Dominant protein interactions that influence the pathogenesis of conformational diseases

Jordan Wright,1 Xiaofan Wang,1,2 Leena Haataja,1 Aaron P. Kellogg,1 Jaemin Lee,1 Ming Liu,1 and Peter Arvan1

1Division of Metabolism, Endocrinology and Diabetes, University of Michigan Medical Center, Ann Arbor, Michigan, USA.
2Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, Boston, Massachusetts, USA.

Misfolding of exportable proteins can trigger endocrinopathies. For example, misfolding of insulin can result in autosomal dominant mutant INS gene–induced diabetes of youth, and misfolding of thyroglobulin can result in autosomal recessive congenital hypothyroidism with deficient thyroglobulin. Both proinsulin and thyroglobulin normally form homodimers; the mutant versions of both proteins misfold in the ER, triggering ER stress, and, in both cases, heterozygosity creates potential for cross-dimerization between mutant and WT gene products. Here, we investigated these two ER-retained mutant secretory proteins and the selectivity of their interactions with their respective WT counterparts. In both cases and in animal models of these diseases, we found that conditions favoring an increased stoichiometry of mutant gene product dominantly inhibited export of the WT partner, while increased relative level of the WT gene product helped to rescue secretion of the mutant partner. Surprisingly, the bidirectional consequences of secretory blockade and rescue occur simultaneously in the same cells. Thus, in the context of heterozygosity, expression level and stability of WT subunits may be a critical factor influencing the effect of protein misfolding on clinical phenotype. These results offer new insight into dominant as well as recessive inheritance of conformational diseases and offer opportunities for the development of new therapies.

Introduction
Several human “conformational diseases” of the secretory pathway are caused by mutations in exportable proteins blocking their export from the 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 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 (Tg) (linked to autosomal recessive disease).

Proinsulin, the major protein synthesized by pancreatic β cells, is cotranslationally 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 mutant INS gene–induced diabetes of youth (MIDY), in which secretion of coexpressed WT 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 β 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 compared with that of β cells that express endogenous proinsulin raises the question of whether WT proinsulin could impact transport of the mutant proinsulin-G(B23)V.

Such a question is also interesting when considering Tg, the major protein product of the thyroid gland that serves as precursor for thyroid hormone synthesis. The large Tg primary structure comprises 3 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 dwarf rats 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 coexpressed, 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 this study, we have investigated the selectivity of interactions between 2 ER-retained mutant secretory proteins and their WT counterparts.

Results
Transdominant 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 (18). We also expressed proinsulin(s) bearing a C-terminal KDEL sequence reported to confer proinsulin retention within the ER (19). 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 (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI67260DS1). hPro-CpepSfGFP-KDEL was retained intracellularly in INS1 β cells (Supplemental Figure 1B), primarily in the same compartment as that marked by an ER-RFP, a red fluorescent protein bearing the KDEL retention signal (6).

Figure 1
Proinsulin-KDEL interacts with and inhibits secretion of WT proinsulin. 293T cells transiently transfected with hPro-CpepMyc were cotransfected 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% ± 12.8% (P = 0.003, n = 6) in cells coexpressing hPro-CpepSfGFP-KDEL compared with that in cells coexpressing WT hPro-CpepSfGFP. (B) Cells lysed in TX-ColP buffer were immunoprecipitated with anti-GFP or anti-Myc and resolved by SDS-PAGE, electrotransfer, and immunoblotting with anti-GFP or anti-Myc as indicated. The top 2 rows demonstrate expression of the indicated proteins, and the bottom row demonstrates CoIP. Gels are representative of 3 independent experiments. (C) Cells transiently expressing WT hPro plus TgGFP were cotransfected with plasmids as indicated. The media collected overnight were analyzed by hPro-specific RIA. TgGFP in the same cell lysates and media was analyzed by SDS-PAGE, electrotransfer, and immunoblotting with anti-GFP. The media/cell ratio of TgGFP bands in cells coexpressing mPro-KDEL exhibited no significant change compared with that from cells coexpressing 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 cotransfected with plasmids expressing mPro-KDEL or mPro. Media were collected overnight, and cell lysates were analyzed by hPro-specific RIA. The data in C and D represent mean ± SEM, each from ≥4 independent transfections. *P < 0.05.
greatly decrease the secretion of WT Tg3xMyc (Figure 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 coexpressed rdw-Tg was recovered in an endo-

