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Improved characterization of the insulin secretory granule proteomes☆

Domitille Schvartz[a,1], Yannick Brunner[a,1,2], Yohann Couté[a,3], Michelangelo Foti[b], Claes B. Wollheim[b], Jean-Charles Sanchez[a,*]

[a]Biomedical Proteomics Research Group, Department of Human Protein Sciences, University Medical Center, Geneva, Switzerland
[b]Department of Cell Physiology and Metabolism, University Medical Center, Geneva, Switzerland

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ABSTRACT

Insulin secretory granules (ISGs) are pivotal organelles of pancreatic ß-cells and represent a key participant to glucose homeostasis. Indeed, insulin is packed and processed within these vesicles before its release by exocytosis. It is therefore crucial to acquire qualitative and quantitative data on the ISG proteome, in order to increase our knowledge on ISG biogenesis, maturation and exocytosis. Despites efforts made in the past years, the coverage of the ISG proteome is still incomplete and comprises many potential protein contaminants most likely coming from suboptimal sample preparations. We developed here a 3-step gradient purification procedure combined to Stable Isotope Labeling with Amino acids in Cell culture (SILAC) to further characterize the ISG protein content. Our results allowed to build three complementary proteomes containing 1/ proteins which are enriched in mature ISGs, 2/ proteins sharing multiple localizations including ISGs, and finally 3/ proteins sorted out from immature ISGs and/or co-purifying contaminants. As a proof of concept, the ProSAAS, a neuronal protein found in ISGs was further characterized and its granular localization proved. ProSAAS might represent a novel potential target allowing to better understand the defaults in insulin processing and secretion observed during type 2 diabetes progression. This article is part of a special issue entitled: Translational Proteomics.

1. Introduction

Insulin is a key player in the adjustment of energy balance, as it is involved in the regulation of glucose homeostasis. This hormone is synthesized, stored and secreted by the ß-cells of the pancreas. It is compacted into dense core secretory vesicles, insulin secretory granules (ISGs), dedicated to its regulated maturation and secretion. In rat ß-cells, there are approximately 10,000 ISGs per ß-cell, and each ISG encloses around 200,000 insulin molecules organized as insulin crystals containing 6 molecules of insulin [1,2]. These vesicles measure about 300–350 nm. Their biogenesis starts with a budding at the trans-Golgi network (TGN), where the proteins are enveloped by TGN membranes. Two models of protein

Abbreviations: ISG, Insulin secretory granules; SILAC, Stable Isotope Labeling with Amino acids in Cell culture; TGN, Trans-Golgi network; PC1/2, Prohormone convertase 1/2; SNAREs, Soluble NSF attachment protein receptors.

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* *Corresponding author at: Biomedical Proteomics Research Group, Department of Human Protein Sciences, Rue Michel Servet 1, CH-1211 Genève 4, Switzerland.* Tel.: +41 22 379 54 86; fax: +41 22 379 55 05.
E-mail address: jean-charles.sanchez@unige.ch (J-C. Sanchez).

[1] Both authors contributed equally to this work.
[2] Present address: Swiss Center for Applied Human Toxicology, HEPIA, Geneva, Switzerland.
[3] Present address: LEDyP (iRTSV / BGE), INSERM/CEA/UJF U1038, F-38054 Grenoble, France.

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sorting at the TGN are debated: the "sorting by entry" or "sorting by retention" hypotheses [3–5]. The first one relies on the fact that proteins are sorted in different specific vesicles directly at the TGN. According to the second hypothesis, proteins at the TGN are incorporated into a unique immature dense core vesicle, from which they are sorted along the maturation processes. In ß-cells, several studies tend to prove that the "sorting by retention" hypothesis might be more plausible [4,6].

Immature ISGs undergo maturations during their progression from the TGN to the plasma membrane. Notably, an acidification of the lumen occurs, which is essential for the vesicle composition. Actually, this acidification allows aggregation of soluble proteins creating the dense core of the vesicles. This aggregation prevents the sorting of the proteins out of the vesicles [7]. Acidification of granular medium leads also to the activation of prohormone convertases (PC), giving rise to the conversion of pro-insulin into insulin by PC1/2 and carboxipeptidase E [8,9]. This maturation process also comprises the removal of the clathrin coat from the surface of the vesicles [10,11].

Once mature, ISGs can release their content by exocytosis under a specific stimulus such as the increase of blood glucose levels. The release of insulin at the plasma membrane is performed through the fusion of the vesicles with the membrane, and exocytosis of the ISG content. The priming, docking and fusion mechanisms rely on the formation of SNARE complexes [12,13], and their regulation by synaptotagmin [14]. Several Rab GTPases and Rab-related proteins also play an important role in vesicle exocytosis, being involved in the targeting and docking of the secretory vesicles to the plasma membrane, as well as the regulation of these steps [15].

