Published in final edited form as:
J Proteome Res. 2007 August ; 6(8): 2978–2992. doi:10.1021/pr0607029.

Proteomic Analysis of Pancreatic Zymogen Granules: Identification of New Granule Proteins

Michael J. Rindler[+*], **Chong-feng Xu**[§], **Iwona Gumper**[+], **Nora N. Smith**[+], and **Thomas A. Neubert**[§]

+*Department of Cell Biology, New York University School of Medicine, New York, NY 10016*

§*Department of Pharmacology and Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, NY 10016*

Abstract

The composition of zymogen granules from rat pancreas was determined by LC-MS/MS. Enriched intragranular content, peripheral membrane and integral membrane protein fractions were analyzed after one-dimensional SDS/PAGE and tryptic digestion of gel slices. A total of 371 proteins were identified with high confidence, including 84 previously identified granule proteins. The 287 remaining proteins included 37 GTP-binding proteins and effectors, 8 tetraspan membrane proteins, and 22 channels and transporters. Seven proteins – pantophysin, cyclic nucleotide phosphodiesterase, carboxypeptidase D, ecto-nucleotide phosphodiesterase 3, aminopeptidase N, ral, and the potassium channel TWIK-2 – were confirmed by immunofluorescence microscopy or by immunoblotting to be new zymogen granule membrane proteins.

Keywords

proteomics; mass spectrometry; LC-MS/MS; pancreas; zymogen granules; acinar cells

Introduction

Pancreatic acinar cells are the sites of synthesis and storage of digestive enzymes released into the pancreatic duct for transport to the duodenum.[1] The enzymes reside in a storage organelle in the cell cytoplasm, the zymogen granule (ZG), and are released by exocytosis into the acinar lumen. The exocytosis process involves the fusion of the ZG membranes with the apical plasma membrane and occurs after acinar cells are stimulated by hormones such as cholecystokinin and acetylcholine. Despite the relevance of the ZG as a mediator of digestive function, its exocytosis is only beginning to be understood at the molecular level.

Exocytosis of zymogen granules at the acinar lumen is dependent in part upon granule membrane proteins such as the SNARE (N-ethylmaleimide-sensitive factor attachment protein receptor) components syntaxin 3 and vamp 8.[2,3] Recently, other proteins playing a role in exocytosis have been documented in pancreas yet were never identified as being present in ZG membranes. One such protein is Noc2, a rab-interacting protein strictly required for ZG exocytosis[4] and known to be present in endocrine and salivary gland granules.[5] As a post-Golgi transport intermediate that can be purified in large quantities, the ZG membrane should be an abundant source of proteins involved in exocytosis and granule biogenesis in exocrine

*Address Correspondence to: Michael Rindler, Ph.D., Associate Professor, Department of Cell Biology, New York University School of Medicine, 550 First Avenue, New York, NY 10016, Tel: 212-263-5812, Fax: 212-263-8139, E-mail address: rindlm01@med.nyu.edu.

pancreas. Many of these proteins are likely to be involved in similar processes in other cell types as well.

Zymogen granule membranes also contain digestive enzymes such as GP-3/pancreatic lipase-related protein 2.[6,7] Some of the membrane proteins are released into the acinar lumen and appear in the pancreatic juice. GP2, the major granule membrane protein, is anchored via a glycosylphosphatidyl inositol linkage and has both membrane and soluble forms[8] as does ITMAP, a granule transmembrane protein.[9] Membranes contain proteins that are thought to aid in granule formation. These include muclin, a sulfated glycoprotein in mouse pancreas;[10] ZG16, a lectin thought to help form a protein scaffold for attachment of granule content proteins to the membrane;[11] and syncollin, which regulates granule size and release.[12,13] ZG membranes also have ion channels and transporters important for maintaining low pH and the ionic milieu in the granule lumen.[14]

The major zymogen granule content proteins have been identified previously by purification of the proteins, conventional N-terminal peptide sequencing techniques and specific immunolocalization of granule components. The granules contain hydrolytic enzymes such as amylase, the most abundant protein, trypsinogens, elastases and chymotrypsinogen.[15] The importance of the granule content proteins for disease processes has been highlighted by the demonstration that individuals with mutations in trypsinogen are predisposed to pancreatitis because of the premature activation of the zymogen trypsinogen to trypsin.[16]

Because of the importance of knowing the composition of the intragranular content as well as granule membrane proteins, we undertook a comprehensive identification of these proteins by LC-MS/MS. Methods for obtaining highly purified secretory granules and their membranes have been reported (summarized in [17]). We prepared highly enriched content and membrane fractions and analyzed their protein composition. More than 500 proteins were identified in the preparation, including 371 with high confidence, of which 84 are known granule proteins. A number of these proteins potentially are important regulators of granule exocytosis. We confirmed seven of the newly identified proteins to be granule membrane proteins.

