent densitometric quantification of bands from (B), expressed as media over total (cell + media) band intensity, n=3. *$P<0.05$ vs. cells lacking any Ero1αMyc6His. †$P<0.05$ vs. cells co-expressing wt Ero1αMyc6His.
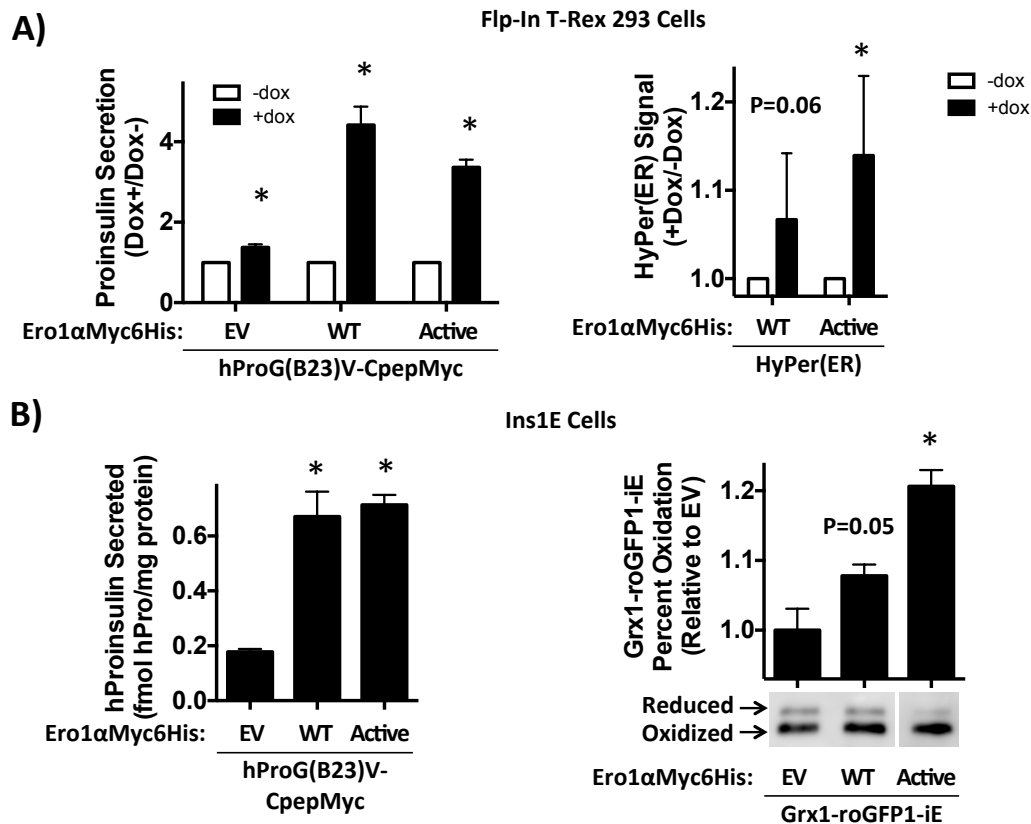
Figure 3.5. Ero1α hyperoxidizes the ER and promotes secretion of proinsulin-G(B23)V in Flp-In T-Rex-293 and Ins1E cells. (A) Stably transfected Flp-In T-Rex-293 cells inducibly expressing empty vector (EV), wt, or Active Ero1αMyc6His were transiently transfected with hProG(B23)V-CpepMyc. 24 hours after transfection, cells were split into 2 separate wells, to be treated with or without 1 ug/ml doxycycline. After 24 hours on doxycycline, cell culture media was collected overnight, and cells were lysed in RIPA buffer. Proinsulin secretion was measured by insulin RIA, normalized to total cellular protein content. HyPer$_{ER}$ oxidation in separately transfected cells was measured as described in Methods**.** Data represents mean ± SEM of three independent experiments, expressed as fold increase of secretion in +dox cells over -dox cells (left panel) or ratio of oxidized over reduced bands relative to –dox cells (right panel). *P<0.05 vs. –dox cells. **(B)** Ins1E cells were co-transfected with hProG(B23)V-CpepMyc and EV or the indicated Ero1αMyc6His variant. 24 hours after transfection, cell culture media was collected overnight, and cells were lysed in RIPA buffer. Proinsulin secretion was measured by human proinsulin specific RIA, normalized to total cellular protein content. Grx1-roGFP1-iE oxidation in separately transfected cells was measured as described in Methods**.** Data represent mean ± SEM from 3-4 independent experiments, expressed as proinsulin secretion normalized to cellular protein (right panel) or ratio of oxidized over reduced bands relative to cells lacking Ero1αMyc6His (right panel). *P<0.05 vs. cells without Ero1αMyc6His.
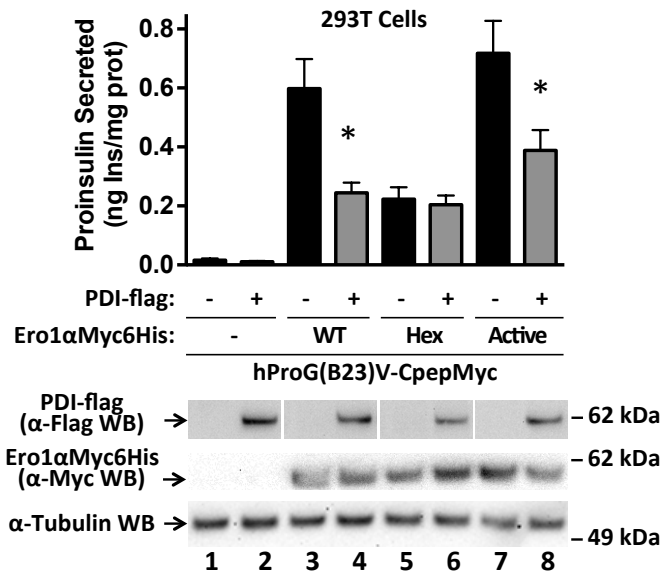
