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Proteomics Analysis of Insulin Secretory Granules*Ⓢ

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Insulin secretory granules (ISGs) are cytoplasmic organelles of pancreatic β-cells. They are responsible for the storage and secretion of insulin. To date, only about 30 different proteins have been clearly described to be associated with these organelles. However, data from two-dimensional gel electrophoresis analyses suggested that almost 150 different polypeptides might be present within ISGs. The elucidation of the identity and function of the ISG proteins by proteomics strategies would be of considerable help to further understand some of the underlying mechanisms implicated in ISG biogenesis and trafficking. Furthermore it should give the bases to the comprehension of impaired insulin secretion observed during diabetes. A proteomics analysis of an enriched insulin granule fraction from the rat insulin-secreting cell line INS-1E was performed. The efficacy of the fractionation procedure was assessed by Western blot and electron microscopy. Proteins of the ISG fraction were separated by SDS-PAGE, excised from consecutive gel slices, and tryptically digested. Peptides were analyzed by nano-LC-ESI-MS/MS. This strategy identified 130 different proteins that were classified into four structural groups including intravesicular proteins, membrane proteins, novel proteins, and other proteins. Confocal microscopy analysis demonstrated the association of Rab37 and VAMP8 with ISGs in INS-1E cells. In conclusion, the present study identified 130 proteins from which 110 are new proteins associated with ISGs. The elucidation of their role will further help in the understanding of the mechanisms governing impaired insulin secretion during diabetes. ***Molecular & Cellular Proteomics 6:1007–1017, 2007.***

Insulin is a key molecule for the organism because it regulates glucose homeostasis (1), lipid and protein metabolism (2, 3), brain function (4), and cell survival (5). Pancreatic β-cells are the functional units responsible for the storage and secretion of insulin (6). These cells are able to adapt their rate of insulin secretion to blood glucose variations (7). Insulin is initially translated as a proinsulin precursor in the endoplasmic reticulum (ER),[1] is then transported from the ER to the Golgi apparatus, and crosses the Golgi network before being sorted into clathrin-coated immature insulin secretory granules (ISGs). The acidification of the lumen of immature ISGs through the action of ATPase proton pumps coincides with the cleavage of proinsulin into insulin and peptide C (8–10). These biochemical modifications trigger the maturation to mature ISGs. After glucose stimulation, mature ISGs release their insulin content into the extracellular space by exocytosis (11). One primary β-cell contains around 10,000 ISGs; however, only 100–200 ISGs are capable to quickly release their insulin content in response to an increased intracellular [Ca^{2+}] (12). The majority of ISGs belong to a reserve pool and must undergo several ATP-dependent reactions to become competent for final exocytosis (11, 13). Suppression of ISG-associated proteins such as vesicle-associated membrane protein 2 (VAMP2) or Rab3a affects insulin secretion demonstrating the functional importance of these proteins in the regulated insulin release (14, 15).

Whether insulin resistance or insulin secretion defects are primary in the development of type 2 diabetes has been a highly debated topic, and there is no consensus. However, it is clear that overt type 2 diabetes only occurs when the insulin output from the pancreatic islets fails to match the insulin requirement as a result of the insulin resistance (16). It is not clear whether the primary decline in insulin secretion results from a reduction in the number of β-cells, a progressive dysfunction of some of the β-cells, or a combination of both (17). The molecular mechanisms leading to insulin secretion impairment may partly be due to the modulation of the expression of several key ISG proteins. Their identification

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[1] The abbreviations used are: ER, endoplasmic reticulum; EGFP, enhanced green fluorescent protein; GDH, glutamate dehydrogenase; ISG, insulin secretory granule; Lamp, lysosome-associated membrane protein; PNS, postnuclear supernatant; SMT, sucrose-MOPS-Tris; Rab, Ras-associated bovine protein; VAMP, vesicle-associated membrane protein; 1-DE, one-dimensional gel electrophoresis; 2-DE, two-dimensional gel electrophoresis; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; v-SNARE, vesicle-associated SNARE; t-SNARE, target membrane SNARE.

would provide a better picture of the events involved in the onset of diabetes. So far only about 30 proteins have been clearly described to be associated with ISGs. However, according to 2-DE gel analysis, Hutton (18) suggested that ISGs potentially contain more than 150 polypeptides. To date the identity of these proteins remains to be determined.

This work presents for the first time a comprehensive picture of the ISG proteome from the rat β-cell line INS-1E. This cell line has previously proven useful for the elucidation of secretory granule protein function (19, 20). The ISG fraction was obtained by a two-step subcellular fractionation strategy, its protein content was separated by SDS-PAGE, and 130 proteins were identified by tandem mass spectrometry. Finally immunofluorescence experiments showed the newly found presence of Rab37 and VAMP8 proteins on ISGs.

EXPERIMENTAL PROCEDURES

Materials—All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich and were of the highest purity available. CH_3CN was purchased from Biosolve (Westford, MA). Percoll solution was obtained from GE Healthcare. Rabbit polyclonal antibody against betagranin was from Eurogentech (Seraing, Belgium), mouse monoclonal antibody against cathepsin L and pig polyclonal antibody against insulin were from Abcam (Cambridge, UK), rabbit polyclonal antibody against VAMP8 was from Synaptic System (Gottingen, Germany), mouse monoclonal antibody against GS-28 was from Calbiochem (EMB Biosciences), rabbit polyclonal antibody against lysosome-associated membrane protein 2 (Lamp2) was from Zymed Laboratories Inc.(Invitrogen), rabbit polyclonal antibody against cathepsin B was from Upstate (Chicago, IL), rabbit polyclonal antibody against calreticulin was a gift from Dr. K. H. Krause (University of Geneva, Geneva, Switzerland), rabbit polyclonal antibody against Glut2 was a gift from Dr. P. Meda (University of Geneva, Geneva, Switzerland), and rabbit polyclonal antibodies against Rab5 and cathepsin D were a gift from Dr. J. Gruenberg (University of Geneva, Geneva, Switzerland). Fluorescent dye-conjugated secondary antibodies (546nm anti-rabbit, 546nm anti-mouse, 488nm anti-rabbit, 488nm anti-mouse, and 488nm anti-pig) were from Fluoprobes (Chemie-Brunschwig, Basel, Switzerland). Insulin content was measured by ELISA (Mercodia, Uppsala, Sweden) according to the manufacturer's instructions.