Materials and Methods

Isolation of Zymogen Granules. The purification of zymogen granules was performed as described previously in detail.[17] In brief, pancreas was removed from male and female Sprague Dawley or Brown Norway rats (for preparation of content fractions only) fed ad libitum, then anesthetized using CO_2 narcosis in compliance with NIH animal care and use guidelines and decapitated. The pancreas was washed three times in homogenization buffer (HB: 0.27 M sucrose, 2 mM MOPS, 0.5 mM $MgSO_4$, pH 6.5–6.7). All solutions contained a protease inhibitor cocktail of 1 μg/ml each of leupeptin and pepstatin (Roche Applied Science, Indianapolis, IN), 10 U/ml aprotinin (Sigma-Aldrich, St. Louis, MO), and either 0.5 mM phenylmethanesulfonyl fluoride (PMSF) or benzamidine (Sigma-Aldrich). The mesentery was trimmed from the pancreas and the tissue minced with scalpels and fine scissors. After homogenization with three strokes of a Teflon-glass homogenizer (Wheaton, Milville, NJ), a post-nuclear supernatant was prepared after centrifugation at 600 *g* for 10 min. The pellet was rehomogenized and centrifuged again. The supernatants were combined and filtered through nylon mesh. An aliquot from the post-nuclear supernatant was saved. The granules were recovered either by centrifugation at 1750 *g* for 20 min. (content protein preparation 1 = C1) or by centrifugation at 3000 *g* for 30 min. through 5 ml cushions of 30% and 60% Percoll (GE Healthcare, Piscataway, NJ) in gradient buffer (GB: 0.26 M sucrose 20 mM MOPS, 1 mM EDTA pH 6.7) (all other preparations). Granule pellets were either washed twice in HB with 1 mM EDTA (HBE) added to remove mitochondria and gently resuspended in HB with 1 mM EDTA (C1) or the loose granule pellets in 60% Percoll were directed resuspended in HBE.

After homogenization (5 strokes in a Dounce homogenizer) the crude granule preparation was loaded onto 60% Percoll in GB and spun for 35 min. at 12,000 *g*. The white granules at the bottom of the gradient were gently resuspended in HBE, homogenized in a Dounce homogenizer and loaded onto 0.6 – 2 M sucrose gradients (10 mM MOPS, 5% Ficoll (Sigma-Aldrich), 1 mM EDTA pH 6.7). After centrifugation for 4 h at 100,000 *g* using an SW41 rotor and an Optima L-90K ultracentrifuge (Beckman Coulter, Fullerton, CA), the granule band near the bottom of the tube was collected and diluted with HBE. Granules were pelleted by centrifugation at 3000 *g* for 20 min.

After resuspension of the granule pellets in HBE, 5–6 volumes of 0.1 M $NaHCO_3$, pH 8.1 were added to lyse the granules. After 45 min. of incubation, the lysed granules were loaded onto 0.5 – 1.1 M sucrose gradients in gradient buffer (GB = 10 mM HEPES. 1 mM EDTA pH 7.0) and the gradients were centrifuged for 13 h at 80,000 *g*. The granule content was collected from the top of the gradient and concentrated by precipitation with trichloroacetic acid (TCA). The granule membrane band was collected and diluted with GB and recovered by centrifugation at 200,000 *g* for 20 min. using a TL100.3 rotor in a TI-100 ultracentrifuge (Beckman Coulter). The membranes were sonicated and washed 2 more times in 0.1 M $NaHCO_3$. Membranes from the post-nuclear supernatants and other steps in the fractionation procedure were collected and washed using the same methods.

To prepare peripheral and integral membrane fractions, the membranes were subjected to carbonate extraction as previously described.[8] Membranes were sonicated in 0.1 M $NaCO_3$, pH 10.8, incubated at 4° C for 30 min. and then centrifuged at 200,000 *g* as described above. The extraction was repeated a second time. Membranes recovered after centrifugation were washed in 10 mM HEPES 1 mM EDTA. The carbonate washes (supernatants) were combined and the proteins recovered by TCA precipitation (peripheral membrane protein fraction). All fractions were frozen and stored at −80° C until use. Protein concentrations were determined using the Bradford method (Bio-Rad, Hercules, CA).