ß-cell function and proper insulin secretion are essential for glucose homeostasis. The characterization of ISG composition and content, as well as the understanding of their biogenesis mechanisms, are indispensable information to understand ß-cell function and its defaults in pathologies such as type 2 diabetes. Thanks to the development of proteomics tools, two independent groups already published data on the ISG proteome using different approaches. Brunner et al. [6] published a list of 130 ISG proteins, resulting of a 2-step gradient purification of ISGs from INS-1E rat ß-cells in culture. Mass spectrometry (MS) data were generated using a MALDI-TOF/TOF instrument. Apart of granular proteins, some proteins known to be localized in mitochondria or lysosomes were also identified. In another study, Hickey and co-workers [16] enriched ISGs from the same cell line but using affinity purification. This allowed the identification of 51 proteins related to ISGs. The set of proteins identified in these 2 independent and complementary works seem to be far from the final figure of ISG related proteins. Indeed these studies notably suffered from the weak sensitivity of the MS analyzers employed. In addition, in a recent review, Suckale highlighted the limitations of the strategies employed for ISG purifications [17]. We propose here an improvement in ISG preparation and present the results obtained when we combined it with modern proteomics tools. For this, we add a third density gradient to our previous 2-step granule preparation method [6]. While associating it with the SILAC strategy [18], this procedure expanded our knowledge on ISG biogenesis, through the segregation of mature granules from immature granules and potential contaminants. Finally, this work pointed out a neuronal enzyme highly enriched in mature ISGs, the ProSAAS. ProSAAS has been used to exemplify the pertinence of the present enrichment procedure to further detect granular proteins associated to insulin processing and ß-cell dysfunction.

2. Experimental procedures

2.1. Cell culture

Rat insulinoma INS-1E cells were grown in RPMI1640 media supplemented with 10% fetal bovine serum. Normal culture conditions include 37 °C and 5% CO_2 in humidified atmosphere. SILAC media were prepared from RPMI-1640 media depleted in Arginine, Leucine and Lysine (Sigma-Aldrich), and supplemented with 10% of dialyzed bovine serum. Cells were grown 4 weeks (approximately 8 doublings) in either "light" or "heavy" media for complete amino-acid incorporation (>98%). "Light" medium was supplemented with Leucine (25 mg/L, Sigma-Aldrich), Lysine (25 mg/L, Sigma-Aldrich), and Arginine (100 mg/L, Sigma-Aldrich). "Heavy" medium was supplemented with $^{13}C_6$-Leucine, $^{13}C_6$–$^{15}N_2$-Lysine (Cambridge Isotope laboratories), and Arginine in the same concentrations.

2.2. Three-step gradient purification procedure

INS-1E cells were grown in the conditions described above. ISGs were enriched according to the protocol published in Brunner et al. [6] before being placed on the top of a continuous sucrose gradient (1–2 M), and ultra-centrifuged 8 h at 110,000 *g*. Nine 1 mL fractions were recovered, and sucrose was removed by a methanol/chloroform precipitation. ELISA assessed the insulin content (Mercodia), and repartition of marker proteins was assessed by Western blot using antibodies raised against Vamp4 (rabbit polyclonal antibody, Sigma), Beta-granin (rabbit polyclonal antibody, Eurogentec), GDH (rabbit polyclonal antibody, Rockland) and Cathepsin L (mouse monoclonal antibody, Abcam). Western blot quantitation was performed with ImageQuant (GE Health Care Life Sciences). Fractions were pooled to obtain a mix of "immature/contaminant" proteins (fractions D and E) and "mature" proteins (fractions G and H). The amount of proteins in each group was determined by ImageMaster 2D Patinium v6.0, on a silver staining of a 1D-gel. For quantitative experiments, light mature and heavy immature pools were mixed in a 1:1 ratio. The reverse experiment was also performed.

2.3. Protein separation by SDS PAGE

Ten μg of proteins of either "immaturesC_{12}/matureC_{13}" or "immaturesC_{13}/maturesC_{12}" mixes were solubilized in Laemmli buffer [19], and heated 5 min at 95 °C, before separation on a 12.5%T, 2.6%C polyacrylamide gel. Proteins were run for 1.5 cm, and the gel was stained with Coomassie Blue R250 (Merck).

2.4. Mass spectrometry analysis

Each SDS-PAGE lane (n=2) was cut into 8 identical slices and proteins contained in each slice were digested *in-gel* by