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PROTEOMICS ANALYSIS OF ROUGH ENDOPLASMIC RETICULUM IN PANCREATIC BETA CELLS

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Abstract

Pancreatic beta cells have well-developed endoplasmic reticulum (ER) to accommodate for the massive production and secretion of insulin. ER homeostasis is vital for normal beta cell function. Perturbation of ER homeostasis contributes to beta cell dysfunction in both type 1 and type 2 diabetes. To systematically identify the molecular machinery responsible for proinsulin biogenesis and maintenance of beta cell ER homeostasis, a widely used mouse pancreatic beta cell line, MIN6 cell was used to purify rough ER. Two different purification schemes were utilized. In each experiment, the ER pellets were solubilized and analyzed by one dimensional SDS-PAGE coupled with HPLC-MS/MS. A total of 1467 proteins were identified in three experiments with 95% confidence, among which 1117 proteins were found in at least two separate experiments and 737 proteins found in all three experiments. Gene ontology analysis revealed a comprehensive profile of known and novel players responsible for proinsulin biogenesis and ER homeostasis. Further bioinformatics analysis also identified potential beta cell specific ER proteins as well as ER proteins present in the risk genetic loci of type 2 diabetes. This dataset defines a molecular environment in the ER for proinsulin synthesis, folding and export and laid a solid foundation for further characterizations of altered ER homeostasis under diabetes-causing conditions.

Keywords

pancreatic beta cell; endoplasmic reticulum; insulin biogenesis; GeLC-MS/MS; organellar proteomics

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CONFLICT OF INTEREST

The authors do not have any conflict of interest to declare.

In pancreatic beta cells, the well-developed ER is responsible for the synthesis, folding and export of proinsulin [1,2]. Therefore, ER homeostasis, the balance between the client protein load and processing capacity of the ER, is vital for normal beta cell function. The disruption of ER homeostasis contributes to beta cell dysfunction and death in both type 1 and type 2 diabetes together affecting an estimated 285 million people worldwide [3,4,5]. Organellar proteomics represents an analytical strategy that combines biochemical fractionation and comprehensive protein identification [6]. The isolation of subcellular organelles leads to the reduced sample complexity and links proteomics data to functional analysis [7]. Because of its central role in the secretory pathway, the ER has been the subject of a number of organellar proteomics studies predominantly in liver and exocrine pancreas [8,9]. In spite of the essential role of ER in normal proinsulin biogenesis and pathogenesis of diabetes, its protein composition has not been systemically analyzed. In this study, we conducted a comprehensive proteomic analysis of the ER isolated from MIN6 cells, a widely used mouse pancreatic beta cell line.

MIN6 cells were cultured at 37°C with 5% CO₂ in DMEM containing 10% fetal bovine serum, antibiotics and 50µM β-mecaptoethanol. Two separate approaches were used to isolate rough endoplasmic reticulum (rER). The first approach used differential ultracentrifugation modified from previously described methods [10,11]. Briefly, confluent MIN6 cells were homogenized in a glass-Teflon motor-driven homogenizer at 900 rpm for 10 strokes. After removing the cell debris, nuclei and mitochondria, the supernatant was centrifuged at 50,000 × g for 30 min at 4 °C to obtain the high density microsome (HDM) fraction which contains most of the rER. The second approach used a step sucrose gradient as previously published by us [12]. Briefly, the homogenate was first centrifuged at 12,000 × g for 15 min at 4 °C to remove all cell debris, nuclei and mitochondria. The rER fraction was collected at the 1.35 to 2M sucrose interface. In both approaches, ribosomes were stripped off the rER using 1 mM puromycin. Highly enriched ER was obtained with minimal contaminations from cytosol or other organelles such as mitochondria and Golgi as demonstrated by the Western blotting analysis using specific organellar markers for the differential ultracentrifugation approach (Fig. 1A) as well as for the step sucrose gradient approach (data not shown) which is consistent with previously published in exocrine pancreas ER [12].

The rER proteins were separated on 4–12% 1D SDS-PAGE and stained with GelCode Blue stain reagent. Then the entire lane was excised in 35 gel slices (Fig. 1B). In-gel tryptic digestion was performed as previously published [13,14,15]. The resulting peptides were separated on a reversed-phase C18 column with a 90 min gradient using the Dionex Ultimate HPLC system at a flow rate of 150 nl/min. MS and MS/MS spectra were acquired on an Applied Biosystems QSTAR XL mass analyzer using information dependent acquisition mode. A MS scan was performed from m/z 400–1,500 followed by product ion scans on two most intense multiply charged ions. The peaklists were submitted to the Mascot server to search against the UniProt database for *Mus musculus* with carbamidomethyl (C) as a fixed modification and oxidation (M), N-acetylation (protein N terminus) as variable modifications, 0 or 1 missed tryptic cleavage, 100 ppm mass tolerance for precursor ions and 0.6 Da for the fragment ions. The Mascot search files were imported

to Scaffold v3.4.3 and a self BLAST procedure was performed to further reduce protein redundancy.