Figure 3.6. PDI antagonizes the ability of Ero1α to promote proinsulin-G(B23)V secretion. 293T cells were triply transfected with hProG(B23)V-CpepMyc with the indicated Ero1αMyc6His mutants with or without PDIflag. After 24 hours, cell culture media was collected overnight, and cells were lysed in RIPA buffer. Proinsulin secretion was detected by insulin RIA, normalized to total cellular protein content. Data represent mean ± SEM from 4 independent experiments. *P<0.05 vs. control cells lacking PDIflag. Ero1αMyc6His was confirmed by anti-Myc immunoblot, with α-Tubulin as a gel loading control.
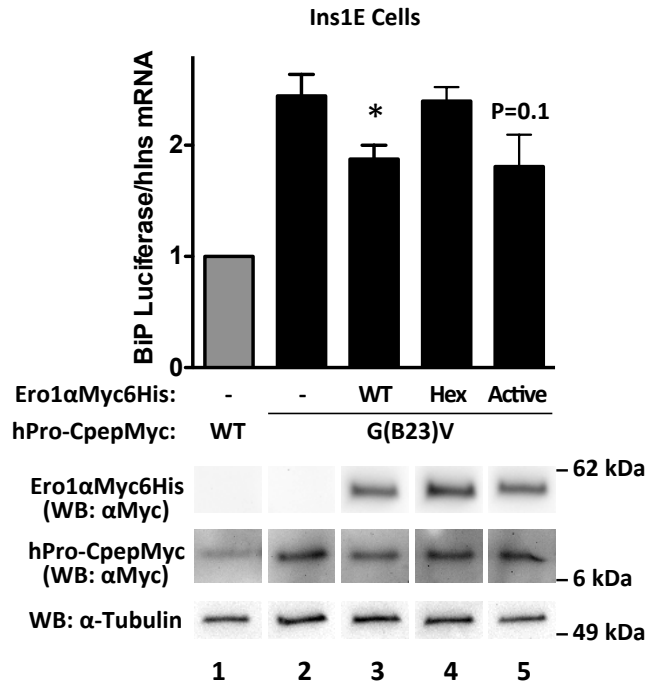
Figure 3.7. Ero1α decreases ER stress activated by proinsulin-G(B23)V. Ins1E cells were triply transfected with plasmids expressing BiP-promoter driven luciferase and the indicated hPro-CpepMyc and Ero1αMyc6His mutants. Transfected cells were split into 3 separate wells, and 48 hours after transfection cells were lysed. Lysates were either used to measure luciferase signal by luminometer, human proinsulin mRNA by RT-qPCR, or hPro-CpepMyc and Ero1αMyc6His content by anti-Myc immunoblot with α-Tubulin as a loading control. Data represent mean ± SEM of 5 independent experiments. *$P<0.05$ compared to cells expressing hProG(B23)V-CpepMyc without Ero1αMyc6His, expressed as fold increase of luciferase signal over human insulin mRNA compared to cells expressing wt proinsulin.
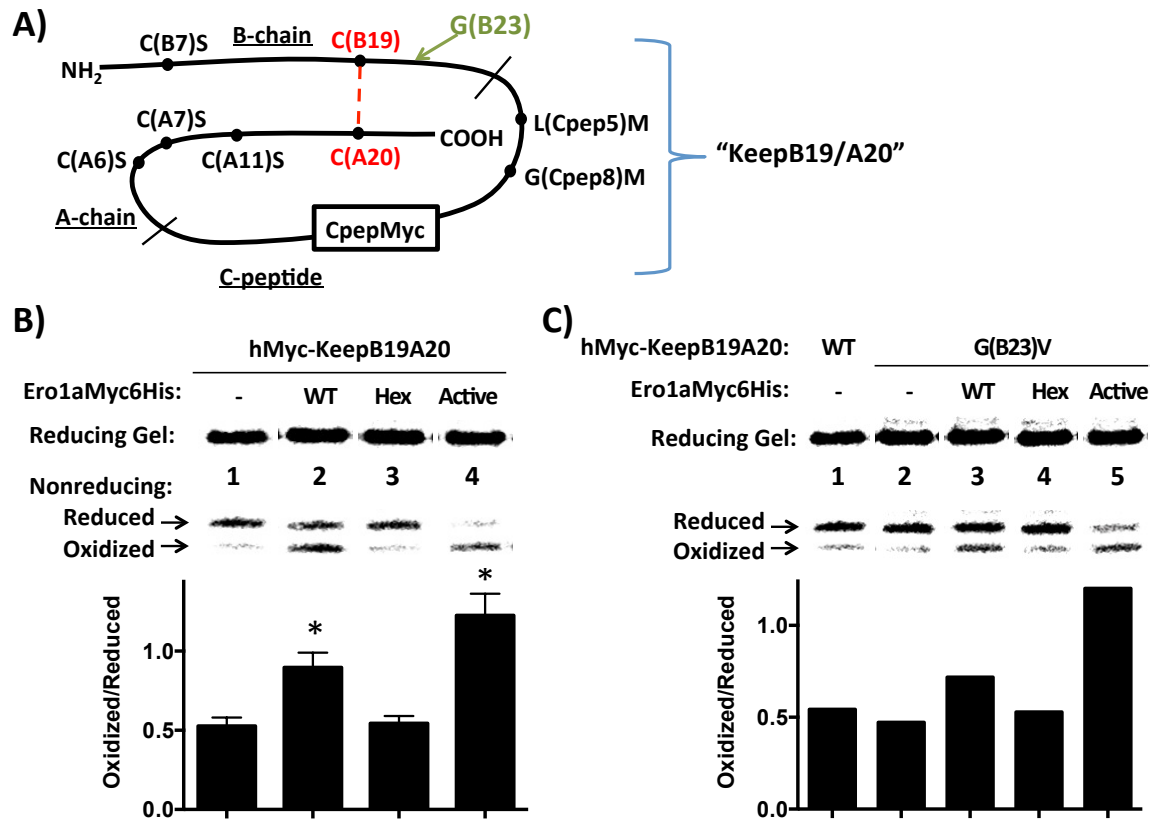
Figure 3.8. Ero1α directly enhances formation of the proinsulin C(B19)-C(A20) disulfide bond. (A) Schematic representation of the KeepB19A20 construct, with the indicated mutations from wt proinsulin indicated. The resulting molecule can make only the C(B19)-C(A20) disulfide bond. (B) 293T cells were co-transfected with KeepB19A20 and the indicated Ero1αMyc6His mutants. After 48 hours, cells were pulse labeled with ^{35}S Cys/Met for 10 min prior to treatment with ice-cold PBS containing 20 mM NEM and lysis in RIPA containing 2 mM NEM. Lysates were immunoprecipitated with anti-Myc antibodies, separate by reducing and non-reducing SDS-PAGE (NuPage), and analyzed by autoradiography. Data represent densitometric quantification of proinsulin bands from 4 independent experiments, expressed as ratio of oxidized over reduced bands. *$P < 0.05$ vs. cells lacking Ero1αMyc6His. (C) 293T cells were co-transfected with wt or G(B23)V KeepB19A20 and with the indicated Ero1αMyc6His mutants. Cells were treated as described in (B). Data represent quantification from 1 experiment.

