Altered β-Cell Prohormone Processing and Secretion in Type 1 Diabetes

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Analysis of data from clinical cohorts, and more recently from human pancreatic tissue, indicates that reduced prohormone processing is an early and persistent finding in type 1 diabetes. In this article, we review the current state of knowledge regarding alterations in islet prohormone expression and processing in type 1 diabetes and consider the clinical impact of these findings. Lingering questions, including pathologic etiologies and consequences of altered prohormone expression and secretion in type 1 diabetes, and the natural history of circulating prohormone production in health and disease, are considered. Finally, key next steps required to move forward in this area are outlined, including longitudinal testing of relevant clinical populations, studies that probe the genetics of altered prohormone processing, the need for combined functional and histologic testing of human pancreatic tissues, continued interrogation of the intersection between prohormone processing and autoimmunity, and optimal approaches for analysis. Successful resolution of these questions may offer the potential to use altered prohormone processing as a biomarker to inform therapeutic strategies aimed at personalized intervention during the natural history of type 1 diabetes and as a pathogenic anchor for identification of potential disease-specific endotypes.

The pancreatic β-cell integrates humoral, metabolic, neural, and paracrine inputs to regulate the production, release, and processing of the hormones insulin and islet amyloid polypeptide (IAPP) (1). In type 1 diabetes, immune-mediated death and dysfunction of pancreatic β-cells lead to reduced circulating levels of insulin and IAPP. The physiological role of insulin as a critical regulator of carbohydrate metabolism is well established. Whereas the biological function of IAPP is not fully understood, studies suggest that it may act centrally to suppress food intake and gastric emptying; and within the islet, IAPP may suppress glucagon secretion (2). Both insulin and IAPP are synthesized as prohormones that undergo sequential processing in the secretory pathway within the β-cell (1,3) (Fig. 1). Multiple studies have identified evidence of increased proinsulin or proIAPP relative to mature insulin or IAPP expression, either in circulation or in the islet, at various stages in the natural history of type 1 diabetes (4–8). For years, conventional dogma suggested that by the time of clinical onset, a large majority of β-cell mass (i.e., 85–90%) has been destroyed, with ultimate progression to complete loss of insulin and IAPP. However, large cohort studies have shown that many individuals continue to secrete low levels of C-peptide for years after type 1 diabetes diagnosis (9). Recent studies have also highlighted persistent secretion of incompletely processed

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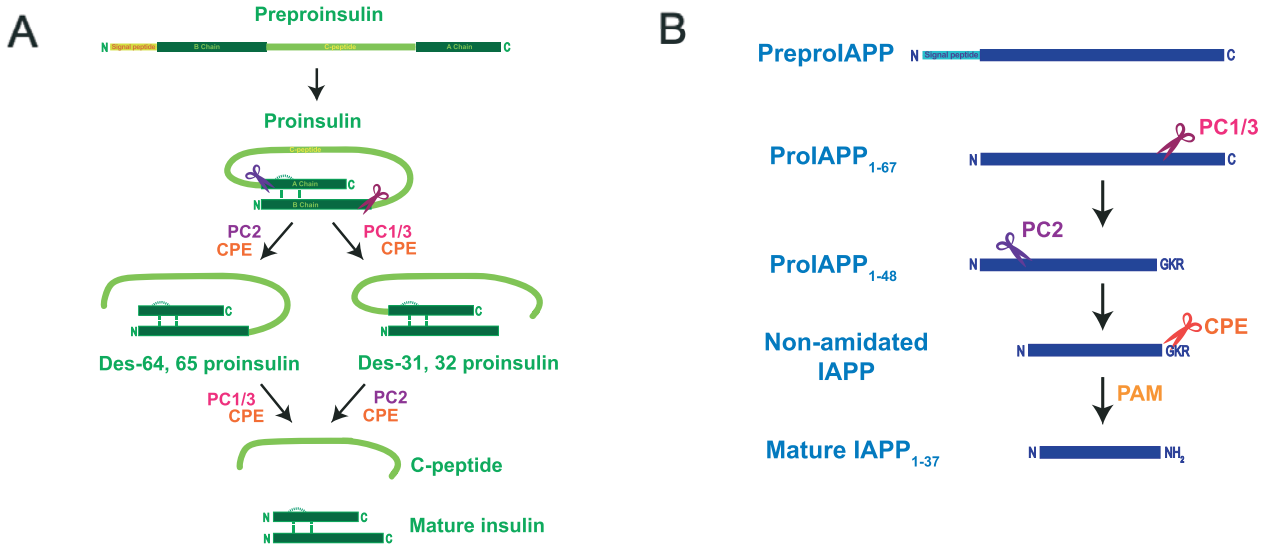

Figure 1—Processing of proinsulin and processing of proIAPP follow similar steps. Insulin and IAPP follow similar pathways of biogenesis, and processing occurs through a mostly common repertoire of enzymes located within the secretory pathway of the β-cell (1,3). *A*: Insulin production begins with translation of preproinsulin mRNA to form preproinsulin, which is a polypeptide that contains an N-terminal signal peptide, A and B chains, and a connecting C-peptide and is 100 amino acids in length. Preproinsulin is subsequently translocated into the lumen of the ER, where the N-terminal signal peptide is cleaved and three disulfide bonds are formed between the B and A chains to yield proinsulin. Proinsulin is transported to the Golgi complex and is sorted into immature secretory granules. Within the secretory granules, proinsulin is proteolytically processed to form C-peptide and the mature insulin molecule. Proteolytic processing of proinsulin is the result of sequential cleavage steps by prohormone convertase 1/3 (PC1/3), prohormone convertase 2 (PC2), and carboxypeptidase E (CPE). The classically described pathway of proinsulin processing involves an initial cleavage by PC1/3 at the junction of the B chain and C-peptide (on the C-terminal side of two basic amino acids, Arg^{31} and Arg^{32}), forming split 32,33 proinsulin. CPE trims dibasic residues at the C-terminal end of the split forms to yield des-31,32 proinsulin, followed by cleavage at the junction of the A chain and C-peptide by PC2 and trimming by CPE to yield insulin and C-peptide. Processing may also begin with PC2 cleavage (C-terminal to Lys^{64}–Arg^{65}) to form split 65,66 proinsulin. CPE trimming yields des-64,65 proinsulin, which is cleaved further by PC1/3 to form insulin and C-peptide. Ex vivo studies as well as analysis of circulating forms of proinsulin in humans, revealing higher levels of the des-31,32 proinsulin intermediate in comparison with des-64,65 proinsulin, support a model where initial cleavage by PC1/3 is strongly favored. *B*: Similar to preproinsulin, the signal peptide of preproIAPP is removed, creating the 67-residue IAPP precursor proIAPP. The C-terminal end of proIAPP is first cleaved by PC1/3 and CPE to produce the $proIAPP_{1–48}$ intermediate, which is then cleaved at its N-terminal end by PC2. The C-terminus of IAPP is amidated by peptidyl α-amidating monooxygenase (PAM). Alternative processing of proIAPP may occur in states of β-cell dysfunction, and the plasma $proIAPP_{1–48}$–to–total IAPP ratio is elevated in subjects with type 1 diabetes (6). Recent analyses have suggested that among human β-cells, PC1/3 may be more critical than PC2 for β-cell proinsulin processing, although impacts on human proIAPP processing remain to be tested (62).

forms of proinsulin and proIAPP in patients with long-duration type 1 diabetes, even in cohorts characterized by the absence of detectable serum C-peptide, suggesting the continued presence of proinsulin-producing cells that do not effectively process prohormones (6,8,10). Analyses of pancreata from organ donors with type 1 diabetes have extended these findings to the tissue level (4,8).