Three separate rER preparations were analyzed by GeLC-MS/MS, two biological replicate experiments (HDM1 and HDM2) using differential ultracentrifugation and one experiment using step sucrose gradient (rER). The numbers of unique proteins identified in each experiment is shown in figure 1C. All together, 1467 unique proteins were identified in all three experiments, among which 737 proteins were found in all experiments and 1117 proteins in at least two experiments. After removing keratin proteins, we annotated the 725 proteins common to all three experiments (Supplemental table 1). For their subcellular localizations (Fig. 2A), the identified proteins included ER resident proteins (28%) and proteins closely associated with ER functions. A small portion of these proteins were likely from contaminating organelles such as mitochondria (6%) and nucleus (2%). Because a wide variety of secretory proteins, so-called cargo proteins, transit through the ER, it is anticipated that these cargo proteins account for a significant portion of the rER proteome. These included secreted proteins (secretory cargo, 5%), lysosomal proteins (3%), Golgi proteins (2%), insulin secretory granule (ISG) proteins (2%) as well as other membrane cargo (22%) with multiple or unspecified subcellular destinations. Other identified proteins also included the cytosolic proteins (12%) and cytoskeleton proteins (4%). Although these proteins are not processed through the ER, many of them play very important roles in the ER functions. These proteins included the COPI and COPII coat proteins, required for retrograde and anterograde ER to Golgi transport respectively, as well as key members of BAG family for the post-translational delivery of tail-anchored membrane proteins to the ER. After excluding the contaminating mitochondrial and nucleus proteins, the remaining 670 proteins were further clustered into functional categories (Fig. 2B). The ER resident and associated proteins fall in several functional categories including protein translation & translocation (13%), protein folding/chaperones (6%), oxidoreductase (4%), ERAD (3%), ER export (7%), membrane trafficking (18%), lipid metabolism (7%) and glycosylation (3%). In addition, a group of proteins in the "Unknown" category (9%) do not have functional information available in the public database or literatures. In an attempt to look for potential beta cell specific/upregulated ER proteins, we compared, by automatic BLAST and then manual confirmations, the current ER protein list with previously published ER proteins which we have compiled and published previously [8]. The results are listed in the column I and J of the Supplemental table.

The ER proteins most relevant to proinsulin folding included the heat shock proteins from Hsp40, Hsp70 and Hsp90 families, protein disulfide-isomerases A1, A3-A6, BiP, oxidoreductases Ero1a and Ero1b which is known to be highly enriched in beta cells, Erp29, Erp44 and Erp46, DnaJ homologs including Dnajc3 (also called p58IPK), Torsin 1-3 and Toll-like receptor-specific co-chaperone Cnpy2,3,4. In the ER export category, a large number of proteins involved in ER to Golgi transport have been identified including COPII coat proteins Sec31a, Sec13, Sec24a, Sar1b and its guanine nucleotide exchange factor mSec12; known or potential cargo receptors such as Lman1, Lman2, p24 family members, Surf4 and Bap31; and most interestingly, two recently discovered COPII cargo adaptors, Mia3 (also called TANGO1) and cTAGE5 required for procollagen secretion primarily in chondrocytes and fibroblasts [16,17]. However, their potential roles in beta cell secretion are

unknown. In addition to the ER chaperones and export proteins, several identified beta cell ER proteins have been associated with type 2 diabetes in the genome-wide association studies [18,19]. These included WFS1, ZnT8 (as a transient cargo) and CDKAL1, among which CDKAL1 was very recently found to be a novel tail-anchored ER protein in beta cells [20]. Additional ER proteins also found as risk loci of type 2 diabetes are listed in the Supplemental table. Among these proteins, three proteins, CDKAL1, ZnT8 and TM163 are of the most interest because they are present in the T2D risk loci as well as being the potential beta cell specific ER proteins. Other potential beta cell specific ER proteins, from our BLAST analysis, revealed an upregulation of machineries involved in proinsulin biogenesis, including the neuroendocrine convertases, protein disulfide isomerases as well as several proteins regulating unfolded protein response (UPR).