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Acknowledgements

CHAPTER 4

PERSPECTIVE AND CONCLUSIONS

Introduction

As insulin is the primary hormone responsible for regulating glucose metabolism, insulin synthesis and secretion is critical for health. As discussed in Chapter 1, the insulin precursor proinsulin folds, forms its three intramolecular disulfide bonds, and non-covalently dimerizes prior to exiting the endoplasmic reticulum (ER). After ER exit, it transits through the Golgi complex to secretory vesicles, where it forms hexamers, is endoproteolytically cleaved to mature insulin, and is stored until secretion. Insulin secretion is tightly coupled to blood glucose and is regulated as well by other nutrients and hormones. Mutations in several of the genes involved in insulin synthesis and secretion have been associated with monogenic diabetes, including mutations in *KCNJ11* and *ABCC8* (which encode subunits of the ATP sensitive potassium channel), *GCK* (which encodes glucokinase, the enzyme responsible for the first step of glucose metabolism), and *SCLC2A2* (the Glut2 glucose transporter) (1).

Mutations in the coding sequence of proinsulin itself also cause disease of varying severity, depending on the nature of the mutation. Multiple translational start site or truncation mutants have been identified to cause insulin deficiency leading to autosomal recessive diabetes in humans (2). Other insulin mutations cause milder and later onset diabetes due to decreased insulin receptor binding (3) or impaired endoproteolytic cleavage of proinsulin (4). Studying these "classical insulinopathies" has shed light on insulin processing and insulin receptor binding and their role in physiology and disease.

For my thesis research, I have focused on a new class of insulin mutations responsible for a syndrome termed mutant Ins-gene induced diabetes of youth (MIDY), with the hope that my research will lead to improved understanding of this disease and novel therapeutic options for

this and other forms of diabetes. In addition, as discussed below, there are many diseases of other organ systems that appear to have deep similarity in terms of molecular pathogenesis, and it is not implausible that work done here could have impact entirely outside of the diabetes arena.

Until quite recently, patients with MIDY were diagnosed with very early onset type 1 diabetes. Unfortunately, even though the etiology of their disease is quite different from type 1 diabetes, they receive identical treatment, namely daily blood glucose monitoring and insulin replacement therapy by subcutaneous injection. While such treatment dramatically improves these patients' life expectancy and quality of life, therapy specifically targeting the underlying problem in these diseases could provide improved glycemic control and quality of life. Unlike type 1 diabetes, which is an autoimmune disease, MIDY is caused by mutations in the coding sequence of an allele of the *Ins* gene. The products of this allele are misfolded and retained in the ER, but their pathogenic mechanism is not due solely to the retention of the mutant molecules alone. Rather, the mutant proinsulin molecules exhibit a toxic gain-of-function based on intermolecular interaction with co-expressed wt proinsulin. Due to this interaction, wt proinsulin is also retained in the ER, which is fundamental to decreased insulin secretion and increased ER stress and beta cell death, leading to hyperglycemia and diabetes (5-7).

Thus, MIDY belongs to a growing class of disorders, the secretory protein conformational diseases (8). Multiple therapies for these diseases have been proposed, including pharmacological modification of the rate of protein synthesis to avoid overloading protein folding capacity (9), manipulation of the intraluminal ER ionic milieu (10, 11) or modulation of ER-associated degradation (ERAD) (12); still others involve pharmacologic chaperones (13) or modulators of endogenous ER chaperone activity (14, 15) including pre-emptive induction of unfolded protein response (16). All of these therapies are general in nature, theoretically affecting all secreted proteins similarly. In Chapter 2 of my dissertation, I investigated a novel approach that may help improve proinsulin secretion specifically, rather than generally. In Chapter 3 of my dissertation, I investigated a second approach to improving proinsulin secretion, by enhancing protein oxidation in the ER.