Mechanistic studies have begun to link β-cell stress, occurring either from extrinsic sources or due to activation of intrinsic molecular pathways within the β-cell, with disruptions in normal prohormone processing (1,11) (Fig. 2). Moreover, T cell autoreactivity has been demonstrated against regions of the incompletely processed forms of proinsulin and proIAPP, suggesting a role for altered prohormone processing in immune activation (12,13). In response to these emerging findings, levels of circulating prohormones relative to mature hormones are actively being studied for their potential as noninvasive indicators of type 1 diabetes risk, as well as biomarkers of therapeutic efficacy, in efforts designed for disease prevention or intervention (14).

Prohormones in the Circulation

A substantial body of work suggests that elevations in relative levels of circulating prohormones can provide insights into β-cell stress at different stages during the evolution of type 1 diabetes. Most studies in individuals at risk for or with a diagnosis of type 1 diabetes have shown elevations in ratios of proinsulin or proIAPP relative to mature insulin/C-peptide or IAPP rather than absolute elevations in circulating prohormones in comparison with control samples (6–8,15,16).

Circulating Proinsulin–to–C-peptide Ratios Predict Disease Progression in Individuals at Risk for Type 1 Diabetes

Analyses from natural history cohorts of islet autoantibody-positive (Aab^+) individuals without diabetes who ultimately develop type 1 diabetes have shown that these individuals exhibit elevated proinsulin–to–C-peptide (PI/C) ratios in comparison with Aab^- control subjects. These efforts also suggest that fasting PI/C ratios are inversely associated with first-phase insulin responses during intravenous glucose tolerance tests or hyperglycemic clamps

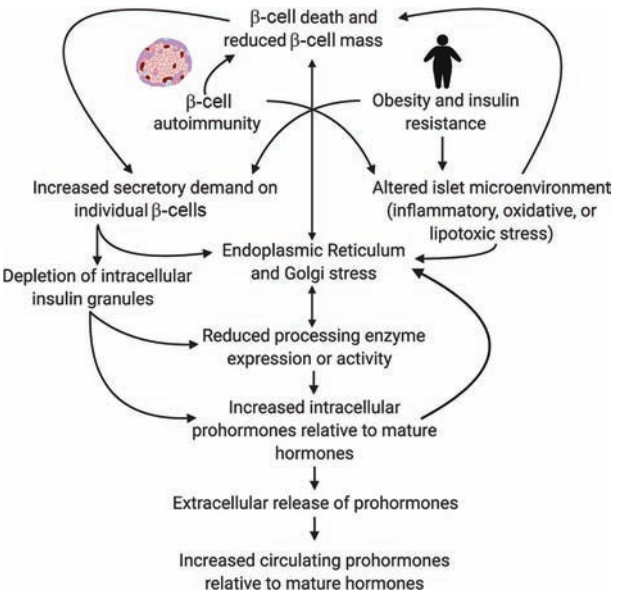

Figure 2—Potential pathologic etiologies of increased circulating immature islet prohormones relative to mature hormones. Potential etiologies arising from sources extrinsic and intrinsic to the β-cell, as well as interactions, are displayed.

(15,16). Among Aab^+ relatives, elevated PI/C ratios are also associated with increased risk of progression to type 1 diabetes and can complement the use of Aab for risk prediction, with longitudinal ratios inversely related to time to diagnosis (7,15,17,18). PI/C ratios appear to be most elevated in younger at-risk children (i.e., age <10 years) who progress to type 1 diabetes, suggesting that this subgroup of individuals may exhibit more severe β-cell stress during disease progression (7).

PI/C Ratio Is Elevated in Many Individuals With New or Recent-Onset Type 1 Diabetes

Not surprisingly, comparisons with Aab^- nonrelative control subjects show elevations of PI/C in individuals with new or recent-onset type 1 diabetes, especially younger children, consistent with high levels of β-cell stress at diagnosis (19,20). However, other efforts have shown differing results regarding whether improvements versus maintained elevations in PI/C occur after exogenous insulin therapy is started, during the clinical remission often referred to as the honeymoon period (19–21). This could be related to varied participant age at the time of diagnosis (youth vs. adult), as well as the degree and/or duration of improved β-cell function over this period (19–21). A recent study testing treatment of children and young adults with recent-onset type 1 diabetes with an IgG1-κ monoclonal antibody specific for human TNF-α showed stable PI/C ratios in drug-treated participants compared with progressive increases over time in placebo (22). Intriguingly, higher PI/C ratio at the time of type 1 diabetes diagnosis was noted in studies decades ago showing an improved response to cyclosporin treatment, suggesting that high PI/C ratios, as a proxy or readout of β-cell stress, could be leveraged to identify individuals who might benefit from a disease-modifying intervention (19). Such findings are consistent with data from other immunomodulatory interventions identifying individuals with active disease and β-cell dysfunction as robust treatment responders (23). Ideally, such biomarkers could also be leveraged to help in selection and optimization of specific interventions.

Elevations in Circulating PI/C and ProIAPP-to-IAPP Ratios Persist in Established Type 1 Diabetes

Very elevated PI/C ratios, suggestive of severe β-cell stress, are consistently present in individuals with long-standing type 1 diabetes and residual detectable C-peptide decades after diagnosis (8,10). Ratios tend to be higher in those diagnosed at very young ages (i.e., <7 years), again suggesting a more severe disease phenotype in younger children (8). Even in those individuals with long-standing type 1 diabetes and undetectable stimulated C-peptide, circulating proinsulin can be identified. The reported percentage of individuals with undetectable C-peptide but persistent proinsulin secretion has varied from 16% to up to 90%, depending on the characteristics of the assays used and cohorts studied (8,10,24,25). In addition, many individuals with undetectable or minimal residual C-peptide, but detectable proinsulin, do not exhibit the expected increase in stimulated circulating proinsulin that is observed in control subjects or those with type 1 diabetes exhibiting higher residual C-peptide (10). While measurements of the levels of the IAPP precursor, proIAPP, relative to mature IAPP have not been performed across the entire natural history of type 1 diabetes, children with long-standing type 1 diabetes and islet transplant recipients with type 1 diabetes exhibit elevated ratios of a proIAPP processing intermediate ($proIAPP_{1–48}$) relative to total IAPP (6). Interestingly, unlike PI/C, ratios of $proIAPP_{1–48}$ to mature IAPP do not appear to be elevated in individuals with type 2 diabetes (6,26).