Cell Culture and Transfection—The INS-1E clone from the insulin-secreting cell line INS-1 was cultured in RPMI 1640 medium and other additions as described by Merglen *et al.* (21). INS-1E cells were transfected with Lipofectamine^{TM} 2000 (Invitrogen) according to the manufacturer's instructions. The mouse Rab37 cDNA (GenBank^{TM} accession number AB232633) was amplified by PCR and subcloned into the BglII/EcoRI site of pEGFP-C1 vector (Clontech) as described previously (22).

Subcellular Fractionation for Insulin Secretory Granules Purification—ISGs were obtained by subcellular fractionation from INS-1E cells. All procedures were performed at 4 °C. Briefly about 8×10^8 INS-1E cells grown in 600-cm^2 dishes were washed once with PBS and scraped in cold PBS. Cells were then homogenized in 15 ml of 0.27 M sucrose, 10 mM MOPS-Tris (pH 6.8) (SMT) by three strokes through a 21-gauge needle followed by three strokes through a 25-gauge needle. Cell debris and nuclei were removed by centrifugation for 5 min at $1000 \times g$. The supernatant was transferred to a new tube, and the remaining pellet was homogenized in 15 ml of SMT by three strokes through a 21-gauge needle followed by five strokes through a 25-gauge needle. The homogenate was centrifuged for 5 min at $1000 \times g$, and the resulting supernatant was pooled with the first one. The pooled supernatant was finally centrifuged for 10 min at $1000 \times g$ to obtain the postnuclear supernatant (PNS). The PNS was then centrifuged at $24{,}700 \times g$ for 20 min to separate organelles from the cytosol. The resulting pellet was resuspended in SMT, loaded on a discontinuous Nycodenz gradient composed of three layers (23.4, 8.8, and 4.4%) and centrifuged at $107{,}000 \times g$ for 75 min. The Nycodenz enriched insulin granule fraction was recovered and loaded on a 27% Percoll solution, which was centrifuged at $35{,}000 \times g$ for 45 min. The Percoll enriched insulin granule fraction was recovered and washed three times with SMT to eliminate colloidal particles of Percoll.

Transmission Electron Microscopy—Fixation of the ISG pellet was performed by incubation in 2.5% glutaraldehyde for 1 h. The fixed pellet was washed three times with PBS, dehydrated, embedded in epoxy resin, and processed for electron microscopy as described previously (23). Ultrathin sections were contrasted with uranyl acetate and lead citrate and observed with a Technai 20 (FEI Co., Eindhoven, Netherlands) electron microscope.

Protein Separation by SDS-PAGE—Prior to SDS-PAGE, proteins were quantified using a Bradford assay (Bio-Rad). Equal amounts of proteins from the total cells and from the different cellular fractions were solubilized in sample buffer (62.5 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 0.1 M dithiothreitol, and traces of bromphenol blue) before separation on a 12.5% T, 2.6% C polyacrylamide gel (71). After electrophoresis proteins were stained with Coomassie Blue R-250 (Merck).

Immunoblotting—Seven micrograms of protein from each cellular fraction was separated by SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Bio-Rad). Immunoreactive bands were revealed by enhanced chemiluminescence using horseradish peroxidase-coupled secondary antibodies (1:2000) (Roche Applied Science).

Protein Identification by LC-MS/MS—A 1-DE gel lane corresponding to insulin secretory granules was cut in 28 consecutive pieces of gel. For each piece of gel "in-gel" digestion was performed as described by Scherl *et al.* (24). LC-MS/MS analysis with the LCQ ion trap (Thermo Finnigan, San Jose, CA) was performed as described by Burgess *et al.* (25). Peak lists were generated using Bioworks 3.1 software (Thermo Finnigan). The resulting .dta files from each analysis were automatically combined into a single text file. The resulting peak lists were searched against the rat International Protein Index (IPI) database version 3.20 (41,546 entries, European Bioinformatics Group) using Mascot operating on a local server (version 2.1, Matrix Science) and Phenyx (GeneBio). Mascot was used with average mass selected, a precursor mass error of 2.0 Da, and a peptide mass error of 1.0 Da. Trypsin was selected as the enzyme with three potential missed cleavages. ESI ion trap was selected as the instrument type, and oxidized methionine and carbamidomethylated cysteine were selected as variable modifications. For Phenyx, ion trap was selected for the instrument type, and LCQ was selected for the algorithm. Two search rounds were used, both with trypsin selected as the enzyme and oxidized methionine and carbamidomethylated cysteine selected as variable modifications. In the first round one missed cleavage was allowed, and the normal cleavage mode was used. This round was selected in "turbo" search mode. In the second round three missed cleavages were allowed, and the cleavage mode was set to half-cleaved. The minimum peptide length allowed was 6 amino acids, and the parent ion tolerance was 2.0 Da in both search rounds. The acceptance criteria were slightly lowered in the second round search (round 1: AC score, 7.0; peptide Z-score, 7.0; peptide p value, 1E$-$7; round 2: AC score, 7.0; peptide Z-score, 6.0; peptide p value, 1E$-$6). Only proteins that were identified with one or more high scoring