Intermolecular Interactions in MIDY

It has been widely observed that many secreted proteins oligomerize intracellularly, even if they function extracellularly as monomers. This observation is also true for insulin and its precursor proinsulin. In Chapter 2, I studied how the intermolecular interactions between homo-dimerization partners can affect trafficking of protein through the secretory pathway. After observing that some mutant proinsulins are more efficiently secreted in cells that co-express wt proinsulin than in heterologous cells lacking endogenous insulin (5-7), I questioned how dimerization between mutant and wt proinsulin may affect trafficking of both partners. Indeed, we had previously found that mutant proinsulin dimerizes with (17)and impairs trafficking of wt proinsulin (5), but the effect of this intermolecular interaction on the mutant protein had never been investigated. I found that dimerization has two simultaneous effects on the partner molecules. Strangely, at the same time that wt proinsulin is impeded by its interaction with mutant proinsulin, wt proinsulin also improves mutant secretion (Figure 2.7A) by stabilizing the misfolded molecule (Figure 2.5A). Though this rescue by dimerization does not apply to all MIDY mutants (Figure 2.8), remarkably, it may be generalizable to other proteins, since mutant rdw-thyroglobulin (Tg) was rescued by co-expression with wt Tg. Similar to proinsulin, mutant Tg and wt Tg dimerize (Figure 2.2D), and a sub-fraction of dimers is secreted while another sub-fraction is retained, leading to secretory rescue of mutant Tg and blockade of wt Tg (Figure 2.7B). For both proteins (Tg and proinsulin), this effect is specific, since wt Tg failed to rescue mutant proinsulin, and wt proinsulin failed to rescue mutant Tg (Figure 2.4A) Thus, any dimer may either be retained in the ER or be transported forward for secretion (Figure 2.9), and the ratio of expression of either partner seems to play a significant role in determining which result predominates (Figure 2.5B-E)

These results raise two important, unanswered questions about the nature of secretory protein dimers. First, how does dimerization allow secretion of a subset of misfolded mutant protein? As far as we can tell, dimerization does not seem to improve folding of the mutant, so rescue of the mutant must involve some mechanism other than simple enhanced folding. Since the mutant is stabilized, it may be reasonable to hypothesize that dimerization masks typical markers for ER-associated degradation (ERAD), preventing binding to the degradation machinery. Similarly, dimerization may simply prevent binding to ER retention factors, allowing escape from ER

quality control. Though the nature of interactions between proinsulin and many ER chaperones remains unclear, some chaperones, including BiP and PDI, have been proposed as proinsulin retention factors. Overexpression or knockdown of these potential proinsulin retention factors, in conjunction with expression of wt proinsulin as a rescue agent for mutant molecules, could be an interesting starting point in addressing this question. The role of individual chaperones and oxidoreductases in proinsulin folding remains an active area of investigation in our lab, and novel findings could provide new insight into these mechanisms. Alternatively, binding of wt proinsulin to some forward-transport cargo receptor may "pull" the misfolded mutant forward out of the ER, though no proinsulin cargo receptor has yet been identified.

Since a single mutant molecule cannot simultaneously be retained and rescued, a second, related question is what determines the fate of a given dimer. Though dimerization does not necessarily improve folding of the mutant proteins, it is possible that the folding state of the wt molecule at the time of dimerization may influence the decision. In this scenario, a well-folded wt protein would rescue its mutant dimerization partner, while a partially-folded wt protein would be retained by its interaction with the misfolded mutant. Alternatively, the decision between retention and secretion may be entirely stochastic, with wt-wt dimers being almost completely secreted, mutant-mutant dimers being almost completely retained, and mixed dimers having an intermediate chance at either outcome (based on the potential mechanisms outlined above). Though interesting, these conflicting hypotheses are exceedingly difficult to test in living cells, and we have discussed collaborations with experts in mathematical modeling to further dissect the behavior of proinsulin dimers. Furthermore, in vitro co-immunoprecipitation experiments to determine the respective strength of interaction between mutant and wt proinsulins could help elucidate the nature and results of dimerization.

Another intriguing aspect of the rescue/blockade described in Chapter 1 is the genetic inheritance patterns of the two diseases involved. Intriguingly, despite the similarities between the molecular behavior of mutant Tg and proinsulin, the rdw Tg gene is a recessive allele, whereas mutant proinsulin acts dominantly. Thus heterozygous expression of the mutant proinsulin causes early onset permanent diabetes, while patients (or animals) expressing one allele of mutant Tg are phenotypically normal. We speculate that the explanation for this difference lies,

at least in part, in the differences in "rescuability" of each mutant. Rescue of mutant proinsulin required higher wt:mutant ratios than did rescue of mutant Tg. Thus, at the theoretical 1:1 ratio found in heterozygotes, mutant Tg may be more efficiently rescued than mutant proinsulin. Of course, other factors may also contribute, including the ability of the thyroid to grow in response to hypothyroidism, compared to the relatively limited growth potential of adult pancreatic beta cells.