Figure 2

Cross-dimerization of mutant/WT proinsulin and mutant/WT Tg. 293T cells were transiently cotransfected with plasmids expressing the indicated proinsulin or Tg variants. (A) At 48 hours after transfection, cell lysates and overnight media were collected; both were immunoprecipitated with anti-Myc (to prepurify the antigen) and then analyzed by SDS-PAGE, electrophoresis, and immunoblotting with anti-Myc. The media/cell ratio of hPro-CpepMyc bands from cells coexpressing mPro-G(B23)V decreased 66.1% ± 1.9% (P < 0.001, n = 4) compared with that of WT mPro. (B) At 48 hours after transfection, cell lysates and overnight media were collected, treated with or without EndoH, and were analyzed by immuno-

WT mutant cross-dimerization offers secretory rescue to the mutant gene product. In INS1 cells, WT hPro-CpepSFGF efficiently reached secretory granules and was well secreted, whereas hProC(A7)Y-CpepSFGF (which harbors the same mutation as that found in the Akita mouse) was retained in the ER (Figure 3A) rather than being secreted (Figure 3A) (20). Interestingly, however, recombinant hProG(B23)V-CpepSFGF 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-CpepSFGF in the mouse pituitary cell line AtT20 (which forms secretory granules containing ACTH but does not express proinsulin). In this cell line, WT hPro-CpepSFGF colocalized with endogenous ACTH in secretory granules that accumulate at the distal tips of cellular processes (Figure 3B). When expressed by itself, hProG(B23)V-CpepSFGF exhibited primarily an ER distribution and did not reach secretory granules (Figure 3B). However, when coexpressed with WT hPro-CpepMyc, the hProG(B23)V-CpepSFGF was partially rescued, becoming visible in secretory granules (Figure 3B). 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 cotransfected with fixed equimolar amounts of hProG(B23)V-CpepMyc and rdw-TgGFP and simultaneously cotransfected with either WT mPro or WT Tg (empty vector was included to keep constant the total DNA in each transfection). Secretory rescue of hProG(B23)V was provided selectively by WT mPro (Figure 4A, top; confirmed by immunoblotting in Figure 4B) but not by WT Tg (Figure 4A, top), as measured by hPro-specific RIA. Conversely, rescue of rdw-TgGFP secretion was conferred upon coexpression of WT Tg (17) but not by WT mPro (lane M, Figure 4A, bottom). 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 4C). Thus, not only can an ER-retained dimerization partner impair secretion of its WT counterpart (Figures 1 and 2), but expression of a WT dimerization partner augments secretion of its misfolded counterpart (Figures 3 and 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 35S-amino acids and measured the fraction of newly synthesized protein remaining at 20 hours after synthesis. hProG(B23)V-CpepMyc stability was significantly increased by coexpression of WT mPro (Figure 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 5B). Rescue of hProG(B23)V-CpepMyc also was observed with increasing concentrations of coexpressed WT mPro (Figure 5C).

Figure 3
Intracellular distribution of mutant proinsulins in regulated secretory cells coexpressing or not coexpressing WT proinsulin. (A) Cultured INS1 pancreatic β 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 (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. 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 cotransfected 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 (data not shown). Enrichment of average GFP intensity in the secretory granule region was compared with average GFP intensity in non-granule regions. Data represent mean ± SEM from 30 to 38 separately imaged cells for each of the 3 respective transfection conditions. *P < 0.05.