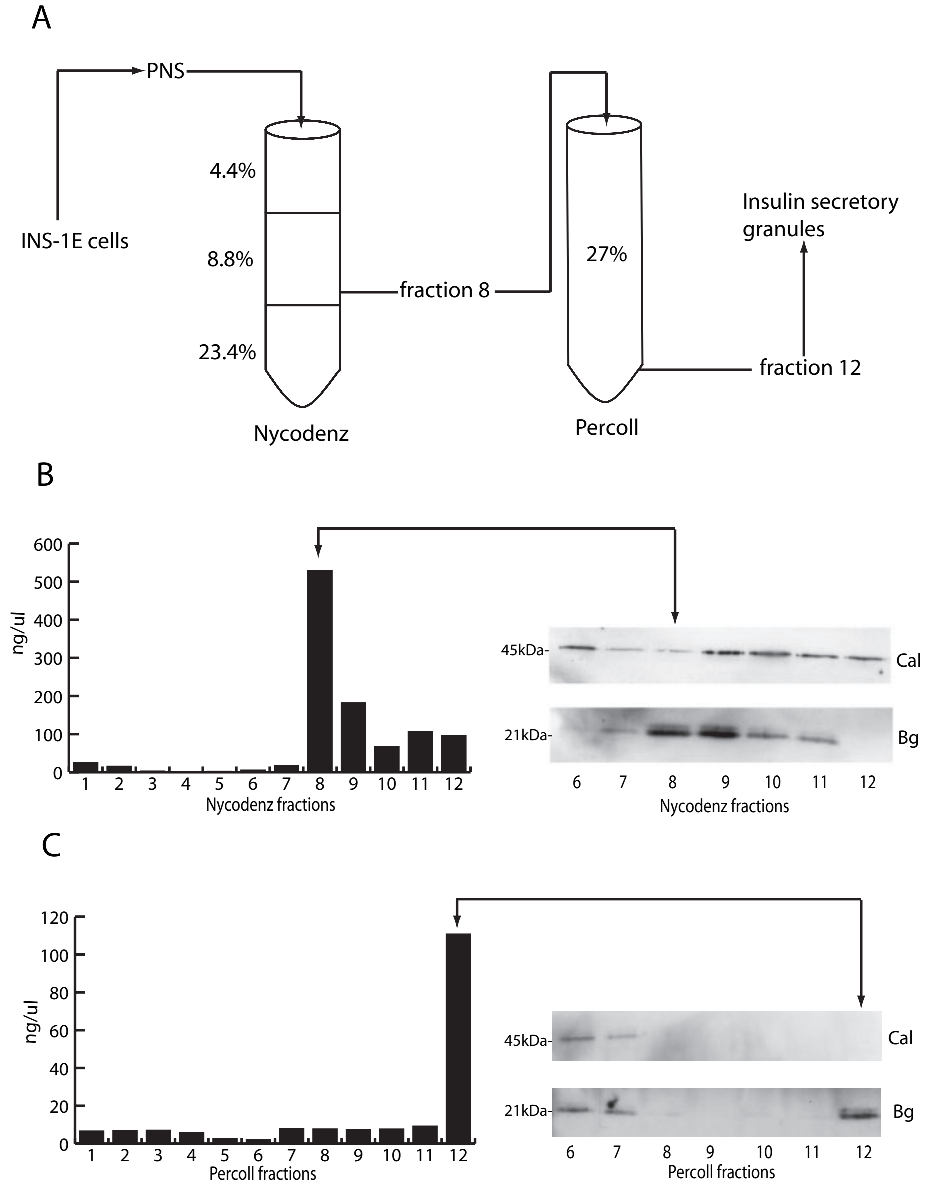

FIG. 1. *A*, schematic to illustrate the isolation of ISGs from INS-1E cells. *B*, *left*, insulin quantification in each of 12 fractions obtained from the Nycodenz gradient. *Right*, Western blot analysis of Nycodenz fractions 6–12 with an antibody directed to calreticulin (*Cal*) and to betagranin (*Bg*). *C*, *left*, insulin was quantified in each of 12 fractions obtained from the Percoll cushion. *Right*, Western blot analysis of Percoll fractions 6–12 with an antibody directed to calreticulin (*Cal*) and to betagranin (*Bg*).

peptides from both Mascot and Phenyx were considered to be true matches. "High scoring peptides" corresponded to peptides that were above the threshold in Mascot ($p < 0.05$) and Phenyx (p value $>1E-7$) searches. Peptides common to different proteins are in italic in Supplemental Table 2. All identified proteins were manually confirmed to contain at least one specific and non-redundant peptide.

Immunocytochemistry—INS-1E cells cultured on coverslips were fixed for 20 min in 3% (w/v) paraformaldehyde in PBS, washed three times with PBS, and exposed for 20 min in 50 mM NH_4Cl to avoid autofluorescence. Cells were then washed three times with PBS, blocked for 15 min in 10% FCS, and exposed for 45 min in PBS containing primary antibodies, 0.1% saponin, and 10% FCS. Cells were then exposed to fluorescent dye-conjugated secondary antibodies (1:100) for 30 min at room temperature. Samples were analyzed using a Zeiss laser confocal microscope (LSM 510 Meta, Carl Zeiss AG, Zurich, Switzerland). Images were taken with a 60× objective.

RESULTS

Efficiency of the Subcellular Fractionation—Efficient organelle proteomics analysis always depends on the quality of subcellular fractionation and organelle enrichment. Here the common continuous sucrose density gradient (0.45–2 M) permitted the separation of the two vesicular populations in INS-1 cells: ISGs and synaptic-like microvesicles (26). However, Western blot analyses of the obtained ISG fractions revealed the presence of calreticulin, indicating an ER contamination (data not shown). Therefore, we established a two-step subcellular fractionation protocol, which is composed of an initial discontinuous Nycodenz gradient followed by a 27% Percoll solution (Fig. 1*A*). To determine the enriched ISG fraction in the Nycodenz gradient, insulin was quantified in 12 fractions. Fraction 8