Bovine pancreatic granules were prepared by a modification of the procedure of Greene et al.[1] Fresh bovine pancreas was obtained from a local slaughterhouse, minced and homogenized as described above for the preparation of rat ZGs except the HB contained 0.8 M sucrose (HBH). Granules were isolated by centrifugation at 1000 *g* for 10 min. This centrifugation was repeated a second time and the white granule pellets were washed to remove mitochondria then were slowly resuspended in HBH with 1 mM EDTA (HBHE) and loaded on Percoll gradients as described above except with 0.7 M sucrose. The loose granule pellets at the bottom of the Percoll gradients were resuspended in HBHE and the granules recovered by centrifugation at 3000 *g* for 20 min. The granule pellets were resuspended in HBHE and lysed with $NaHCO_3$ as described above. The membrane and content fractions were also isolated as described above for rat pancreas.

LC-MS/MS Analysis. Four independent ZG preparations from rat pancreas were analyzed by LC-MS/MS. Proteins from two intragranular content preparations, one integral membrane and one peripheral membrane preparation were separated by SDS/PAGE on 10% gels after heating to 100°C. for 2 min. in the presence of 50 mM dithiothreitol. Fifty µg of protein was loaded on each of two adjacent lanes. Gels were fixed in 46 % methanol, 7 % acetic acid, and 0.1% Coomassie Blue R-250, destained in this solution without Coomassie, and stored in 1% acetic acid until use. Silver staining of parallel samples using the method of Wray et al[18] was used for documentation purposes only.

Twenty to 30 contiguous slices were excised from each gel. Each slice was digested using mass spectrometry grade trypsin (Promega, Madison, WI) at 12.5 to 25.0 ng/µL in 25mM NH_4HCO_3 buffer. For the peripheral membrane and integral membrane sample, 10% AcN was

added to the digestion buffer. The resulting peptides were extracted and dried under vacuum, then resuspended in 10 µl of 0.1% formic acid. Four to 6 µl of peptide mixtures were analyzed using nanoflow LC/ESI-MS/MS. The content extracts were analyzed with a CapLC HPLC coupled directly to a QTOF Micro MS (Waters, Milford, MA) and the membrane extracts were analyzed with a nanoAcquity UPLC coupled directly to a QTOF Premier MS (Waters).

For all preparations, a C18 pre-column was used to load the sample to a 75-µm × 15-cm fused silica C18 analytical column. In the case of CapLC, PepMap100 was used as the analytical column (LC Packings, Dionex, Sunnyvale, CA), whereas Atlantis columns (Waters) were used for UPLC. A gradient of 2–40 % ACN in 0.1% formic acid was delivered over 80 or 120 min at a flow rate of 200 nL/min through a fused silica distal end-coated tip nano-electrospray needle (New Objective, Woburn, MA). The data acquisition involved MS survey scans and automatic data-dependent MS/MS acquisitions, which were invoked after selected ions met preset parameters of minimum signal intensity of 8 counts per second, ion charge state 2+, 3 +, or 4+, and appropriate retention time. Survey scans of 1 s were followed by MS/MS of the three most intense ions for up to 6.6 s each, or until 6,000 total MS/MS ion counts per precursor peptide were obtained. The raw MS data were subsequently processed using ProteinLynx software (Waters), which generated DTA or PKL files from each MS/MS spectrum, which were merged into a single file containing all spectra from all of the gel bands from a single lane (i.e. one PKL file for each granule preparation).