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 when the amount of WT Tg3xMyc was raised (Figure 5D).
We also wished to determine whether 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 (22), with deletion of either one allele or homozygous knockout of endogenous Ins2 to progressively decrease WT proinsulin expression. We then used mPro-specific immunofluorescence to examine the intracellular distribution of the remaining endogenous (primarily Ins1) gene product. In WT, Ins2<sup>−/−</sup>, and Ins2<sup>−/−</sup> mice lacking the mutant transgene, most pancreatic β cells exhibit strong proinsulin immunostaining in the Golgi region, consistent with previous reports (20, 23). However, in the presence of the mutant hProC(A7)Y-CpepGFP (which itself is entrapped in the ER; ref. 22), more cells began to appear with endogenous mPro in an ER-like pattern (colocalizing with calnexin; Figure 6B, top 2 rows). There is published evidence that endogenous proinsulin synthesis/secretion is heterogeneous in the population of islet β cells (24, 25). Nevertheless, in Ins2<sup>−/−</sup> 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 6B). These findings demonstrate that, in tissues from actual conformational disease models, both mutant secretory protein rescue (Figure 6A) and WT secretory protein blockade (Figure 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 mPro and hPro secretion by species-specific immunoassay. Secretion of WT mPro was significantly diminished when the ratio favored the presence of the mutant hProG(B23)V (Figure 7A). Remarkably, from the same cells, hProG(B23)V secretion was improved by the coexpression of WT mPro (Figure 7A). By contrast, in thyroid tissue of WT Ins2<sup>+/−</sup> mice lacking the mutant transgene, secretion of WT mPro was significantly diminished (Figure 7B), whereas WT Tg3xMyc was markedly improved (Figure 7B), whereas WT Tg3xMyc was markedly diminished when the ratio favored hProG(B23)V secretion (Figure 7B). Intermediate ratios showed both rescue and blockade (Figure 7B). Both sets of data in Figure 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

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**Figure 4**

Cross-dimerization as a basis for secretory rescue of mutant proinsulin or Tg is specific to respective WT partners. (A) 293T cells transiently expressing both mutant proinsulin and mutant Tg were cotransfected with either WT mPro or WT Tg. The media were collected overnight, and cells were lysed; proinsulin secretion was quantified by hPro-specific RIA. Data represent mean ± SEM relative to cells lacking mPro or WT Tg (P = 0.07, n = 3). From the same cell lysates and media, secretion of rdw-TgGFP was analyzed by SDS-PAGE, electrotransfer, and immunoblotting with anti-GFP. (B) At 48 hours after cotransfection, overnight secretion of mutant hProG(B23)V-CpepMyc (in duplicate) was measured by immunoprecipitation and immunoblotting with anti-Myc. The results shown in A and B are representative of 3 separate experiments. Empty vector, EV. (C) At 48 hours after transfection, cells cotransfected as indicated were either untreated or treated with 5 μg/ml brefeldin A (BFA). After 5 hours, the media were collected and analyzed by hPro-specific RIA. The data shown are mean values ± range from 2 independent measurements.
natural cysteine partners (4). To see which class of mutants could be rescued by coexpression of WT proinsulin, we compared secretion of a series of coexpressed 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 coexpression of WT proinsulin (Figure 8). By contrast, secretion of several other misfolded MIDY proinsulins [e.g., A(SP24)D, H(B5)D, G(B8)S] was 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 1B and Figure 2C) or Tg (Figure 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 (26), and degradation of exportable proteins (27), 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 (28), others involve manipulation of the intraluminal ER ionic milieu (29, 30) or modulating ER-associated degradation (ERAD) (31), and still others involve pharmacologic chaperones (32) or modulators of endogenous ER chaperone activity (33, 34), including preemptive induction of unfolded protein response (35). Each of these therapies is 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 (36). 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 (37). Oligomerization limits chaperone rebinding and helps to relieve ER entrapment (38, 39), often by limiting exposure of unpaired Cys residues that can frequently be associated with ER retention (40–42). Typically, monomer folding precedes oligomerization (43), but there are examples in which oligomer formation may be a very early folding step (44). Either way, for many exportable proteins, achieving an oligomeric state is a critical decision point in determining anterograde transport versus ERAD (45).