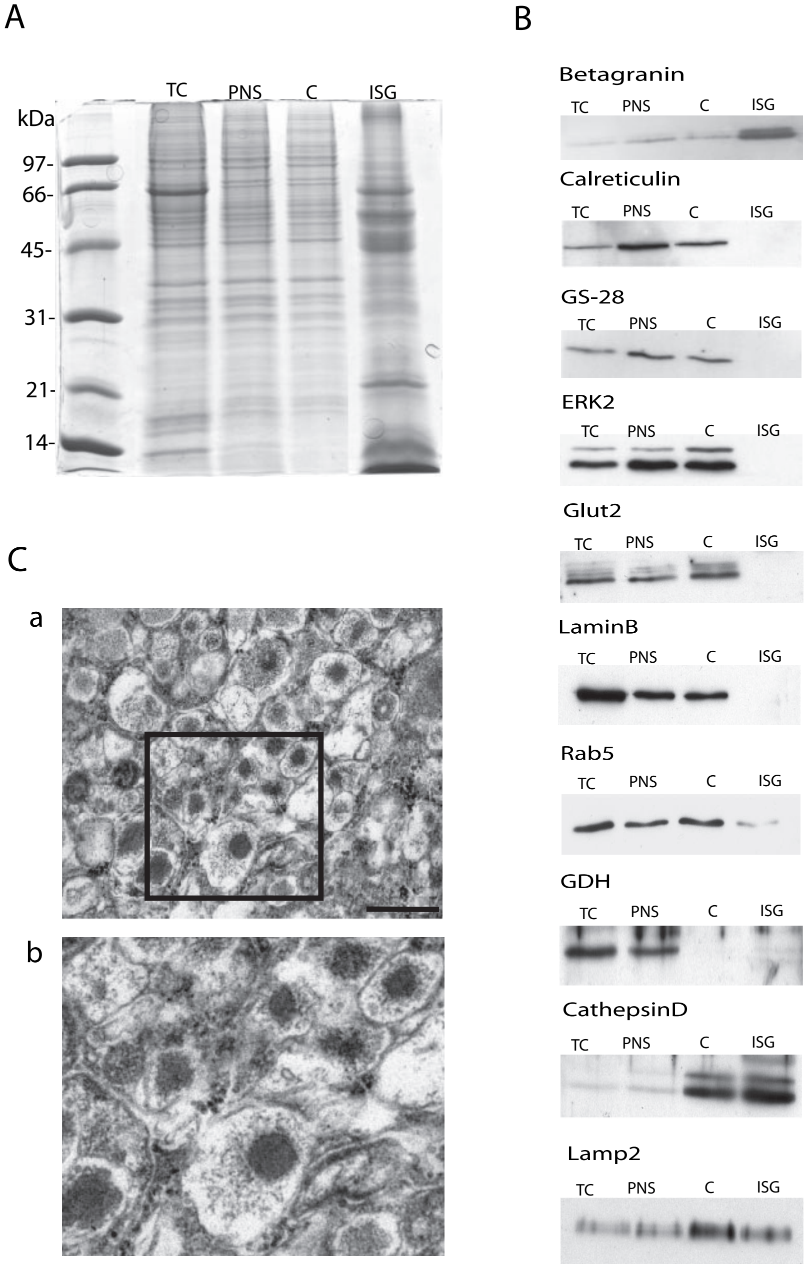

FIG. 2. *A*, insulin secretory granule protein separation by SDS-PAGE. Shown is the 1-DE separation of proteins extracted from total cells (*TC*) and from subcellular fractions as indicated on the *top* of the gel (*C*, cytoplasm). Each fraction (25 μg of proteins) was separated on a 12.5% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. *B*, immunoblotting assessment of insulin secretory granule enrichment. Western blot analyses of total cells (*TC*), PNS, cytoplasm (*C*), and ISGs using antibodies against betagranin, calreticulin, GS28, ERK2, Glut2, LaminB, Rab5, GDH, cathepsin D, and Lamp2. Each lane was loaded with 7 μg of proteins. *C*, *a*, representative electron micrograph of an ISG-enriched fraction. The insulin secretory granule fraction purified from INS-1E cells was fixed and processed for electron microscopy analysis as described under "Experimental Procedures." *Scale bar*, 500 nm. *b*, magnified image of the *boxed area*.

exhibited the largest level of insulin (Fig. 1*B*, *left panel*). Western blotting performed with antibodies against betagranin (N-terminal fragment of chromogranin A) or calreticulin (ER-resident protein) showed that fraction 8 contains higher levels of betagranin and lower levels of calreticulin (Fig. 1*B*, *right panel*). Thus, fraction 8 was chosen for subsequent separation on a Percoll solution. Analysis of the different fractions showed that in fraction 12 of this Percoll solution calreticulin was absent, whereas insulin and betagranin were present in high amounts (Fig. 1*C*). This fraction, referred to as the ISG fraction, was selected for subsequent proteomics analysis.

Subcellular Fractionation Validation by 1-DE Gels, Immunoblotting, and Electron Microscopy—To evaluate the ISG enrichment, 25 μg of proteins from each cellular fraction was separated by SDS-PAGE and stained with Coomassie Blue (Fig. 2*A*). The band patterns obtained from total cells, PNS, and cytoplasmic fractions were very similar. However, the band pattern of the ISG fraction was clearly different. Similar results were observed by silver staining (data not shown). These data demonstrate that this fraction is highly enriched in specific proteins.

To further investigate the enrichment of the ISG preparation, we performed Western blotting using antibodies against markers of various cellular compartments such as ISG (betagranin), endoplasmic reticulum (calreticulin), Golgi network (GS28), cytoplasm (ERK2), plasma membrane (Glut2), nucleus

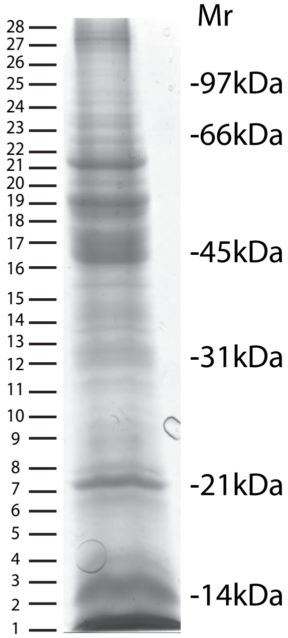

FIG. 3. **The insulin secretory granule lane was cut in 28 consecutive gel pieces for protein analysis by mass spectrometry.**

(LaminB), late endosome (Rab5), mitochondria (GDH), and lysosomes (Cathepsin D and Lamp2) (Fig. 2*B*). Betagranin, used as a positive control, was mainly recovered in the ISG fraction. In contrast, except Cathepsin D and Lamp2, the tested markers were absent or barely detectable (Rab5 and GDH) in the ISG fraction, indicating only minor contamination.

Consistent with these data, electron microscopy analysis revealed that the ISG fraction was highly enriched in ISGs. Furthermore ISGs maintained an intact morphology with the typical insulin dense core surrounded by filamentous structures (Fig. 2*C*) (27).