Protein Identification Criteria. Stringent criteria for determining whether protein matches are genuine have been previously established and applied by us to the analysis of the neuronal post-synaptic density.[19] We used similar criteria in these studies. The DTA or PKL files were used to search the NCBI and Swissprot nonredundant protein databases (updated on June 7, 2006) using the Mascot search engine (Version 2.1.0, Matrix Science, Boston, MA). The search parameters included peptide mass tolerance of up to 1.5 Da, MS/MS mass tolerance of up to 0.5 Da, and variable oxidation of methionines with up to one missed tryptic cleavage allowed. The Mascot algorithm was used to determine peptide and protein expectation values. Each protein in the peptide summary report that met the probability-based Mowse threshold [$p(x) < 0.05$] determined by Mascot were analyzed by a stricter set of criteria to determine the number of unique peptide matches. To pass our threshold, peptides had to have a minimum 6 residues, a Mascot probability-based score >15, an expectation value of <0.5, a precursor ion mass error of <0.5 Da, no missed cleavages and a rank of 1. In addition, the peptide could not appear in other proteins of higher score, and for each protein, only the highest scoring peptide (lowest expectation value) with a given amino acid sequence was considered. In general, each protein match was required to have at least two nonredundant unique peptides meeting these criteria, but not also found in other proteins identified unless they were isoforms of the same protein. Exceptions were made for proteins previously shown to be in ZGs, in pancreatic juice, or in granules of parotid gland, which are close relatives, or to be highly similar to known granule proteins. For proteins with closely related family members sharing peptides, one unique peptide and one shared peptide was considered sufficient for assignment purposes. Spectra of peptides from proteins scoring 50 or below were visually inspected to assure that the peptide assignments were reliable.

Functional annotation and organelle assignments were made using ProteinCenter (Proxeon Biosystems A/S, Odense, Denmark) together with the linked Uniprot, Genbank and Unigene (for tissue-specific mRNA expression) databases. Additional annotation was incorporated from literature searches, using the references provided in the tables in the text and Supplemental Information. Proteins localized to more than one organelle were assigned to the endoplasmic reticulum or endosomes/lysosomes categories if that was their predominant localization. Since some proteins whose predominant localization is to the plasma membrane or the Golgi

apparatus are known to be present in secretory granules, these proteins are not listed as separate categories. Otherwise, proteins are characterized as enzymes, membrane, or secreted proteins.

Antibodies. Monoclonal antibody directed against ral was purchased from BD Pharmingen. Rabbit anti-pantophysin was a gift of Dr. Rudolf Leube, (Johannes Gutenberg University, Mainz, Germany).[20] Rabbit antibody to aminopeptidase M/N was a gift of Dr. Ann Hubbard (Johns Hopkins School of Medicine, Baltimore, MD) who together with Dr. Michele Maurice (INSERM Saint-Antoine Medical Faculty, Paris, France), also provided the monoclonal anti-ectonucleotide phosphodiesterase 3 (ENPP3).[20,21] Rabbit antibodies to carboxypeptidase D and cyclic nucleotide phosphodiesterase were gifts of Drs. Lloyd Fricker (Albert Einstein College of Medicine, Bronx, NY) and David Colman (McGill Univ., Montreal, Canada), respectively.[22,23] Affinity purified antibody to TWIK-2 was provided by Dr. Anant Mhatre (NYU School of Medicine).[24] Antibody to ribophorin 1 was provided by Dr. Gert Kreibich (NYU School of Medicine)[25] and to endolyn-78 was a gift of Drs. Melvin Rosenfeld and David Sabatini (NYU School of Medicine).[26] Antibody to ral was purchased from BD Biosciences, to mitochondrial hsp60 from Stressgen, and to amylase from Sigma. Antibody to GP2 was made by our laboratory as described previously.[8]

Immunofluorescence Microscopy. Rat pancreas was cut into 2 mm blocks and fixed for 3 h in 8% paraformaldehyde in 60 mM PIPES, 25 mM HEPES, 2 mM $MgCl_2$, 10 mM EGTA, pH 6.9 (PHEM). The blocks were washed in PHEM buffer and PBS with 20 mM glycine. The blocks were infused sequentially in PBS containing 2% and 10% gelatin and then equilibrated with 2.3 M sucrose in PBS overnight at 4° C. After freezing at −80° C, thin (0.5μ) frozen sections were prepared on a Leica cryomicrotome (Bannockburn, IL) and bound to Superfrost Plus Gold slides (Electron Microscopic Sciences, Hatfield, PA) with silicone isolators. Sections were stored at 4° C in 2.3 M sucrose in PBS until use. After washing 5 times in PBS and once in PBS with 1% nonfat milk, antibodies were applied overnight (1:250–1:1000). After washing in PBS, Alexa 488 goat anti-rabbit or mouse secondary antibodies (Molecular Probes) were applied along with Texas-red conjugated phalloidin (Molecular Probes) to label actin filaments at the luminal surface of the acinar cells. DAPI was also present in the mounting medium (Prolong Gold DAPI, Invitrogen, Carlsbad, CA) for orientation purposes. Samples were photographed on a Zeiss LSM510 confocal microscope (Carl Zeiss, Thornwood, NY). Isolated granules were prepared from the Percoll gradients, diluted in HB and allowed to settle on polylysine-coated coverslips for 1 h at 4° C. They were fixed in 4% paraformaldehyde, 20 mM HEPES, 0.3 M sucrose for 30 min. After washing with Hank's PBS, they were stored at 4° C. Before use, the granules were permeabilized with 0.2% TX-100 for 1 min. Staining was otherwise conducted as described above for pancreatic sections. Samples were photographed on a Zeiss Axiophot microscope equipped with a Hamamatsu camera (Bridgewater, NJ).