In this study, we compared two different approaches to isolate rER from a pancreatic beta cell line, MIN6 cells. We found both methods yielded highly enriched rER fractions and identified similar numbers of proteins. Either method can be used in future studies to characterize the alteration of ER homeostasis under diabetes-causing conditions. A fairly detailed molecular model starts to emerge from this comprehensive profiling which include potential key players regulating proinsulin folding, degradation and ER export. The specific roles of these players at each step of proinsulin processing and ER homeostasis will be gradually unveiled in subsequent quantitative proteomic and functional studies to perturb beta cell ER homeostasis using combined genetic and biochemical approaches.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium [21] via the PRIDE partner repository with the dataset identifier PXD001081 and DOI 10.6019/PXD001081

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ERAD	ER associated degradation
HDM	High density microsomes
ISG	Insulin secretory granule
LC	liquid chromatography
MS/MS	tandem mass spectrometry
rER	Rough endoplasmic reticulum

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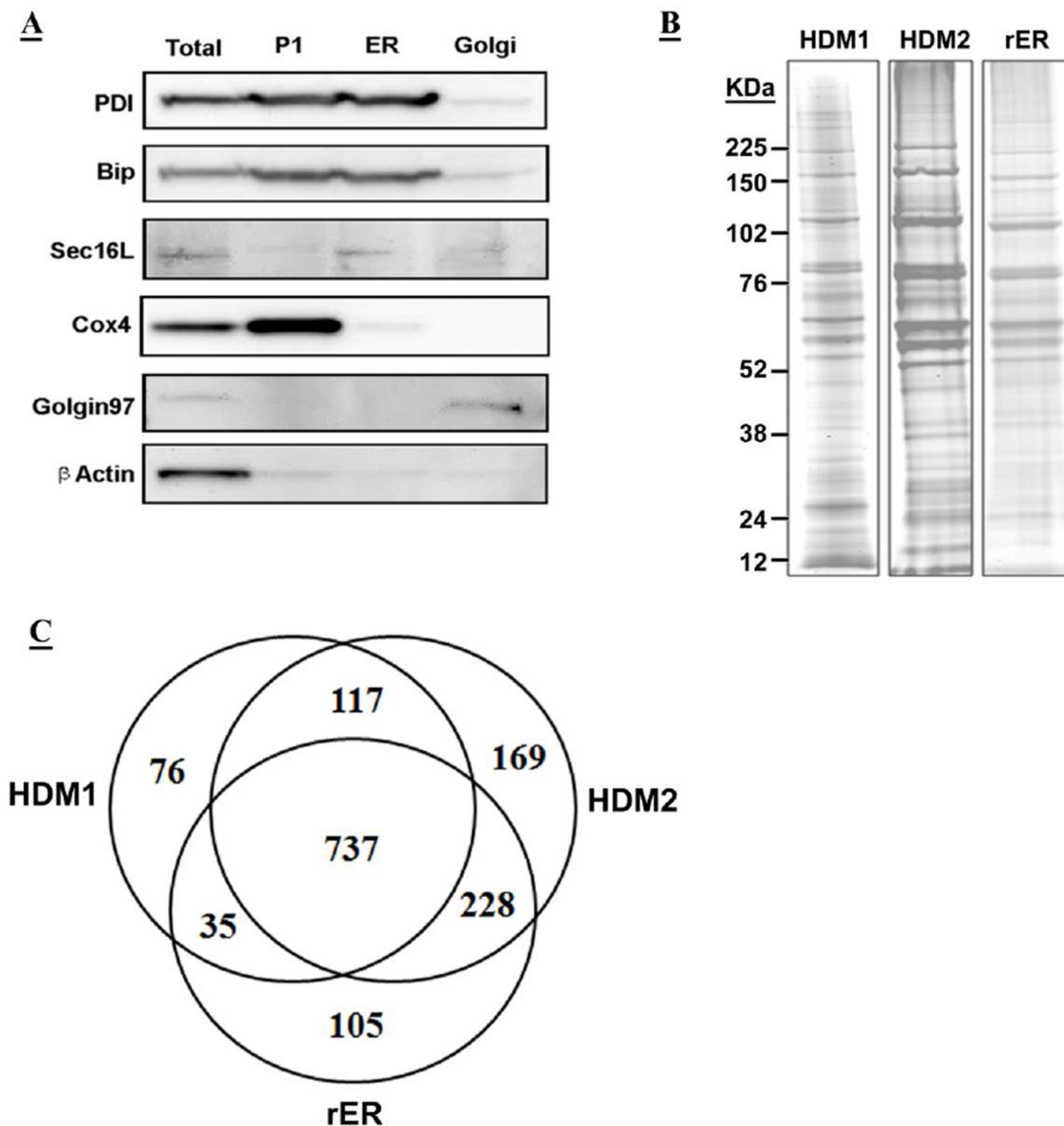
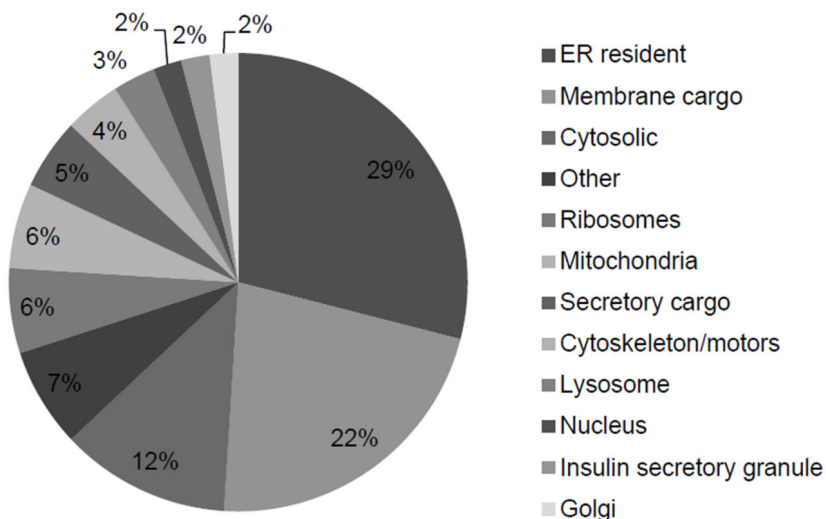