Separation and Identification of ISG Proteins—To characterize the ISG proteome, 28 consecutive SDS-PAGE gel bands were cut from the insulin granule lane (Fig. 3). LC-MS/MS identified 130 rat proteins (Table I and Supplemental Table 2). According to the literature we grouped the identified proteins in four structural classes as follows (Fig. 4): C1, intravesicular proteins ($n = 57$) such as chromogranin A (28), betagranin (29), or insulin (30); C2, membrane proteins ($n = 59$) including cysteine string protein (12), Rab3a, -b, -c, and -d (26), granuphilin (31), or VAMP2 and -3 (14); C3, novel proteins ($n = 4$) that are a small number of proteins with unpredictable function and cellular location; and C4, other proteins ($n = 10$) represented by proteins with various functions not previously described in insulin-secreting cells.

Immunocytochemistry Validation—Because numerous lysosomal hydrolases were identified in the C1 class, we first evaluated the cellular distribution of cathepsin B, D, and L by immunofluorescence in INS-1E cells (Fig. 5). Confocal microscopy images showed that all three isoforms are expressed in compartments containing immunoreactive insulin. These proteins were also detected in nuclear near regions devoid of insulin (Fig. 5, *A*–*C*).

Regarding the C2 class, we next assessed the distribution of the Lamp2. Consistent with cathepsin localization, Lamp2 was observed in both areas close to the membrane containing insulin and in the perinuclear region without the hormone (Fig. 6*A*).

Because the C2 class contains numerous vesicle-associated proteins including Rabs and VAMPs, we focused on Rab37 and VAMP8. Double labeling experiments showed that VAMP8 mainly colocalizes to ISGs (Fig. 6*B*); the protein was also concentrated in perinuclear organelles devoid of insulin. Given that Rab37 antibody was not appropriate for immunofluorescence studies, we transiently overexpressed EGFP-tagged Rab37 in INS-1E cells. Fig. 6*C* shows that EGFP-Rab37 was predominantly associated with ISGs.

DISCUSSION

Isolation of Insulin Secretory Granules

Subcellular fractionation has been widely used for proteomics analyses of different intracellular organelles and complexes such as mitochondria (32), Golgi network (33), lysosome (34), phagosome (35), endosome (36), peroxisome (37), or nucleoli (38). The main inconvenience of this approach is the potential presence of other subcellular structures that could share similar physical properties and comigrate in density gradients. Therefore, a complete purification of an organelle by subcellular fractionation is hardly possible. However, this approach is still the most efficient strategy to obtain enriched organelles of interest for further proteome studies (39). Regarding ISG purification, we first tested a continuous sucrose density gradient (0.45–2 M) that showed a poor separation of ISGs from the endoplasmic reticulum. Therefore, a two-step fractionation strategy with an initial gradient of Nycodenz followed by a Percoll solution was applied. This method generated a highly enriched fraction of ISG proteins as demonstrated by Western blot and electron microscopy experiments.

Proteomics of Insulin Secretory Granules

To date about 30 proteins have been reported as being associated with insulin granules. However, Guest *et al.* (40) have shown that a few hundred polypeptides could be detected by 2-DE gel analyses of an enriched ISG fraction. The main disadvantage of using 2-DE gels is the inability to analyze membrane proteins, which could be of primary importance in vesicles such as ISGs. In contrast, 1-DE gels coupled to liquid chromatography permitted a good extraction and separation of both soluble and insoluble proteins (24) and was therefore used in the present study. MS analyses were performed on the tryptic digests coming from 28 consecutive gel slices. This strategy identified 130 different proteins. Twenty of them have been reported previously to colocalize with ISGs, whereas further validation is required for the remaining 110.

Table I

Proteins identified in insulin secretory granules by LC-MS/MS analysis

GM2, GalNAcβ 4(Neu5Acα 3)Galβ 4GlcCer; SPARC, secreted protein acidic and rich in cysteine; ADAM, a disintegrin and metalloproteinase; GLI, glioma.

Protein name	ISG proteins	Refs.
A) Intravesicular proteins		
Hydrolase		
α-*N*-Acetylgalactosaminidase precursor		
Arylsulfatase B		
β-Hexosaminidase α chain precursor		
β-Hexosaminidase β chain precursor		
Carboxypeptidase E precursor	x	58
Carboxypeptidase N catalytic chain precursor		
Cathepsin B precursor	x	8
Cathepsin D precursor		
Cathepsin F		
Cathepsin L precursor	x	8
Di-*N*-acetylchitobiase precursor		
Dipeptidyl-peptidase 2 precursor		
Galactosamine (*N*-acetyl)-6-sulfate sulfatase		
Glucosamine (*N*-acetyl)-6-sulfatase		
Glucosidase, α; acid		
Mannosidase 2, α B1		
N^4-(β-*N*-Acetylglucosaminyl)-L-asparaginase precursor		
N-Acylsphingosine amidohydrolase 1		
Neuroendocrine convertase 2 precursor	x	59
Neuroendocrine protein 7B2 precursor		
Palmitoyl-protein thioesterase 1 precursor		
Plasma α-L-fucosidase precursor		
Predicted: similar to α-galactosidase A precursor		
Predicted: similar to α-*N*-acetylglucosaminidase		
Predicted: similar to angiotensinase C-like		
Predicted: similar to β-galactosidase precursor		
Predicted: similar to sialate *O*-acetylesterase precursor		
ProSAAS precursor		
Protective protein for β-galactosidase		
Retinoid-inducible serine carboxypeptidase precursor		
Sialidase-1 precursor		
Tissue α-L-fucosidase precursor		
Tripeptidyl-peptidase 1 precursor		
Secreted		
Chromogranin A precursor	x	60
Clusterin precursor		
Ependymin-related protein 2		
Fibronectin precursor		
Insulin-1 precursor	x	61–63
Insulin-2 precursor	x	61–63
Isoform PAM-3B of peptidyl-glycine α-amidating monooxygenase precursor		
Neuronal pentraxin-1 precursor		
Nucleobindin-2 precursor		
Predicted: similar to brain link protein 2		
Prx IV (peroxiredoxin 4)		
Secretogranin-1 precursor	x	64, 65
Secretogranin-2 precursor		
Secretogranin-3 precursor	x	66, 67
Stanniocalcin-1 precursor		
Sulfated glycoprotein 1 precursor		
Transcobalamin-2 precursor		
Wnt inhibitory factor 1 precursor		
Other		
10-kDa heat shock protein, mitochondrial		
Cystatin C precursor		
Malate dehydrogenase, mitochondrial precursor		
Protein-disulfide isomerase A3 precursor		
Superoxide dismutase		
Vitamin D-binding protein precursor		
B) Membrane proteins		
Rab		
Ras-related protein Rab1a		
Ras-related protein Rab1b		
Ras-related protein Rab2a		
Ras-related protein Rab3a	x	26
Ras-related protein Rab3c	x	26
GTP-binding protein Rab3d	x	26
Small GTP-binding protein Rab5		
Predicted: similar to Rab5b, member Ras oncogene family		
Predicted: similar to Ras-related protein Rab5c		
Ras-related protein Rab7		
Rab10, member Ras oncogene family		
Ras-related protein Rab14		
Ras-related protein Rab35		
25-kDa protein (Rab37)		
Vacuolar ATPase		
ATPase, H^+-transporting, lysosomal 34 kDa, V1 subunit D		
ATPase, H^+-transporting, lysosomal 50/57 kDa, V1 subunit H		
ATPase, H^+-transporting, V0 subunit D isoform 1		
Predicted: similar to ATPase, H^+-transporting, lysosomal accessory protein 2		
Predicted: similar to ATPase, H^+-transporting, V1 subunit A	x	9
Predicted: similar to vacuolar H^+-ATPase G1		
Vacuolar ATP synthase subunit B, brain isoform		
VAMP		
Vesicle-associated membrane protein 2B	x	68
Vesicle-associated membrane protein 3	x	14
Vesicle-associated membrane protein 7		
Vesicle-associated membrane protein 8		
Other		
112-kDa protein (receptor-type tyrosine-protein phosphatase N2 precursor)	x	69
ADP-ribosylation factor-like protein 8B		
Annexin A4		
ATP synthase α chain, mitochondrial precursor		
CD81 antigen		
DnaJ homolog subfamily C member 5	x	12
Ectonucleotide pyrophosphatase/phosphodiesterase 5 precursor		
GM2 ganglioside activator protein		