To further investigate this phenomenon in animal disease, we are currently generating a knock-in mouse expressing an inducible Ins2$^{G(B23)V}$ allele. Since mice express two insulin genes, Ins1 and Ins2, these B23V mice are expected to express proinsulin at a wt:mutant ratio of approximately 3:1 (though actual ratio is likely lower since Ins2 expresses more strongly than Ins1 [18]). By crossing these mice to Ins1 knockout mice, Ins2 knockout mice, and compound Ins1/Ins2 knockout mice (which we have in our lab), we will be able to generate mice expressing proinsulin wt:mutant ratios ranging from 0:1 to 3:1. Since the Ins2$^{G(B23)V}$ allele is a myc-tagged human preproinsulin gene, we will be able to distinguish processing and secretion of mutant from wt proinsulins. In conjunction with my results in Chapter 2, I expect mice with low wt:mutant ratios to develop insulin-deficient hyperglycemia earlier and more severely, as well as impaired subcellular trafficking of both wt and mutant proinsulins, compared to mice with high wt:mutant ratios. Differences in onset of diabetes between mice expressing more or less wt proinsulin could potentially be explained simply by relative deficiency of wt insulin secretion. At its most extreme, animals lacking any wt insulin die within 48 hours of birth, so mice with a 0:1 ratio may not contribute to experiments involving blood glucose measurement, but comparison between mice with more moderate ratios may help determine the significance of rescue of mutant proinsulin by wt proinsulin in living animals. Furthermore, by isolating islets from these mice, we will be able to control for external effects such as hyperglycemia to monitor how efficiently mutant and wt proinsulin is trafficked and secreted from these cells.

As a further extension of this project, I would like to use chemical compounds to interfere with intermolecular interactions between proinsulin molecules and observe their effect on secretion and cell health. Using in silico screening of compounds that can fit in the interface between proinsulin dimerization partners, our lab has identified several potential dimerization inhibitors.

I would like to measure wt and mutant proinsulin secretion in the presence of these compounds, to see if preventing dimerization attenuates mutant rescue, improves wt secretion, or affects cell health.

Another tool that would be valuable in continuing this project would be non-dimerizing proinsulin molecules. Indeed, monomeric insulins are used extensively to treat type 1 diabetic patients, due to their improved kinetics of bioactivity. All of these monomeric insulins are artificially synthesized, and their in vivo behavior as proinsulin is unknown. A monomeric proinsulin would allow me to directly test our hypothesis that dimerization is what accounts for secretion of proinsulin-G(B23)V and some other MIDY mutants. Surprisingly though, all of the "monomeric proinsulins" that I have made, including P(B28)D and PK(B28, 29)KP (based on Insulin-Aspart and Insulin-LysPro, respectively), co-immunoprecipitated with wt or mutant proinsulin (Figure 4.1). Though two of the "non-dimerizers" had decreased co-immunoprecipitation and thus may be promising, the fact that the proinsulins based on monomeric insulins do form dimers may suggest that proinsulin dimerization is a critical, unavoidable step in proinsulin maturation. Regardless, our lab continues efforts to generate monomeric proinsulins using crystallographic information about the dimer interface and based on other monomeric insulins, with hopes that this will be a valuable research tool.

ER Oxidation in MIDY

ER stress and activation of the unfolded protein response are often associated with increased oxidative stress (19-21), which may contribute to beta cell dysfunction and cell death in diabetes. Multiple markers of oxidative protein damage have been observed in islets of type 2 diabetics (22, 23), and antioxidant treatment of various rodent models of diabetes ameliorated the development of hyperglycemia and prevented the loss of beta cells (24-27). Nevertheless, the extent to which ER-derived reactive oxygen species, such as the H_2O_2 produced by Ero1, contributes to this oxidative stress remains unclear. In yeast (28) and in *C. elegans* (29), partial attenuation of Ero1 activity is beneficial in the context of pharmacologically-induced ER stress. In mammalian cells, siRNA-mediated knockdown of Ero1α in murine macrophages decreased susceptibility to pharmacologically induced ER stress and apoptosis (30), and knockdown of Ero1β seemed to be cytoprotective in Min6 cells stably expressing the mutant proinsulin-

C(A7)Y (31), though this result seems contradictory to another study finding that Ero1β knockdown in Min6 cells increased phospho-JNK and susceptibility to ER-stress-induced apoptosis (32). Furthermore, a recent study implicated Ero1α activity as a major contributor to cell death induced by tunicamycin (a potent ER stressor) or by forced expression of ATF4 and CHOP (the principle transcription factors believed to be responsible for ER-stress induced apoptosis) (33). Despite a prevailing view that ER-derived reactive oxygen species damage cells, in vivo models have failed to support the hypothesis that Ero1 activity contributes to beta cell dysfunction in diabetes. In fact, mice lacking Ero1β actually develop a diabetic phenotype (i.e., more Ero1β is better) and mice expressing mutant proinsulin-C(A7)Y and only one allele of Ero1β develop diabetes earlier and more severely than mice with two functional Ero1β alleles (31). Thus, while some in vitro evidence suggests that Ero1 activity is detrimental to cell health, in vivo studies seem to suggest the opposite.

Based on the foregoing logic, it is not unreasonable to hypothesize that enhanced Ero1 activity might actually be beneficial to insulin secretion and beta cell health in the context of misfolded mutant proinsulin. In Chapter 3, I investigated how Ero1α overexpression affects mutant and wt proinsulin secretion and ER stress response in beta cells. I found that overexpression of Ero1α improved wt proinsulin secretion in the presence of mutant proinsulin (Figure 3.1). Ero1α also directly improved secretion of a significant subset of the MIDY mutants (Figure 3.2). This rescue of mutant proinsulin is likely due to improved proinsulin oxidation (Figure 3.3), especially of the C(B19)-C(A20) bond (Figure 3.8), though non-oxidative effects might also play a role since a redox-inactive Ero1α mutant also moderately improved secretion of mutant proinsulin (Figure 3.4). Consistent with a previous report (34), Ero1α's activity on proinsulin was not mediated by proinsulin disulfide isomerase (PDI), since PDI overexpression actually antagonized the effect of enzymatically-active Ero1α (Figure 3.6). Importantly, increased Ero1α may be beneficial to beta cell health since mutant proinsulin-induced ER stress response was decreased by Ero1α overexpression (Figure 3.7).