Elevated PI/C Ratios May Be Present in Some Aab^- Relatives of Individuals With Type 1 Diabetes and in Aab^+ Nonprogressors

Several older studies in relatives of individuals with type 1 diabetes testing negative for islet autoimmunity have described elevations in circulating absolute and normalized proinsulin in comparison with unrelated control subjects, suggesting that some individuals may inherit a predisposition to abnormal prohormone processing that predates detectable autoimmunity. Islet cell cytoplasmic antibody $(ICA)^+$ and ICA^- individuals without diabetes with an identical twin affected by type 1 diabetes showed an absolute increase in fasting proinsulin in comparison with unrelated control subjects (27). Despite normal insulin and glucose values, testing in groups of Swedish and Swiss first-degree relatives of patients with type 1

diabetes who were ICA$^+$ and ICA$^-$ also showed HLA-independent increases in fasting absolute proinsulin values in comparison with control subjects, although ICA$^-$ relatives showed less pronounced elevations (28,29). An important caveat to these results is the use of older, less sensitive assays for assessment of islet autoimmunity. In contrast, more recent testing in first-degree relatives from the Belgian Diabetes Registry demonstrated similar random PI/C ratio values among Aab$^+$ relatives that did not progress to diabetes during follow-up, Aab$^-$ relatives, and a small group of nonrelative Aab$^-$ control subjects (17).

Prohormones in Islets

Increased PI/C ratios in the circulation months to years before clinical diagnosis (7,15,17,18) point to a potential functional β-cell defect appearing very early in the type 1 diabetes disease process. However, until recently, whether this relative increase in circulating proinsulin could be linked to abnormal proinsulin expression in the pancreas remained largely unknown. Recent access to human pancreas samples through the Network for Pancreatic Organ donors with Diabetes (nPOD) and the Exeter Archival Diabetes Biobank (EADB) coupled with improvements in image analysis methodologies has provided a unique opportunity to study these phenomena in samples from control subjects without diabetes (Fig. 3) as well as across multiple phases of type 1 diabetes (4,5,8) (Figs. 4 and 5). Here, analysis has shown that islets from Aab$^+$ individuals exhibit an increase in absolute pancreatic proinsulin area and in the proinsulin-to-insulin ratio without a significant reduction in insulin area or β-cell mass (5) (Fig. 4*A*). Moreover, islets from Aab$^+$ donors show changes in the subcellular localization of proinsulin, which was predominantly localized in secretory vesicles in Aab$^+$ individuals with multiple Aab, as opposed to the normal juxtanuclear localization. Taken together, these findings suggest that proinsulin maturation might already be defective in the prediabetic pancreas, independently of the loss of β-cell mass. The same analysis reported elevations in the proinsulin-to-insulin ratio in pancreatic samples from living individuals with recent-onset type 1 diabetes collected through the Diabetes Virus Detection Study (DiViD) (5). Continued elevation of ratios despite exogenous insulin therapy suggests that β-cells remain dysfunctional at the time of diagnosis, even when their workload is diminished.

Further studies demonstrated that residual β-cells containing insulin and proinsulin could be found in individuals with short and long duration of type 1 diabetes (4). Islets from donors with type 1 diabetes exhibit overall reductions in insulin, proinsulin, and C-peptide in pancreatic protein extracts in comparison with nondiabetic control subjects. However, the levels of proinsulin were higher than expected and comparable with those of Aab$^+$ individuals. Both the proinsulin-to-insulin and PI/C ratios were significantly elevated in individuals with type 1 diabetes. Furthermore, insulin mRNA was low but detectable in almost all subjects with type 1 diabetes (4). In agreement with these studies, populations of proinsulin-enriched, insulin-depleted cells have been described in a subset of individuals with long-standing type 1 diabetes (30) (Fig. 5*A*). This depletion of insulin-positive granules might suggest β-cell exhaustion (degranulation) with (or perhaps even without) a dramatic reduction in β-cell mass.

Abnormal Proinsulin and Insulin Colocalization May Be Associated With Specific Endotypes of Type 1 Diabetes

Recent work has focused on the influence of age at diagnosis on the expression and distribution of insulin and proinsulin in the pancreas (8). Analysis of samples from the EADB and the nPOD collections showed that in children diagnosed early in life (before 7 years), most β-cells exhibited a high degree of insulin and proinsulin colocalization (8) (example in Fig. 4*B*). This population also displayed a hyperimmune profile characterized by abundant islet-infiltrating CD20$^+$ and CD8$^+$ cells. The authors proposed that these features represent one particular "endotype" coined as type 1 diabetes endotype 1 (T1DE1) (6). By contrast, in samples from individuals diagnosed at or after the age of 13 years, >70% of islets displayed little colocalization and had a lower proportion of CD20$^+$ cells in inflamed islets. It was also noted that, irrespective of age at diagnosis, for the islets of people in whom β-cells persisted for >5 years after diagnosis there were low rates of proinsulin-to-insulin colocalization. Such islets were most prevalent in subjects diagnosed at age ≥13 years. Taken together with work described above, these findings suggest that there is a strong interplay among abnormal proinsulin processing, β-cell function, and immune activity in children diagnosed very early in life. Continued study of pancreata from children and adults at multiple stages of disease should help to confirm whether disease endotypes exist (rather than a continuously variable set of pancreatic phenotypes) and may suggest routes to personalized therapeutic intervention.

Prohormone Processing Is Altered in Type 1 Diabetes

The studies described above demonstrate that proinsulin is elevated in islets before and after onset of type 1 diabetes and that relative circulating levels of proinsulin remain increased in individuals with long-standing disease. From these findings, it seems very likely that the efficiency of prohormone processing is unable to keep up with prohormone production during the course of type 1 diabetes. Potential etiologies of these findings are further discussed in Table 1. A likely contributing etiology is increased insulin secretory demand or rapid insulin release in association with reduced β-cell mass (31), resulting in mature secretory granule depletion and resultant depletion of processing enzymes. It is also possible that an intrinsic prohormone processing defect may arise during the course of the disease. Expression patterns of proinsulin and proIAPP processing enzymes (prohormone convertase