TABLE I—*continued*

Protein name	ISG proteins	Refs.
Guanine nucleotide-binding protein $G_i/G_s/G_t$ subunit β 2		
Lipid phosphate phosphohydrolase 1		
Lipolysis-stimulated lipoprotein receptor precursor		
LRRGT00199 (ATP synthase γ chain, mitochondrial)		
Lysosome membrane protein 2		
Lysosome-associated membrane glycoprotein 1 precursor		
Lysosome-associated membrane glycoprotein 2 precursor		
Nucleobindin-1 precursor		
Phosphate carrier protein, mitochondrial precursor		
Phospholipase D_3		
Predicted: similar to ADAM 22 precursor		
Predicted: similar to annexin A10		
Predicted: similar to GLI pathogenesis-related 2		
Predicted: similar to glucosylceramidase precursor (β-glucocerebrosidase)		
Predicted: similar to SPARC/osteonectin, CWCV and Kazal-like domains proteoglycan 2		
Predicted: similar to transmembrane protein 63a		
Predicted: similar to vesicle transport through interaction with t-SNAREs 1B homolog		
Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3 precursor		
Proteolipid protein 2 (by similarity)		
Ras-related protein Rap-1A precursor (or Rap-1B)		
Receptor-type tyrosine-protein phosphatase-like N precursor		
Secretory carrier-associated membrane protein 1		
Synaptotagmin 5	x	20
Synaptotagmin-like protein 4 (granuphilin)		
Thy-1 membrane glycoprotein precursor		
Voltage-dependent anion-selective channel protein 1	x	9
C) Other proteins		
14-3-3 protein ζ/δ		
14-3-3 protein γ		
14-3-3 protein θ		
Actin, cytoplasmic 1		
Amplified in osteosarcoma (OS-9)		
Fructose-bisphosphate aldolase A		
Glyceraldehyde-3-phosphate dehydrogenase	x	70
Mama		
Peptidyl-prolyl cis-trans isomerase A		
RDCR-0918-3 protein		
D) Novel proteins		
Hypothetical protein MGC72560		
Hypothetical protein LOC498284 (mouse: intelectin-1a 59)		
Hypothetical protein RGD1305246		
Predicted: hypothetical protein		

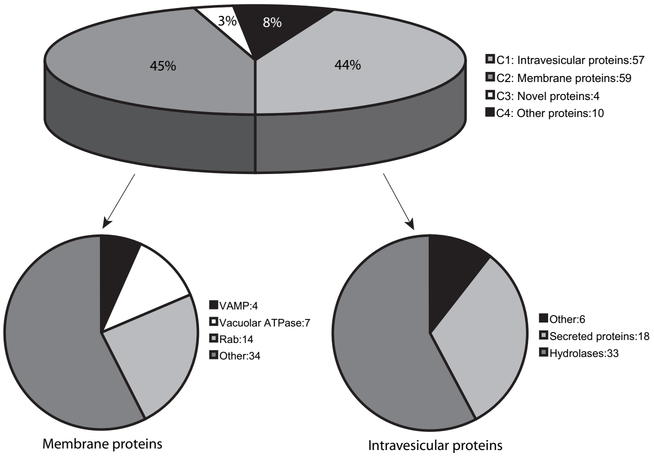

FIG. 4. **Illustration in four functional classes of the 130 insulin granule proteins identified in this study.** See the list in Supplemental Table 2. The number (*n*) and the percentage of each class are shown.

The Structural Classification of ISG Proteins

According to the literature we could group the identified proteins in four structural classes: C1, intravesicular proteins ($n = 57$); C2, membrane proteins ($n = 59$); C3, novel proteins ($n = 4$); and C4, other proteins ($n = 10$). These four classes are discussed below.