To further advance this work, I would like to better understand the mechanism by which Ero1α overexpression rescues secretion of mutant proinsulin. Clearly, enhanced oxidation contributes to its mechanism, since wt Ero1α and hyperactive Ero1α-Active had significantly more effect

than the redox-inactive Ero1α-Hex. To determine if the effect is predominantly oxidative or non-oxidative, I first would like to determine the oxidation state of the secreted mutant molecules when co-expressed with the Ero1α variants. If the molecules rescued by Ero1α-Hex exhibit enhanced oxidation (as measured by the assay in Figure 3.3B), Ero1α may have a previously unidentified oxidative mechanism, which could be probed using site-directed mutagenesis of other combinations of its 15 cysteine residues (35). Ero1α is known to form homo-dimers, and overexpression of any Ero1α variant may also stabilize and enhance activity of the endogenous Ero1α molecules. This hypothesis could be tested by pulse chase experiments with Ero1α immunoprecipitation, to measure the half-life of newly synthesized Ero1α in the presence or absence of exogenous Ero1α. Furthermore, Ero1α forms complexes with multiple ER oxidoreductases, including PDI. Since these oxidoreductases may act as proinsulin reductases, Ero1α-mediated rescue may involve sequestration of the proinsulin-reducing enzymes, indirectly causing enhanced oxidation. This hypothesis is currently being tested by measuring the ability of Ero1α variants, including Ero1α-Hex, to form complexes with the various ER oxidoreductases. Similarly, non-oxidative effects of Ero1α overexpression may involve sequestration of ER retention factors, independent of their ability to oxidize or reduce proinsulin. Moreover, since Ero1α activity depends on the cofactor FAD to couple oxidation of disulfide bonds with reduction of molecular oxygen (36), Ero1α overexpression may affect other FAD-dependent redox reactions in the cell. Accordingly, I would like to measure FAD, FADH2, and other related metabolites in cells expressing the various Ero1α mutants.

If Ero1α modulation is to become a potential therapeutic option in MIDY and other related diseases, we need to identify small-molecule Ero1α activators that can be tested in cell culture and animal models. A high-throughput assay for Ero1α has already been used to identify pharmacologic inhibitors (37), and a similar approach could be used to screen for activators of both Ero1 isoforms. Furthermore, structural biology may be helpful in identifying candidate compounds. For Ero1α, one of the active site disulfide pairs (C94 and C99) lies on a mobile loop, the position of which is regulated by formation of a regulatory disulfide between C94 and C131 (38), which stabilizes the enzyme's inactive form, preventing oxidation of substrate proteins. The known crystallographic structure of the protein could potentially be used to predict small molecules that would bind Ero1 and prevent formation of this regulatory disulfide,

effectively making a hyperactive Ero1α. Less is known about regulation of Ero1β, though identification of a specific activator of the β form would be beneficial since its expression is limited largely to pancreatic beta cells and some secretory stomach cells (39, 40), so its activation would have more limited effects than the broadly expressed Ero1α.

As discussed above, the notion that increasing oxidation in the ER may be a therapeutic approach in some forms of diabetes runs counter to some current beliefs (27), since the catalytic cycle of Ero1α is coupled to hydrogen peroxide production. Recently, however, an ER oxidoreductase, peroxiredoxin 4 (PRDX4), was identified that couples hydrogen peroxide consumption with de novo disulfide bond formation (41). Other ER resident proteins may also contribute to disulfide bond formation while consuming reactive oxygen species (as discussed in Chapter 1). Therefore, hydrogen peroxide produced by Ero1 may be rendered inconsequential compared to the beneficial effects of improved folding and/or clearance of misfolded proinsulin molecules. With that in mind, I would like to investigate the role that other ER oxidants have on proinsulin oxidation. Multiple pathways, including PRDX4, QSOX, VKOR, and GPX7/8 may contribute to disulfide bond formation in the ER. All of these genes are expressed quite highly in human, mouse and rat islets, as well as Min6 and Ins1 beta cell lines (See Figure 4.2) (42), and may therefore contribute to proinsulin maturation. I would like to overexpress these genes individually in cells expressing mutant or wt proinsulin and observe their affect on proinsulin folding, processing, and secretion and their effect on cell heatlh. The peroxidases (GPX7, GPX8, and especially PRDX4) are particularly interesting as potential targets for improving proinsulin folding in diabetes because of their ability to catalyze hydrogen peroxide. Though the exact source of peroxides for these enzymes remains an active area of investigation (43), stressed cells are likely to generate hydrogen peroxide from multiple sources, including increased ER oxidation and mitochondrial respiration, as well as peroxisome and NADPH oxidase activities. Interestingly, pancreatic beta cells express superoxide dismutases, which convert oxygen radicals to H_2O_2, but lack catalase, the enzyme that typically breaks down H_2O_2 (44). Therefore, the ER resident peroxidases may serve an important dual role, coupling protein folding with diminution of oxidative stress.