It has been shown that expression of misfolded mutant proteins has the potential to cause WT (bystander) dimerization partners to be retained in the ER (5, 18, 46–49). Protein-specific rather than general enhancement — lowering the levels of the mutant partner (50) 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 (51, 52). In this 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 1 and 5–7). In this

Figure 6
Rescue of mutant Tg and blockade of WT 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 posttranslational iodination were labeled by incubation of thyroid lobules with 1.0 μCi/μl Na125I for 30 minutes, as described in Methods. The thyroid lobules were then lysed and immunoprecipitated with anti-Myc. The immunoprecipitates were either mock-digested or digested with EndoH, as in Figure 2B, and then analyzed by SDS-PAGE and autoradiography. *P < 0.05. (B) Pancreata from 6-week-old mice, with the genotypes indicated, were fixed in paraffin, sectioned, deparaffinized, and immunostained with antibodies specific to mPro (red) and calnexin to mark the ER (green). From confocal microscope images (scale bar: 10 μm), a blinded reader scored the localization of WT mPro in each β cell as either a predominant juxtanuclear crescent of increased intensity (Golgi, consistent with previous reports, refs. 20, 23; e.g., see arrows) or mainly colocalized with calnexin (ER; e.g., see arrowheads). Quantitation of these data is shown as mean ± SEM from n = 5 mice with 5 islets per mouse. BG, blood glucose. *P < 0.05.
Moreover, expressing the MIDY (Figure 9), producing insulin deficiency from the WT allele proinsulin appears to account for the dominant inheritance of (Figure 1C and data not shown). This dominant effect on WT neither associating with nor blocking WT Tg in the same cells (Figure 2C) and blocks secretion of that partner (Figure 2A) while be nonspecific (54), we have found that expression of the proinsulin formation of misfolded protein complexes was once thought to retention of the misfolded gene product is the rule (53). While on a similar underlying cell biological principle: in the case of blockade, demonstrating specificity (Figure 1C). We have every WT Tg secretion continues unimpeded in the face of proinsulin to allow escape from this blockade (Figure 1D). In the same cells, addition of WT proinsulin competes with the proinsulin-KDEL cogle/cog mice, rdw-Tg3xMyc acquired endoH resistance and even became iodinated in animals making endogenous WT Tg (Figure 6A). Moreover, rescue is also observed for proinsulin mutations that are ordinarily transmitted with autosomal dominant inheritance. In pancreatic β cells, partial rescue of proinsulin-G(B23)V to secre- tion of the 2 protein partners) results in progressive blockade of remaining WT proinsulin, independent of changes in random blood glucose (Figure 6B).

Despite an autosomal recessive pattern of inheritance, we found surprisingly similar molecular behavior for the genetically unrelated Tg 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 2D). Remarkably, we found that expression of the recessive rdw-Tg can also dominantly block export of its WT Tg partner (Figure 2B), and this phenotype is linked to lowering the WT/mutant protein ratio (Figure 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 tissues of mice expressing a mutant rdw-Tg3xMyc transgene in a WT genetic background: unlike in cog/cog mice, rdw-Tg3xMyc acquired endoH resistance and even became iodinated in animals making endogenous WT Tg (Figure 6A). Moreover, rescue is also observed for proinsulin mutations that are ordinarily transmitted with autosomal dominant inheritance. In pancreatic β cells, partial rescue of proinsulin-G(B23)V to secretory granules was already in evidence (Figure 3A), conferring enhanced secretion under both basal and stimulated conditions (Figure 5E and ref. 6), and such an effect can be directly attributed to coexpression of WT proinsulin (Figure 3B). Moreover, rescue of mutant proinsulin-G(B23)V or rdw-Tg is also protein specific: in cells coexpressing both mutants, secretory rescue of report, we have studied proinsulin mutants causing MIDY and Tg mutants causing congenital hypothyroidism as 2 genetically unrelated representatives of a broad class of congenital 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 β 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 this 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 1, A and C) in conjunction with direct physical association between the partners (Figure 1B). Also, further addition of WT proinsulin competes with the proinsulin-KDEL to allow escape from this blockade (Figure 1D). In the same cells, WT Tg secretion continues unimpeded in the face of proinsulin blockade, demonstrating specificity (Figure 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 (53). While formation of misfolded protein complexes was once thought to be nonspecific (54), we have found that expression of the proinsulin-G(B23)V mutant selectively coprecipitates its WT partner (Figure 2C) and blocks secretion of that partner (Figure 2A) while neither associating with nor blocking WT Tg in the same cells (Figure 1C and data not shown). This dominant effect on WT proinsulin appears to account for the dominant inheritance of MIDY (Figure 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 with homozygous loss of endogenous Ins2 (i.e., changing the relative expression of the 2 protein partners) results in progressive blockade of remaining WT proinsulin, independent of changes in random blood glucose (Figure 6B).
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proinsulin-G(B23)V was accomplished exclusively by WT proinsulin, whereas secretory rescue of rdw-Tg was accomplished exclusively by WT Tg (Figure 4).