Intravesicular Proteins—A large fraction of the 57 proteins is represented by secreted proteins ($n = 18$), which include insulin, chromogranin A, betagranin, and secretogranin. A substantial number of proteins are represented by hydrolases ($n = 33$) including different forms of cathepsin (B, D, and L) or glucuronidase. By immunofluorescence experiments, we located cathepsin B, D, and L in ISGs and in other vesicles most likely corresponding to lysosome. Several authors (18, 41) have previously described the presence of hydrolases within ISGs. Kuliawat *et al.* (42) showed by electron microscopy that both cathepsins B and L are present in immature β-granules, whereas only the immunolabeling for cathepsin L (but not B) persists in mature granules. The authors could demonstrate that the sorting of these enzymes from the immature granules follows the same mechanisms as that in lysosomes, *i.e.* involving the mannose 6-phosphate receptor. These results might be consistent with the "sorting by retention" hypothesis in primary β-cells (43). In this hypothesis the immature secretory granules are the sorting point for proteins targeted either to mature granules or to lysosomes. On the contrary, in the "sorting for entry" hypothesis, the trans-Golgi network is the unique sorting point for ISG proteins and for proteins of other organelles (44). Alternatively to the above hypothesis, lysosomal proteins could come from the crinophagy process. Effectively the insulin content of β-cells is balanced through its biosynthesis, secretion, and degradation. Intracellular degradation of insulin is performed by crinophagy through the fusion of secretory granules with lysosomes (45). The three

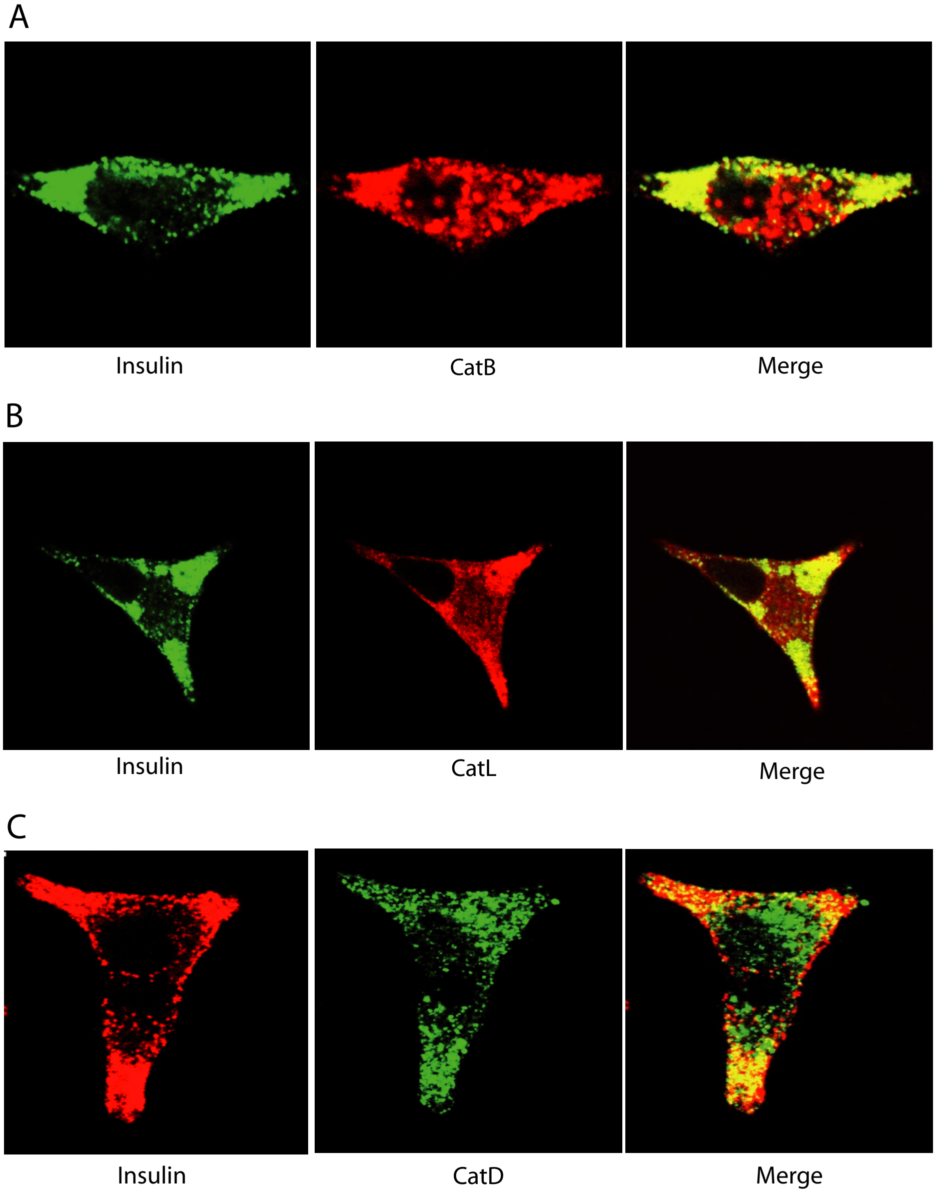

FIG. 5. **Intracellular localization of cathepsins.** INS-1E cells were analyzed by confocal microscopy after double labeling with an antibody against insulin and with cathepsin B (*CatB*), cathepsin L (*CatL*), or cathepsin D (*CatD*) antibodies; the cells were then exposed to FITC- or rhodamine-conjugated secondary antibodies. Merged images are shown in the *right panels*.

alternative hypotheses are not exclusive because it is possible that the sorting by retention hypothesis only occurs in cells having regulated secretion such as β-cells (43). All together our results strongly support the sorting by retention hypothesis. Further enrichment of immature secretory granules will be required to investigate the role of well selected hydrolases in the early biogenesis of ISGs.

Membrane Proteins—A second large class of identified proteins is represented by membrane or membrane-associated proteins (n = 59). We identified 14 different Rab proteins. Rabs are monomeric GTPases of the Ras superfamily that regulate different steps of vesicular trafficking. Although the role of Rab proteins in vesicle fusion is fairly well recognized, the precise task of such Rabs in transport is still unknown (46). Among these proteins only the Rab3 isoforms (26) and Rab27a (47) were shown to be associated with ISGs. Both proteins are involved in the regulation of insulin exocytosis together with their specific effectors such as Noc2, granuphilin, or the Rab3-interacting molecule RIM (48–51). Among the 14 Rabs, we identified Rab12, which is associated with secretory granules of atrial myocytes and Sertoli cells (52, 53), as well as Rab37, which is found on secretory granules in mast cells (54). Our immunofluorescence experiments revealed that overexpressed EGFP-Rab37 is associated with ISGs in INS-1E cells suggesting its functional role in the regulation of insulin secretion.