Finally, I would like to employ a non-biased approach to identify other genetic modifiers of insulin synthesis and secretion. Since the onset of diabetes caused by some MIDY mutants varies widely (45), other genes are very likely involved in modulating proinsulin folding and a cell's response to misfolded proinsulin. As has been done previously for other secreted proteins (46), I would like to use yeast overexpression and deletion libraries to screen for effects on secretion of mutant and wt proinsulins. After identifying potential genes from the yeasts relatively simple genome, we could then test the effects of their mammalian homologs in mammalian cell lines. This approach could help identify novel therapeutic targets for treatment of secretory protein conformational diseases.

Summary

In this dissertation, I have outlined two distinct mechanisms by which secretion of mutant proinsulin can be improved. In the first mechanism, wt proinsulin dimerizes with the mutant, stabilizes it, and allows it to exit the ER for secretion. In the second mechanism, Ero1α overexpression enhances oxidative folding of the mutant molecule, allowing anterograde transport out of the ER. Interestingly, both of these mechanisms seem to act on separate subsets of molecules, since their effects are additive when combined (Figure 3.3A). Both of these studies point to the potential to enhance ER exit of misfolded proteins as a therapeutic mechanism for secretory protein conformational diseases such as MIDY.
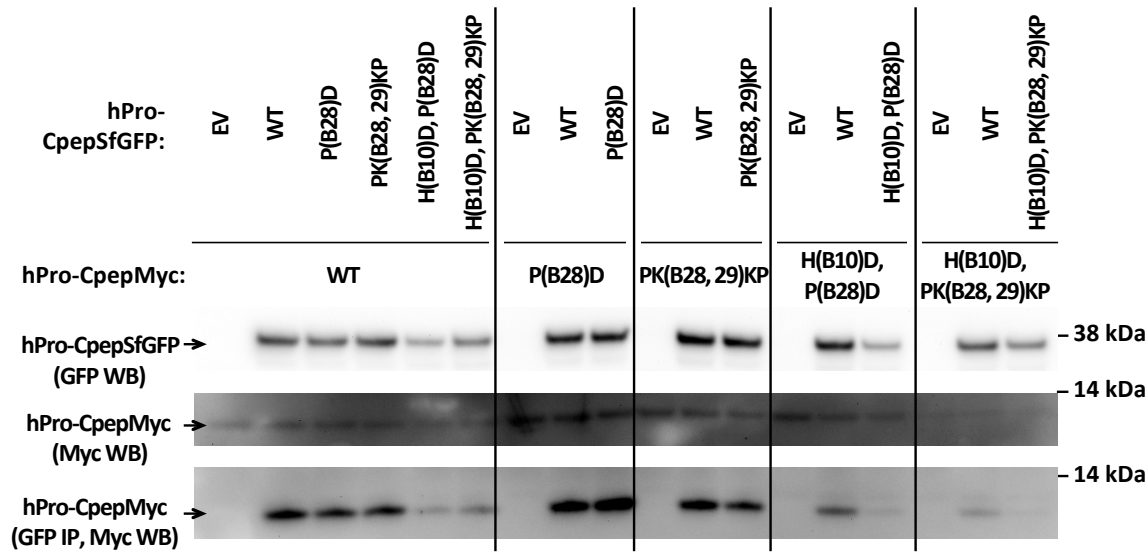
Figure 4.1 Co-immunoprecipitation of "monomeric" proinsulin
293T cells were transiently transfected with the indicated combinations of GFP-tagged (hPro-CpepSfGFP) and myc-tagged (hPro-CpepMyc) proinsulins. Cells were lysed in CoIP buffer (100mM NaCl, 25mM Tris, 0.1% TritonX-100, and 5mM EDTA). Lysates were either immunoprecipitated with anti-GFP antibodies (lowest gel) or directly separated by SDS-PAGE and analyzed immunoblot using GFP or Myc antibodies. The top and middle gels show expression of hPro-CpepSfGFP and hPro-CpepMyc, respectively, and the bottom gel demonstrates co-immunoprecipitation between the proinsulin molecules.
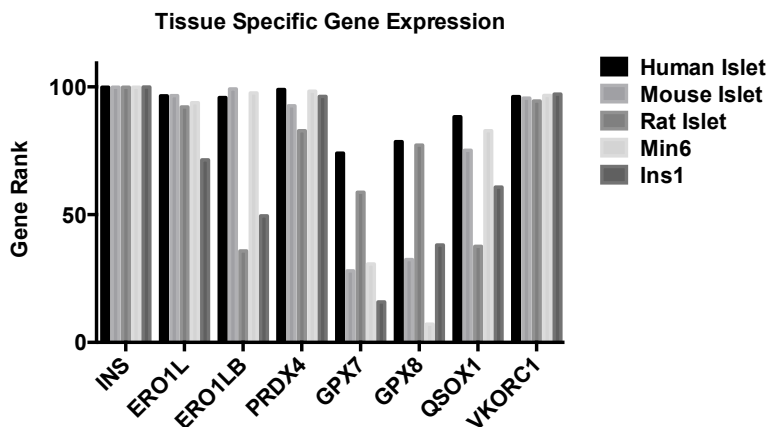
Figure 4.2 Expression of ER oxidases in various tissues and cell lines.
mRNA levels of insulin, Ero1α, Ero1β, Prdx4, Gpx7, Gpx8, QSOX1, and VKOR in human islet, mouse islet, and rat islets, as well as the mouse insulinoma cell line Min6, and the rat insulinoma cell line Ins1. Taken from the Beta Cell Gene Atlas at t1dbase.org(42).