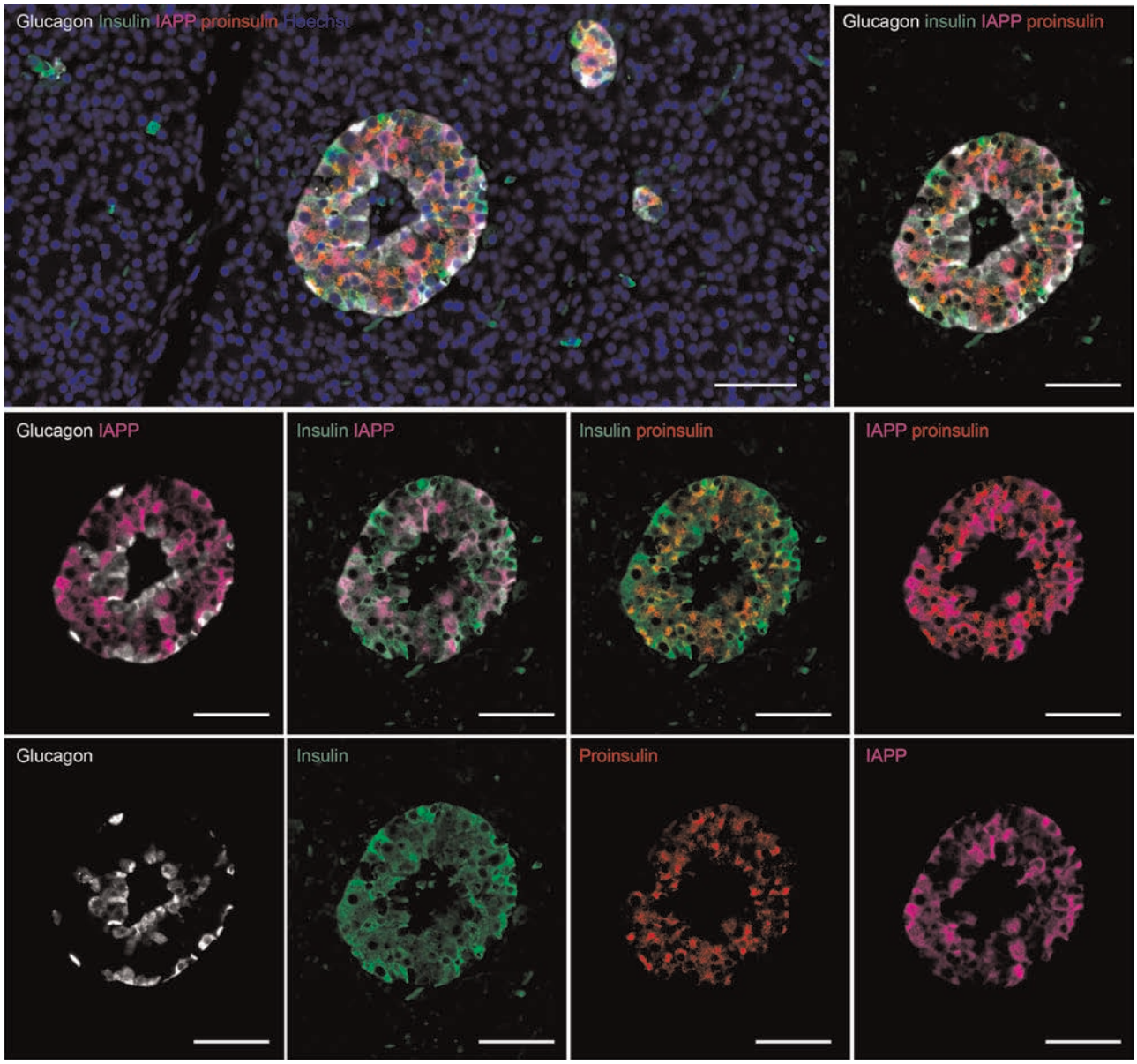

Figure 3—Immunofluorescent staining showing distribution of proinsulin, insulin, and IAPP in islets from a control donor without diabetes. Scale bars = 50 μm in the low-magnification image and 25 μm in the rest. Anti-proinsulin antibody (GS-9A8; Developmental Studies Hybridoma Bank) (detects the B-C junction and so cross-reactive with intact proinsulin, 65,66 split proinsulin, and des-64,65 proinsulin [validated in 63]) and anti-IAPP antibody (HPA053194; Atlas) were used.

1/3 [PC1/3], prohormone convertase 2 [PC2], and carboxypeptidase E [CPE]) have been investigated in islets from individuals with short and long disease duration. One study found significant reductions in PC1/3 mRNA but not in PC2 or CPE (4). Similarly, in another study, with use of laser-capture microdissection and mass spectrometry (MS), significant reductions were reported in PC1/3 and a tendency to lower CPE but no difference in PC2 (30).

Mechanistic studies testing human islet prohormone processing in the context of type 1 diabetes are fairly limited in number. Cytokine treatment for 24 h of isolated islets from donors without diabetes was associated with a significant reduction in mRNA expression levels of PC1/3, CPE, and PC2 (30). Longer (48–72 h) in vitro exposure of human islets to cytokine combinations resulted in reduced protein concentrations of PC1/3 and PC2 in association with an increase in medium proinsulin-to-insulin ratio, supporting the idea that islet inflammatory stress might be associated with intrinsic reductions in prohormone processing (32). Accumulation of misprocessed proteins can have dramatic effects on β-cell survival and mRNA translation through activation of cell-intrinsic stress pathways. Indeed, β-cells exposed to proinflammatory cytokines are