The VAMPs are members of the v-SNARE family, which participates in diverse intracellular docking/fusion events by pairing with their cognate t-SNAREs on the target membrane (55). We identified four VAMPs. VAMP2 and -3 are associated

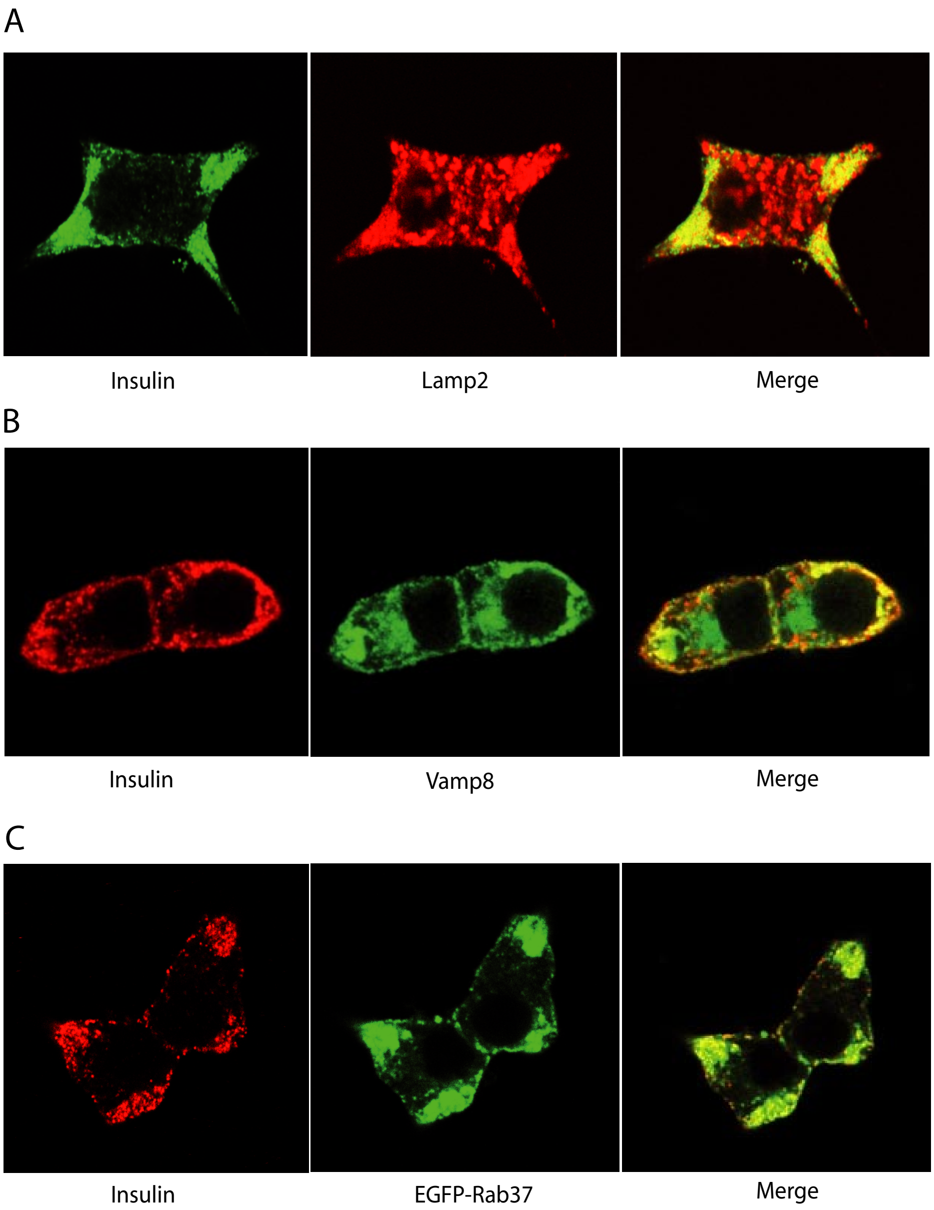

FIG. 6. **Subcellular distribution of Lamp2, VAMP8, and Rab37.** *A* and *B*, INS-1E cells were double labeled with an antibody directed to insulin together with anti-Lamp2 or anti-VAMP8 and further exposed to FITC- or rhodamine-conjugated secondary antibodies. *C*, EGFP-Rab37 was transiently transfected in INS-1E cells. Three days later the cells were stained with an anti-insulin antibody coupled to rhodamine. Overlapping images are shown in the *right panels*.

with ISGs (14), whereas VAMP7 and -8 are colocalized with late endosomes (56) and to zymogen granules of pancreatic acinar cells (57), respectively. Our confocal microscopy analysis demonstrated that VAMP8 is located on ISGs and other organelles probably corresponding to early endosomes as reported for the insulinoma MIN6 (57). We found other membrane proteins involved in the regulated exocytosis such as synaptotagmin 5 or annexin A4 as well as different forms of the vacuolar ATPase or voltage-dependent anion channel.

The finding of known lysosomal proteins in our preparation was not limited to hydrolases because Lamp2, a lysosome-associated membrane protein, was identified as well. Using immunofluorescence, Lamp2 was detected on ISGs and other vesicular structures corresponding most likely to lysosomes.

Novel Proteins—For four of the 130 identified proteins we could not assign any biological function using sequence similarities, domain composition, or pathway information.

Other Proteins—Finally the function of a relative small proportion ($n = 10$) of identified proteins like the fructose-bisphosphate aldolase A or the peptidyl-prolyl cis-trans isomerase A has never been described in β-cells. Therefore, the validation and the association of these proteins with ISGs should be further investigated.

In conclusion, the present proteomics analysis of ISGs revealed 130 proteins. Confocal microscopy analysis of some selected proteins demonstrated their presence on ISGs. The validation of hydrolases and lysosomal proteins within ISGs supports the sorting by retention hypothesis. The association of Rab37 and VAMP8 proteins with ISGs was also highlighted for the first time suggesting an even more complex mechanism for vesicle trafficking than described previously in β-cells. In summary, these results clearly demonstrate that our strategy has determined a new subset of insulin secretory granule proteins that will further help to unravel the complex molecular anatomy and functional structure of insulin secretory granules.

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