Western Blotting. Immunoblotting was performed after SDS/PAGE of membrane samples from the post-nuclear supernatants and the granules. After transfer to nitrocellulose and blocking in 5% milk, 0.5% Tween-20 in PBS, antibodies were applied overnight (1:200–1:2000). Secondary antibodies directed against rabbit or mouse IgG and coupled to horseradish peroxidase (Jackson Immunoresearch, West Grove, PA) were used with a chemiluminescence substrate (Western Lightning Plus, Perkin Elmer, Boston, MA or ECL Advance, GE Healthcare, Piscataway, NJ) to detect the immunoradioactivity. The film was digitally scanned and processed using Adobe Photoshop. All quantitation was performed using NIH Image software.

Results

Zymogen granules were purified from rat pancreas. Based on quantification of Coomassie Blue and silver stained polyacrylamide gels of the preparation (Figure 1A) and amylase activity

measurements (data not shown), an increase of 4.7 fold of amylase specific activity and a similar increase in the levels of the other major granule proteins was obtained. This is comparable to the 4–4.6 fold purification previously reported by ourselves[17] and others.[27, 28] Since ZG proteins constitute as much as 30% of total acinar cell protein, our preparations are close to the theoretical limit of purity.[29] The granules were substantially pure and visible contamination was minimal when the fraction was examined by electron microscopy.[17]

Peripheral and integral membrane protein fractions were prepared from membranes incubated with sodium carbonate.[8] Although there was some overlap in the protein patterns, for the most part the major peripheral and integral membrane proteins appeared distinct (Figure 1A). Using specific antibodies to other organelles, we determined that lysosomal membrane proteins were detectable in the preparation while mitochondria and endoplasmic reticulum were not detectable in granules when compared to equal amounts of the post-nuclear supernatant (Figure 1B). The presence of lysosomes was expected since some are similar in density and size to ZGs.

Proteins from intragranular content, peripheral membrane, and integral membrane protein fractions were separated by one-dimensional SDS/PAGE. The gels were cut into slices, the proteins digested with trypsin, and the resulting peptides were analyzed by LC/MS-MS. The resulting spectra were used to search NCBInr and Swissprot databases to identify the proteins present. From the four content and membrane preparations, a total of 371 proteins were identified with high confidence (see Methods for a discussion of criteria used in this analysis). Of these, 84 were known granule proteins (23% of the total). The functional distribution of the proteins is depicted in graphical form in Figure 2. Another 140 proteins were identified with less confidence based on a single unique peptide match (see Supplemental Table 1 for a complete list). To verify our database search criteria, we also searched a rat NCBI real/reverse combined database using the peptide sets from each ZG preparation. Even using our low stringency criteria, these searches yielded a total of only one false protein assignment, which was based on a single peptide match, confirming the validity of our methods. Moreover, this false positive result would not have survived the filter used to establish our high confidence identifications.

Table 1 includes a list of the 23 previously known granule content proteins that we identified. Many of these proteins (e.g., amylase, trypsinogens, chymotrypsinogen, elastases) are well-characterized ZG proteins. Others are not necessarily granule specific but have been shown to be present in granules. Among these is protein disulfide isomerase, a protein predominantly localized to the endoplasmic reticulum but present in granules as well.[30] Presumably the appearance in granules occurs when molecules occasionally escape from the endoplasmic reticulum (ER) and traverse the secretory pathway.