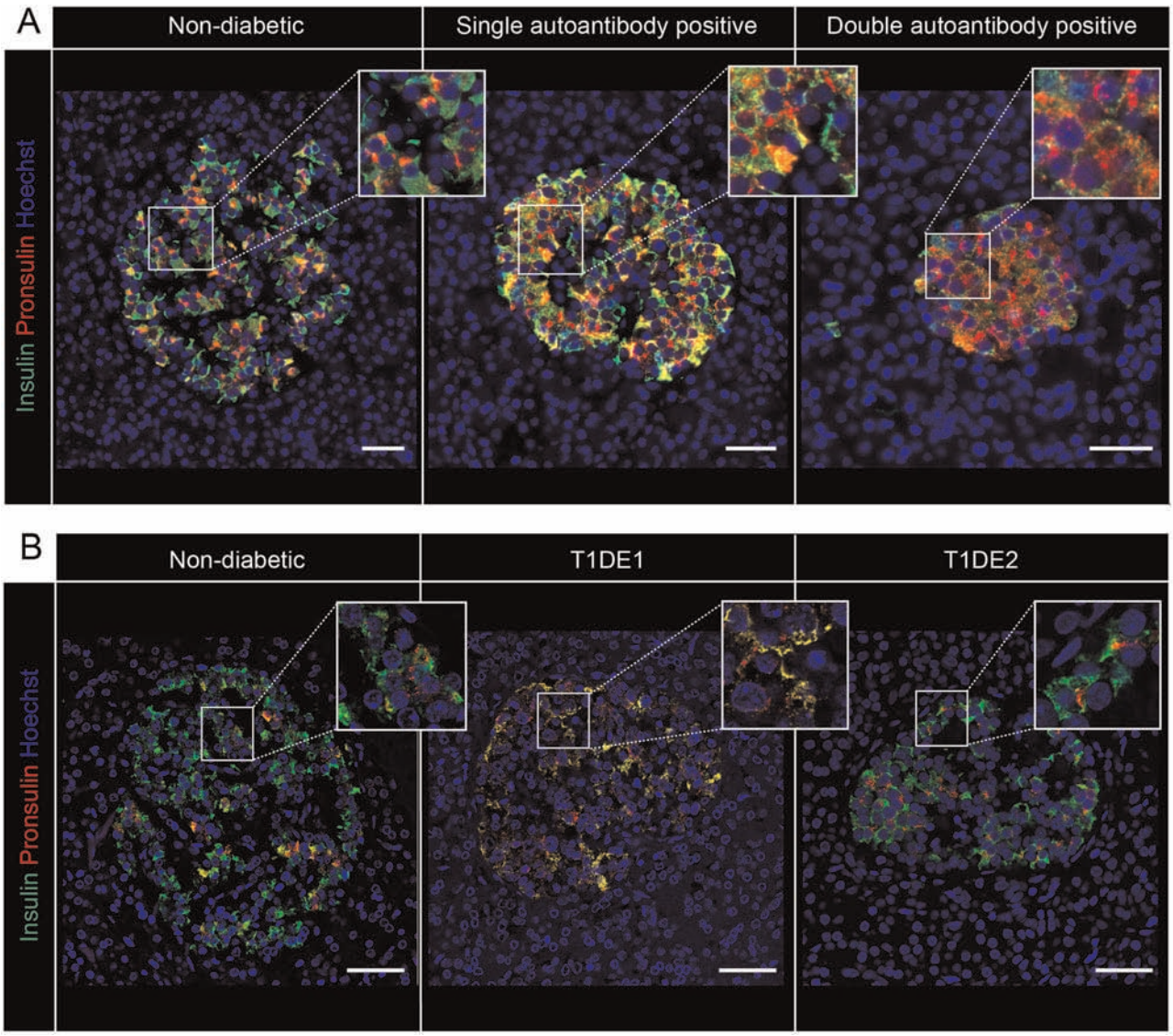

Figure 4—Examples of islet prohormone phenotypes. *A*: Pancreas sections obtained from nPOD from three individuals (no diabetes, single islet Aab positive, and double islet Aab positive) showing representative examples of increases in proinsulin-positive area (staining further described and quantified in multiple donor samples in 5). Anti-proinsulin antibody was used (GS-9A8; Developmental Studies Hybridoma Bank) (detects the B-C junction and so cross-reactive with intact proinsulin, 65,66 split proinsulin, and des-64,65 proinsulin [validated in 63]). Sections were imaged with a Zeiss Axio Scan.Z1 slide scanner. Scale bars = 25 μm. *B*: Representative pancreas sections from three individuals obtained from the EADB and nPOD (no diabetes and donors with T1DE1 and T1DE2) showing increased colocalization of insulin and proinsulin in T1DE1 in comparison with no diabetes and T1DE2 (staining further described and quantified in multiple sections in 8). Anti-proinsulin primary antibody was used (ab8301; Abcam) (detects the B-C junction and so cross-reactive with intact proinsulin, 65,66 split proinsulin, and des-64,65 proinsulin). Sections were imaged with use of high-resolution confocal (Leica DMi8, SP8) microscopy. Scale bars = 50 μm.

prone to endoplasmic reticulum (ER) stress and apoptosis (33), while islets from individuals with type 1 diabetes have increased levels of the ER stress markers BIP and CHOP, the latter of which has been associated with β-cell death (34). Defects in protein processing could theoretically contribute to β-cell death in type 1 diabetes, perhaps through exacerbation of increased secretory demands or in ways that are analogous to some monogenic forms of diabetes that occur due to proinsulin misfolding and ER stress (35,36). Misfolded proinsulin has not been identified in secretory granules; however, β-cells under conditions of ER stress independent of cytokines could also potentially contribute unprocessed proinsulin and/or proIAPP to the circulation via "leakage" or cell death. Aberrant processing of proIAPP to mature IAPP has been hypothesized to contribute to IAPP aggregation and amyloid formation (37), which has recently been detected in pancreata of some donors with long-standing type 1 diabetes (38) (Fig. 5*B*). As IAPP