Figure 1. Purification and GeLC-MS/MS analysis of rER in MIN6 cells

A, MIN6 cells were homogenized (total) and after clearing cell debris, nucleus and mitochondria at 14,000g (P1), the high density microsomes fractions containing the rER were collected by ultracentrifugation (ER). The lighter fraction containing the low density microsomes was also collected with an additional higher speed spin (Golgi). Same amount of proteins were loaded for WB to test for the ER markers: PDI, BiP and Sec16L; the mitochondria marker: Cox4; the Golgi marker: Golgin97; the cytosolic marker: beta-actin. **B**, In three separate experiments using two different purification schemes, 40 μ g of

solubilized ER proteins were separated in each lane. Thirty five gel slices were excised from each lane for in-gel tryptic digestion. HDM1 and HDM2 were replicate experiments using differential ultracentrifugation purification and rER was the experiment using a step sucrose gradient. C, The numbers of unique proteins identified in three separate experiments are shown in a Venn diagram. In three separate experiments, 965, 1251 and 1105 unique proteins were identified with >95% confidence in HDM1, HDM2 and rER experiments respectively.

A



B

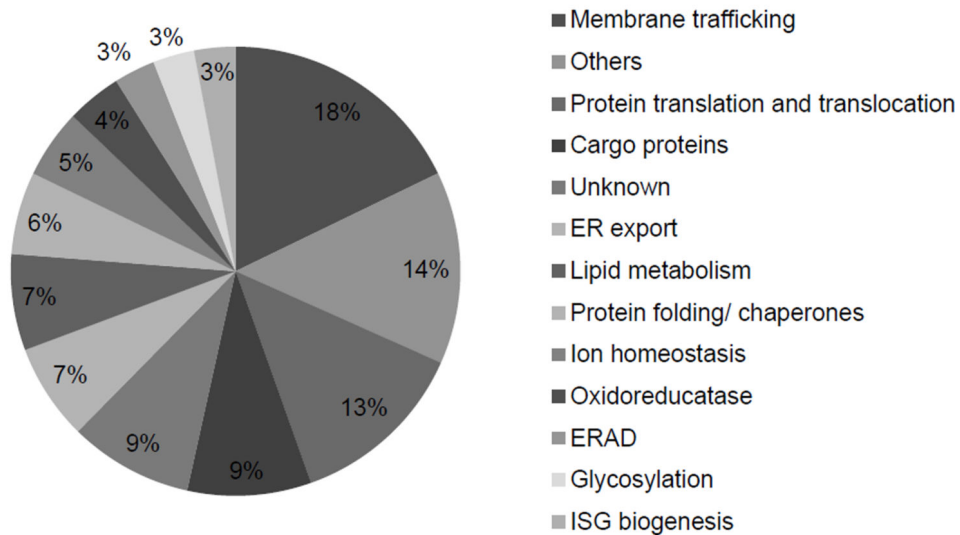


Figure 2. Classification of the identified proteins from purified rER in MIN6 cells
A, Subcellular localization of proteins identified in all three experiments were analyzed and presented as percentage of the total 725 proteins. **B**, 670 identified rER and associated proteins were classified in thirteen functional categories. The percentage of total proteins in each category is illustrated in the pie chart. In both charts, the categories are sorted from the highest percentage to the lowest percentage.