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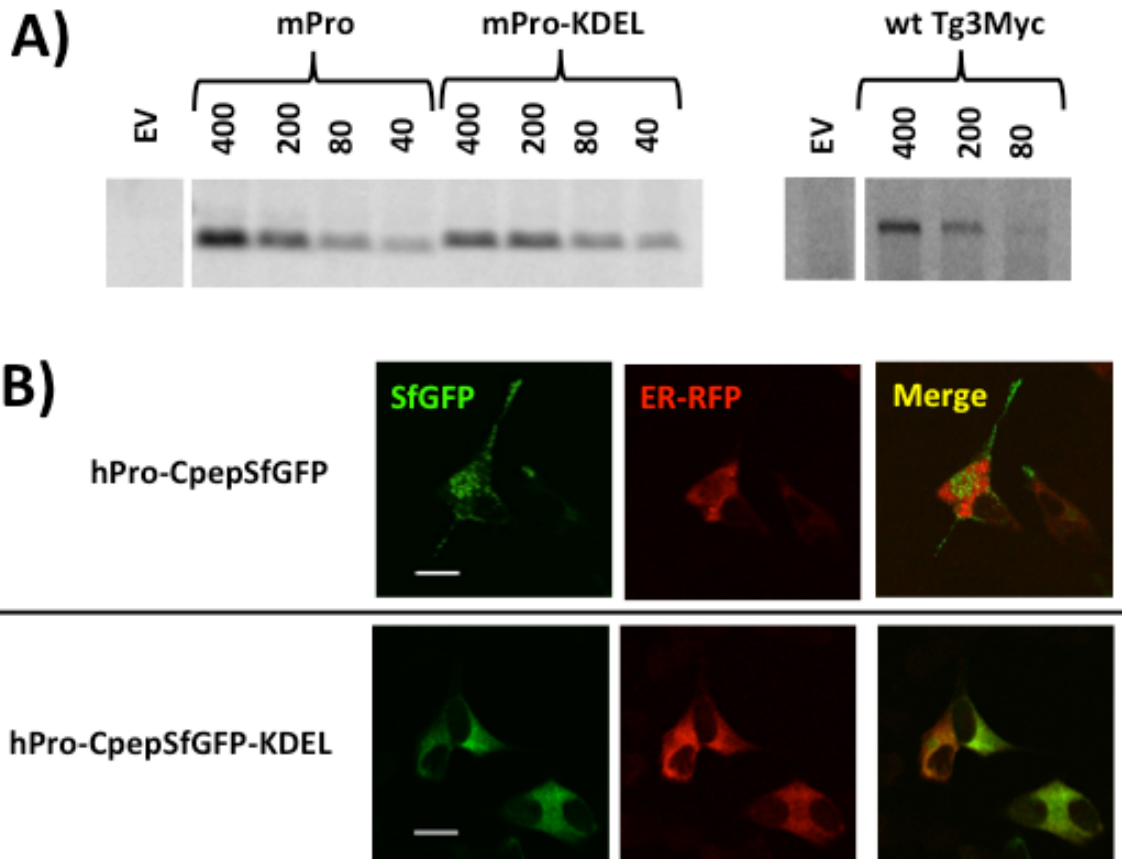
Supplemental Figure S1. Synthesis of proinsulin and thyroglobulin is proportional to the amount of plasmid transfected; a KDEL-tag retains proinsulin in an ER-like compartment. A) 293T cells were transiently transfected in 12-well plates with the plasmids and amounts (ng/well) indicated. The transfected cells were pulse labeled with 35S-Cys for 10 min and lysed in RIPA buffer. Cell lysates were immunoprecipitated with either anti-insulin (left panel) or anti-myc (right panel) and analyzed by reducing SDS-PAGE and autoradiography. EV=Empty Vector B) INS1 cells were co-transfected with ER-RFP (red fluorescence) and either wt hPro-CpepSfGFP (upper) or hPro-CpepSfGFP-KDEL (lower) (green fluorescence), fixed in 4% formaldehyde, and imaged by confocal miscriscopy. Scale bar = 20 mm.
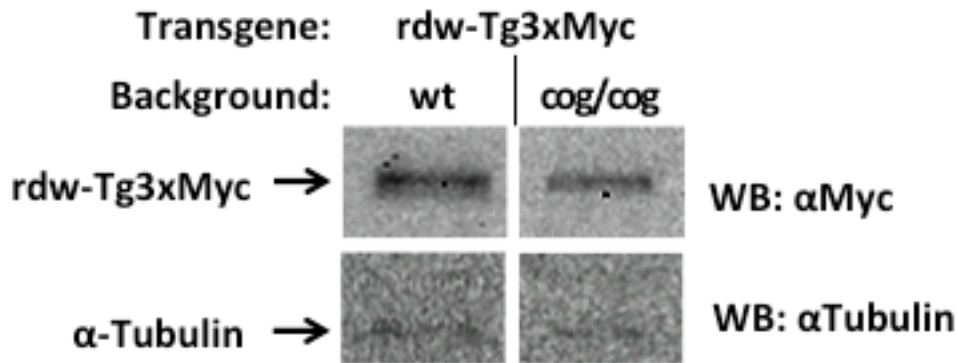
Supplemental Figure S2. Expression of rdw-Tg3xMyc protein in the thyroid glands of rdwTg3xMyc transgenic mice in a C57BL/6-wt or C57BL/6-cog/cog background. Thyroid glands from mice of the indicated genotypes were lysed by sonication in lysis buffer. Lysates, normalized to total DNA content, were analyzed by immunoblotting with anti-myc antibodies.