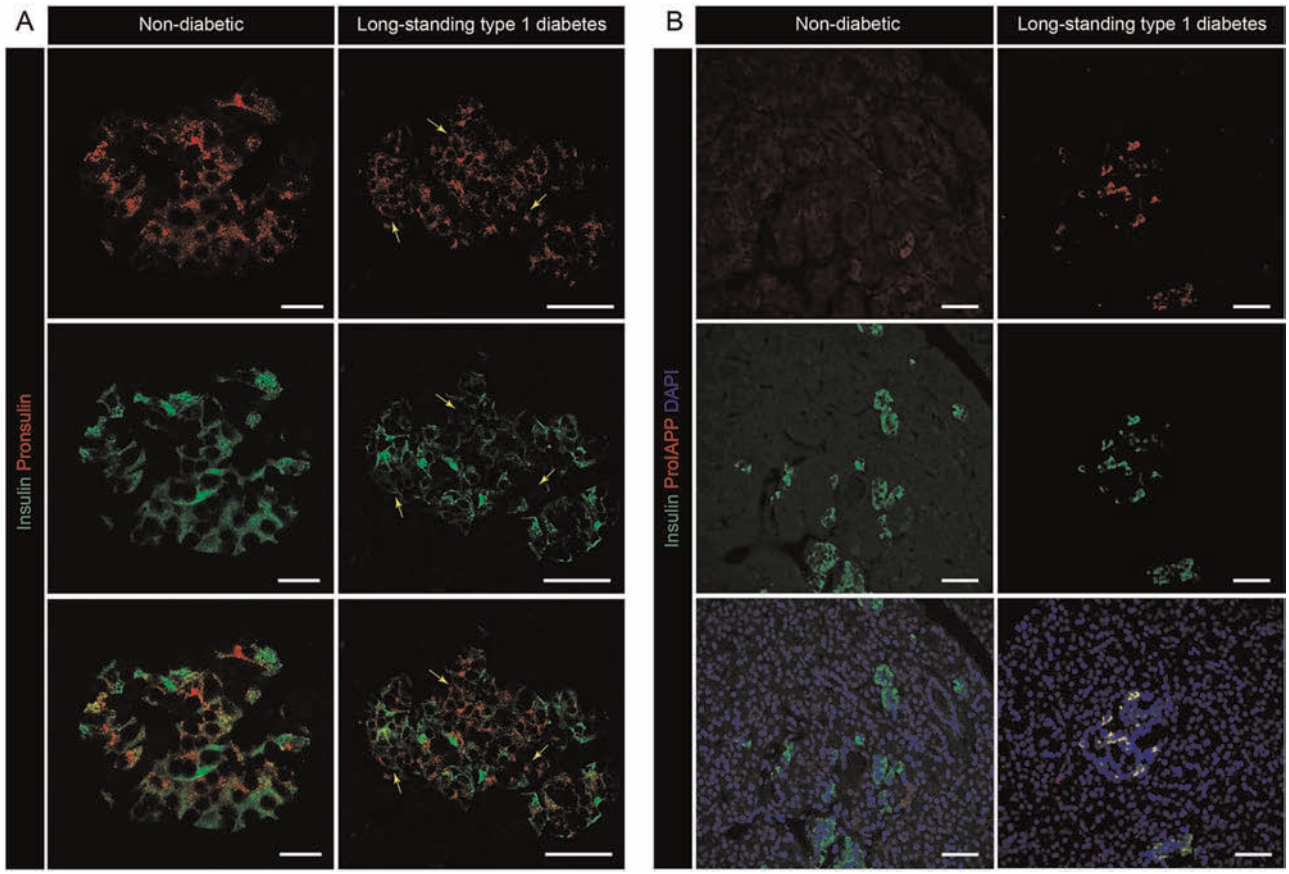

Figure 5—Examples of islet prohormone phenotypes. *A* and *B*: Immunostaining of pancreas sections, obtained from individuals through nPOD, showing islets. *A*: From a donor without diabetes (scale bars = 20 μm) and a donor with type 1 diabetes with islets showing increased numbers of proinsulin-enriched, insulin-poor cells, indicated with yellow arrows (scale bars = 50 μm). *B*: From a donor without diabetes and a donor with type 1 diabetes with islets exhibiting staining for C-terminally extended proIAPP. Sections were imaged with a ZEISS LSM 700 confocal microscope (*A*) and a Leica SP5 confocal microscope (*B*). Scale bars = 50 μm. Staining in *A* is further described and quantified in 30. Anti-proinsulin antibody (GS-9A8; Developmental Studies Hybridoma Bank) (detects the B-C junction and so cross-reactive with intact proinsulin, 65,66 split proinsulin, and des-64,65 proinsulin [validated in 63]) and anti-proIAPP antibody (F063; gift from Medimmune) (raised to an epitope in the C-terminal flanking of peptide of human proIAPP and predicted to detect intact proIAPP) were used.

aggregates are a trigger for inflammation and β-cell stress (6,37), it is plausible that impaired handling of proIAPP or IAPP could contribute to β-cell dysfunction and loss in type 1 diabetes, as is suggested in the case of type 2 diabetes (38).

In addition, accumulating evidence indicates a link between prohormone processing and T cell–mediated autoreactivity (12). T cell repertoire studies have identified autoreactivity against multiple epitopes of preproinsulin, proinsulin, and proIAPP, while analysis of the β-cell HLA-I peptidome has confirmed that many of these epitopes can be presented by the β-cell (12,39,40). Specifically, in the case of insulin, subcellular targeting and protein modifications impact processing pathways, as well as efficiency and regulation of antigen presentation (41). Errors in processing can lead to posttranslational modifications of the native protein; posttranslationally modified insulin-derived epitopes have improved HLA-I binding capacity and induce T cell activation (42). Moreover, the physiologic release of granule contents directly into the bloodstream may allow for an opportunity to interact with distant antigen-presenting cells outside of the pancreas (12). Importantly, islet autoantibodies are absent at diagnosis of inherited forms of diabetes related to protein misfolding and ER stress, pointing away from widespread activation of islet autoimmunity under conditions of β-cell stress (35,36). However, it is possible that β-cell stress is linked to increased antigen presentation and T cell–mediated autoreactivity in individuals with a genetic predisposition to autoimmunity.

Lingering Questions Regarding Prohormones in Type 1 Diabetes

The existing body of work quantifying circulating and tissue levels of proinsulin and proIAPP has provided convincing data that during the natural history of type 1 diabetes, prohormone processing cannot keep pace with prohormone production. PI/C ratios are increased in many at-risk individuals before, at, and after diagnosis and are associated with disease progression, particularly in young

Table 1—Key exploratory questions regarding the pathophysiology of disproportionate β-cell prohormone levels and secretion

Key question	Important points to consider or test
What are the specific pathologic etiologies leading to elevations in circulating and β-cell expression of prohormones relative to mature hormones in type 1 diabetes?	• Are relative elevations in circulating prohormones compared with mature hormones due to a defect in proinsulin processing or a simple reflection of β-cell exhaustion, resulting in release of immature granules with more unprocessed prohormone? ◦ Elevated levels of prohormones relative to mature hormones observed in at-risk populations and after clinical diagnosis, rather than elevations in absolute prohormone levels, may point more toward a β-cell exhaustion phenotype with mature granule depletion over primary defects in proinsulin processing (4–8). ◦ Absolute increases in islet proinsulin area along with findings of reduced expression of islet prohormone processing enzymes in islets from donors with type 1 diabetes support the idea of defective prohormone processing, but processing enzyme levels could also theoretically be impacted by reduced β-cell counts per islet or granule depletion (4,5,30). ◦ Proinflammatory cytokine–induced increases in media proinsulin-to-insulin ratio in association with reduced islet processing enzyme expression support the idea of an intrinsic defect associated with β-cell proinflammatory stress (30,32). • Similar to increased PI/C ratios after partial pancreatectomy (31), could altered prohormone processing in type 1 diabetes result in part from the impact of reduced β-cell mass on insulin secretory demand on individual β-cells? • Could increases in β-cell death due to ER stress result in leakage of prohormone contents from cells leading to increased levels in circulation? • Do changes in the human islet microenvironment impact prohormone processing and relative secretion in concert? • Systematic comparisons of circulating prohormones as well as tissue prohormone and processing enzyme expression performed with the same reagents in samples from individuals with or at risk for type 1 and type 2 diabetes could help with identification of distinct pathological contributors to β-cell stress resulting in differing timing or phenotypes of altered prohormone processing and secretion. • Do some individuals with a high genetic risk inherit β-cells with an intrinsic functional defect, either at baseline or in response to environmental stressors? ◦ The rs2611215 single nucleotide polymorphism that has been linked to increased type 1 diabetes risk could theoretically affect CPE expression, and, thus, prohormone processing may be impacted in this subset of individuals (57). ◦ Answers to this question could be informed by genetic studies in type 2 diabetes cohorts, where clustering analysis has identified single nucleotide polymorphisms potentially leading to impaired proinsulin processing (58). • Could reduced prohormone processing serve as a readout of other particular immune phenotypes or disease endotypes beyond the constellation of features suggested as the T1DE1 endotype (8)?
How do differences in the immune system's interactions with β-cells under conditions of abnormal prohormone expression contribute to the pathophysiology of type 1 diabetes?	• Do altered interactions with the immune system occur early in the disease process due to formation of neoantigens, via misfolding, alternative translation, or posttranslational modifications (reviewed in 12)? If so, does this occur in predisposed individuals at baseline or in response to an environmental exposure, such as a viral infection (59)? • What mechanisms link $CD20^+$ B cells to impaired prohormone processing? Do insulitis profiles associated with large numbers of $CD20^+$ B cells yield increased β-cell stress? Or do stressed β-cells somehow attract more severe insulitis with a higher proportion of $CD20^+$ cells (8)? • Does increased susceptibility to β-cell stress exacerbate β-cell failure and death once autoimmunity is established? • Is reduced expression of processing enzymes and subsequent reduced prohormone processing part of a dedifferentiated β-cell phenotype that is able to evade autoimmunity (45,46)?
Which prohormone forms (i.e., intact/unprocessed prohormones vs. partially processed forms) are relevant to β-cell stress in type 1 diabetes?	• Although both intact/unprocessed proinsulin and partially processed des-31,32 proinsulin forms are elevated in type 2 diabetes, specific quantification of relative proportions of intact vs. partially processed prohormone forms of either proinsulin or proIAPP has not been reported in type 1 diabetes (47). • What are the relationships between increased relative proportions of proinsulin and proIAPP in circulation and pancreas tissue?