All evidence points to the idea that secretory rescue is a consequence of intracellular stabilization of the mutant gene product: this was true for both proinsulin-G(B23)V (Figure 5A) and rdw-Tg (Figure 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 5, C and D). Most remarkable of all, rescue (of mutant) and blockade (of WT) secretion occurred simultaneously in the same cells (Figure 7). Thus, for both proteins, there is a dynamic bidirectional balance between retention and anterograde transport of mutant cross-dimers (Figure 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 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 required higher WT/mutant ratios than the rescue of rdw-Tg secretion (Figure 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 Tg (55), 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 for 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 (56, 57), and its accumulation may contribute to β cell failure (58, 59). 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.

Methods

Materials. Lipofectamine 2000, 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-Aldrich. EndoH was from New England Biolabs. Complete protease inhibitor cocktail was from Roche. Citrisolv was from Fisher Scientific. Rabbit anti-Myc and 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 M. Low (University of Michigan). hPro-specific RIA was from Millipore. mPro-specific ELISA and mPro-specific antibody was from Alpco. Trans35S label and Na125I 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, 10 mM HEPES, penicillin (100 U/ml), streptomycin (100 μg/ml), and 50 mM 2-mercaptoethanol. Proinsulin and Tg variants were expressed in 293T cells transiently cotransfected with plasmids expressing the indicated hPr and either empty vector or WT mPro were incubated overnight in growth medium beginning at 24 hours after transfection. Media were collected, and hPro secretion was measured by RIA. The data shown (fold increase in mutant proinsulin secretion as a consequence of expressing WT mPro over empty vector) are mean values ± range from 2 independent experiments. X, undetectable.
pCDNA3.1 or pTarget mammalian expression vectors. The hPro-CpepSf-GFP-KDEL vector was a gift from E. Snapp (AEOM). 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 hours after transfection and lysed in 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 protease 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 minutes. Fresh basal media (2.8 mM glucose) was then collected for 90 minutes, followed by stimulation media (21 mM glucose, 1 mM tolbutamide, 1 mM IBMX) for 90 minutes, 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 heterozygous/homozygous disruption of endogenous Ins2, were as previously described (22). A full description of rdw-Tg3xMyc transgenic mice is forthcoming: briefly, a transgene consisting of the bovine Tgn promoter (60) 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 CS7BL/6 mice. These rdw-Tg3xMyc transgenic mice in a Tgn+/+ background were crossed and then backcrossed with Tgn+/cog mice to generate Tgn+/+ mice and then Tgn+/- mice with or without the rdw-Tg3xMyc transgene. 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 permeabilized 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 (1:5,000 dilution) and rabbit anti-ACTH (1:25,000). Thereafter, slides were rinsed and incubated with secondary antibody conjugates, mounted with ProLong Gold with DAPI and by confocal epifluorescence as above. Handling and euthanizing of mice were performed in accordance with the Committee on Use and Care of Animals at the University of Michigan.

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Address correspondence to: Peter Arvan, Division of Metabolism, Endocrinology and Diabetes, University of Michigan, Brehm Tower Rm 5112, 1000 Wall St., Ann Arbor, Michigan 48105, USA. Phone: 734.936.5505; Fax: 734.936.6684; E-mail: parvan@umich.edu.

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