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Table 1—Continued

Key question	Important points to consider or test
	• Do different pathophysiologic etiologies of β-cell stress result in differing elevations in relative levels of prohormone forms? • Why are PI/C and proIAPP$_{1–48}$–to–total IAPP ratios similarly elevated in type 1 but not type 2 diabetes (6)? • How do differences in clearance of prohormone species in comparison with mature hormones impact circulating prohormone–to–mature hormone ratios in type 1 diabetes?

children. Even many years after diagnosis, persistent secretion of proinsulin and proIAPP has been documented, reflecting the existence of severely dysfunctional β-cells in long-standing disease. However, several lingering questions regarding the natural history and physiology of circulating prohormones remain. These will be important to answer moving forward in order to implement use of circulating prohormones as biomarkers to guide type 1 diabetes prediction, progression, and treatment. Similarly, despite the increasing number of studies in islet tissue on absolute or relative prohormone accumulation and processing, there are still conceptual gaps in our understanding of prohormone conversion dynamics and storage, both in "normal" β-cells of humans without diabetes and in cells influenced by different types of stressors.

Natural History of Circulating Prohormones in Health and Disease

Studies often show significant elevations in PI/C ratios in comparisons with control subjects, but in many cases, values overlap with those of control subjects. This might be related to several factors, including heterogenous phenotypes or endotypes of type 1 diabetes, which do not uniformly involve high levels of intrinsic β-cell stress (43). Overlap with values of control subjects could also reflect disease heterogeneity due to physiologic differences in "normal" PI/C ratios during different life stages, especially for pediatric age-groups advancing through puberty, or groups of adults compared with children. Both adults and pubertal children can exhibit increases in insulin resistance relative to prepubertal children, and knowing the expected "normal" range for PI/C ratios and proIAPP-to-IAPP ratios more definitively during growth and aging will be important for understanding what constitutes an "abnormal" value (44).

Pragmatically, another important hurdle in the implementation of prohormone measurements as biomarkers for use in prediction and intervention efforts will be development of a better definition of the expected natural history of increasing prohormones/mature hormones as type 1 diabetes develops. Longitudinal data are needed in adequately powered studies of diverse at-risk populations that allow for improved characterization of patterns of change over time. While proIAPP has been measured in established type 1 diabetes, it is not clear how patterns of secretion change during earlier disease stages or whether there is concordance between proIAPP and proinsulin secretion at different disease stages. Such analyses will allow for a better understanding of whether certain groups of individuals are at risk for abnormal values, either at baseline or in association with environmental stressors. Along these lines, older data in ICA$^-$ first-degree relatives are intriguing but must be followed up using newer biochemical Aab assays. Additionally, ratios may increase progressively over time or could change in a relapsing-remitting pattern, in association with periods of active autoimmune disease or intermittent environmental stressors. Such factors have important implications for the timing of screening and prediction approaches—but will also be informative in tracking of the disease course in individuals. In addition, samples from successful intervention studies should be tested to identify whether circulating prohormones can be used as an early marker of successful treatment response. Such analyses would also serve to identify whether circulating prohormones could be used to identify reversible β-cell stress and predict treatment "responders" to immunomodulation.

Pathologic Etiologies and Consequences of Altered Prohormone Processing

Several important unanswered questions revolve around the pathophysiologic relevance of increases in circulating prohormones in type 1 diabetes (outlined in Table 1). First, given that multiple pathways could theoretically increase the relative proportion of β-cell prohormone expression and secretion, what are the specific pathologic etiologies leading to these outcomes in type 1 diabetes? Exploration of this question will lead to a better understanding of whether defects in prohormone processing can serve as a readout of particular immune phenotypes and/or disease endotypes (8).

Work from several investigators suggests that the immune system could react differently to β-cells under situations in which abnormal prohormone expression occurs (reviewed in 12). Alternatively, increased susceptibility to β-cell stress could exacerbate β-cell failure and death once autoimmunity is established. In contrast, recent preclinical work suggests a model wherein partially dedifferentiated β-cells may be selected for their ability to evade autoimmunity. Along these lines, loss of processing enzyme

Table 2—Optimizing measurements of proportional elevations of prohormones in type 1 diabetes

Factor	Important considerations
Timing of sampling	• Samples from all participants should be optimally obtained at consistent timing relative to fasting vs. glucose or mixed-meal stimulation. • For normalization, prohormones and mature hormones should be measured in samples obtained from the same time point. • Fasting PI/C ratios reportedly elevated in individuals at risk for or diagnosed with type 1 diabetes. • Stimulated PI/C ratios may be less impacted relative to fasting values in longer-duration type 1 diabetes, but definitive understanding of timing of peak PI/C ratios in these populations requires more robust study that includes multiple stimulated time points.
Prohormone measurements	
• Proinsulin assay	• Commercially available assays include radioimmunoassays and ELISA. • Does assay of interest test intact (unprocessed) proinsulin or total proinsulin (unprocessed + different combinations of partially processed split products depending on assay)? Cross-reactivity with partially processed proinsulin species is common in most commercial proinsulin and insulin assays. No commercial assays are currently available that quantify des-64,65 proinsulin or des-31,32 proinsulin individually. • Antibody-based assays are well established but often not well characterized. Because standards for partially processed forms of proinsulin are not widely available for external validation, there is uncertainty regarding which proinsulin products commercially available antibodies recognize. • Has assay of interest been externally validated to show reproducible results? • Is assay of interest calibrated based on 84/611 (older) or 09/296 (newer) intact proinsulin standard (60)? Due to diminishing supply, in 2014 the original World Health Organization intact proinsulin standard was switched to the 09/296 standard (60). Because of this, although newer intact and total proinsulin assays are calibrated based on the 09/296 standard, several older assays are calibrated against the prior 84/611 standard, thereby limiting direct comparison of results, given that values obtained with assays based on different standards are not quantitatively equivalent. • While partially processed split products do not add discriminatory capability above that of intact proinsulin for samples from control subjects and patients with type 2 diabetes, this has not been tested in type 1 diabetes (47). • Targeted MS-based techniques may allow for antibody-independent measurements and validation of antibody-based assays. But such assays are still in the developmental stage at research laboratories.
• ProIAPP assay	• Externally validated proIAPP$_{1–48}$ ELISA is available through research laboratories only (6). • Assays for intact proIAPP$_{1–67}$ and other intermediate forms are currently under development. • Targeted MS-based techniques are currently under development.
Mature hormone measurements	• Understanding of proportional elevations in prohormones requires careful quantification of levels relative to mature hormones. Thus, optimizing mature hormone measurement is also a key goal.
• C-peptide or insulin assay	• Elevations in both PI/C and proinsulin-to-insulin ratios have been described in circulation for certain populations relevant to type 1 diabetes (7,28,29,61). • PI/C ratio outperforms proinsulin-to-insulin ratio to predict incident diabetes in insulin-resistant populations, as circulating insulin values can reflect altered hepatic insulin clearance (26). • Measurement of endogenous insulin levels may be confounded by use of insulin analogs. • International standards are available for both C-peptide and insulin. • Certain immunoassays for C-peptide and insulin may cross-react with proinsulin species. • MS-based based assays for C-peptide and intact insulin are available through commercial laboratories (e.g., Quest Diagnostics).
• IAPP assay	• Commercially available assays include radioimmunoassays and ELISA. • Commercial assays have not been externally validated. • Externally validated immunoassay for mature, C-terminally amidated IAPP is available through research laboratories (5). • No international standard exists for cross calibration of assays. • MS-based techniques are currently under development.

expression and the reduced ability to complete prohormone processing could be viewed as part of a dedifferentiated β-cell phenotype (45,46). How these potentially conflicting paradigms contribute to type 1 pathophysiology is not clear and should be a focus of future studies.

Although both intact/unprocessed proinsulin and partially processed des-31,32 proinsulin forms are elevated in type 2 diabetes (47), the relative proportion of intermediate and intact forms of proinsulin and proIAPP is still unclear in type 1 diabetes. Separate quantification of both intact and partially processed prohormone forms in the

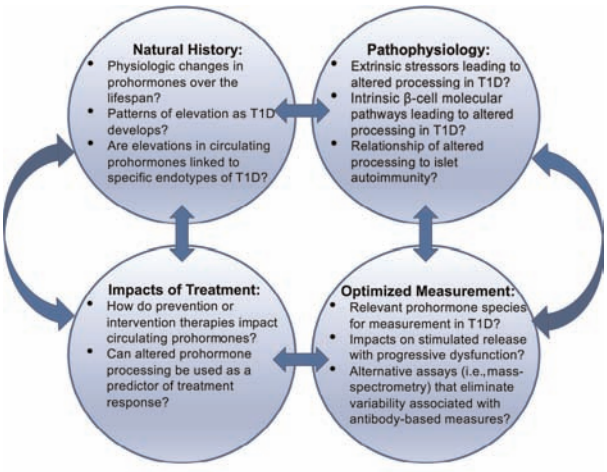

Figure 6—Key next steps in the field moving forward. Key next steps involve determining the natural history of abnormal relative prohormone expression in the islet and circulation during the progression of type 1 diabetes (T1D), better understanding the pathophysiology leading to these findings, defining the impact of disease treatment, and optimizing prohormone measurement.

circulation and pancreas from individuals with type 1 and type 2 diabetes, and of relationships between increased relative proportions of proinsulin and proIAPP, may clarify the pathophysiologic etiologies of altered ratios.

Studies testing both pancreas tissue and serum from the same individual would be optimal to address these questions, but such samples are challenging to obtain from living individuals. However, recent advances have provided the opportunity to study live pancreatic tissue from organ donors and therefore correlate anatomical and physiological data. Slices of whole pancreas tissue with preserved endocrine, exocrine, and immune tissue compartments can be obtained from donors with or without type 1 diabetes (48). With use of these, it may be feasible to measure basal and stimulated prohormone secretion as well as to evaluate enzymatic prohormone processing in both nondiabetic and diabetic tissue environments. A preliminary study in pancreas slices from a donor with type 1 diabetes has confirmed that, in some cases, significant β-cell mass is preserved at disease onset and that insulin deficiency can result from β-cell dysfunction (5,48). These findings corroborate data generated from islets, isolated from living donors with recent-onset type 1 diabetes in DiViD, that were dysfunctional initially but recovered glucose responsiveness during culture. Expanding these studies will be key to defining the role of β-cell dysfunction in type 1 diabetes.

Optimizing Prohormone Measurement

Strategies and challenges in optimizing prohormone measurement are outlined in Table 2. Of note, the use of highly specific and reliable assays for multiple forms of prohormone (e.g., intact, des-64,65, and des-31,32 proinsulins) will be critical to gaining a more detailed understanding of prohormone processing in type 1 diabetes. Immunoassays have been developed for detecting intact proinsulin (49), total proinsulin (which includes intact + partially processed proinsulin forms) (50,51), C-peptide, insulin, and proIAPP$_{1–48}$ (6). Unfortunately, the performance characteristics of prohormone immunoassays, which are limited by the inherent exclusive dependence on reproducible antibody reagents, are often not well documented (52). For proinsulin, obtaining optimal specificity can be particularly challenging due to the structural similarities among proinsulin, its partially processed forms, insulin, and C-peptide and the low concentration of proinsulin in comparison with insulin/C-peptide (52). Specifically, proinsulin and mature insulin or C-peptide immunoassays often cross-react with intact proinsulin as well as a combination of proinsulin split products, resulting in a degree of inaccuracy in quantification of proinsulin or insulin levels from clinical data. Although to date there are no widely accepted guidelines or standardized approaches, careful independent validation is needed in confirming consistency of findings (53,54).

A promising alternative means to develop highly specific assays for prohormone products is the use of targeted MS. These techniques (55,56) provide direct measurements of surrogate peptides from any given protein with well-characterizable specificity and multiplexing capacity but without the need for affinity reagents. In principle, it is highly feasible to develop targeted MS assays for specific forms of prohormone processing products in serum, tissue, and secretome (10); however, the current limitations of targeted MS assays lie in the achievable sensitivity and analytical throughput. Further developments are needed to achieve the sensitivity required for such detection. The rigor and reproducibility of immunoassays could be substantially improved if the specificities and performance are validated and standardized with use of antibody-free targeted MS-based assays along with improved calibration standards.

Conclusions: Key Next Steps in the Field Moving Forward

In summary, analysis of pancreatic tissue and data from clinical cohorts indicate that prohormone processing insufficiency is an early and persistent component of type 1 diabetes pathogenesis. The body of work outlined herein has identified a number of exploratory questions for the type 1 diabetes research community and suggests several key next steps that should be taken to clarify the role of altered prohormone expression and secretion in type 1 diabetes (Fig. 6). These questions largely focus around continued and longitudinal testing of relevant clinical populations, studies that probe the genetics of impaired prohormone processing in type 1 diabetes, combined functional and histologic testing of human pancreas tissues, and continued interrogation of how prohormone processing intersects with immune activation and autoreactivity. A key tenet of this work is the need for reliable

and well-validated assays that can detect and distinguish intact prohormones and processing intermediates. Successful resolution of these questions may offer the potential to use altered prohormone processing as an anchor that can subsequently be used to cluster different endotypes of disease enveloping a large set of variables (e.g., age, genetic background, severity of insulitis and its composition, and rate of progression). Ultimately, such an approach could inform clinical therapeutic strategies aimed at intervening in the natural history of type 1 diabetes.

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