We also identified 61 membrane proteins previously reported to be in pancreatic zymogen granule membranes (Table 1). Here again, some are predominantly localized to granules, such as GP2, the major granule membrane protein,[8,31] and ITMAP.[9] GP2, ITMAP, and some of the other proteins also appear in content. Proteins predominantly localized to the apical plasma membrane, such as dipeptidase I and gamma-glutamyl transpeptidase, were identified. These proteins have also been shown to be present in granule membranes.[32,33] Sixteen GTP binding proteins, both conventional G protein subunits and small ras-related GTPases, which had been previously detected in pancreatic ZGs, were also identified in our studies. The vacuolar H^+-ATPase is known to acidify organelles. ZGs are acidic and several subunits have been previously shown to be present on their membranes.[34] We identified 10 of the 14 known subunits.[35]

Other secreted proteins were identified in the preparation (Table 2). These include proteins that are likely to be present in rat pancreatic ZGs. For example, trypsinogen V (via a single unique peptide) and trypsinogens 8 and 9 were identified. Given the composition of ZGs, these are likely to be granule proteins. In addition four proteins found in the pancreatic juice – chymopasin, clusterin, cystatin C, and gamma-glutamyl hydrolase – are likely granule candidates. Among the other secreted enzymes identified was lipoprotein lipase, a secretory protein made in many tissues and whose mRNA is present in pancreas.[36]

Membrane proteins were also identified that had not previously been found in zymogen granules (Table 3). These include two proteins known to be present in parotid secretory granules -- rab26 and noc2. A number of transporters and channels were also identified. These included aquaporin-8, the potassium channel TWIK-2/KCNK6, ATPases, anion and cation channels, and amino acid transporters. In addition, GTP binding proteins, enzymes, and many other membrane proteins were identified. Some of the proteins are primarily found in other organelles, such as lysosomes, which are likely present in our ZG preparations, and the endoplasmic reticulum (Table 3 and Supplemental Tables 1 and 2 in Supporting Information). Despite the large number of proteins we identified, at least 19 proteins reported in the literature to be present in granules were not detected in our LC/MS-MS analysis (Supplemental Table 2).

Four of the new proteins in Table 3 were confirmed to be ZG proteins by immunofluorescence microscopy (Figure 3 and Figure 4). These included the membrane protein pantophysin, a tetraspan membrane protein and homolog of synaptophysin thought to be involved in vesicle trafficking.[37] Pantophysin was localized to ZGs on pancreatic sections (Figure 3) and on isolated granules (Figure 4). Similarly, cyclic nucleotide phosphodiesterase (CNP) was found in granules when sections or isolated granules were stained with a specific antibody (Fig. 3 & Fig. 4). Ecto-nucleotide pyrophosphatase 3 (ENPP3) was localized to the apical membrane of pancreatic acinar cells using a monoclonal antibody (Figure 3) that also labeled the regions where the granules are found and isolated granules (Figure 4). Carboxypeptidase D (CPD) is a protein known to be present in the trans Golgi network in other cell types.[22] It localized primarily to regions deeper in the cell than most granules (Figure 3). Immunofluorescence labeling of isolated granules using antibodies to CPD revealed intense staining of large vesicle structures and a lower, but detectable level on some of the granules (Figure 4). This would be consistent with a scenario whereby some CPD enters immature granules from the TGN but is transported out as the granules mature.

Three additional proteins were found to be enriched in granule membrane fractions as determined by immunoblotting but had low levels of labeling by immunofluorescence microscopy (Figure 5). These included the small ras-related GTPase ral and the 2P domain K + channel TWIK-2 (rKCNK6), The antibody to aminopeptidase N, which is known to be an apical membrane protein in many cell types, including pancreatic duct and acinar cells,[38] was observed to crossreact with rat GP2 (data not shown) and so bovine membranes were used for immunoblotting. Aminopeptidase was also enriched in the pancreatic membrane fractions as compared to total membranes from the post-nuclear supernatant (Figure 5).

Discussion

Of the 371 proteins that met our criteria for high confidence identification by mass spectrometry, 84 had been previously reported to be in secretory granules. Many of the new proteins identified are secreted proteins and proteins known or predicted to be membrane-associated (Table 2 and Table 3). There was considerable redundancy in the protein composition of the different fractions. This was expected because many of the integral

membrane proteins are also present in the content of the granules and the major granule content proteins are thought to be present in granule membranes as well.

Most of the secretory proteins identified by us were known zymogen granule proteins. However, a few are enzymes that had been identified in pancreas or pancreatic juice but never shown previously to be in granules, including gamma-glutamyl hydrolase[39,40] and chymopasin, a chymotrypsin-like enzyme.[41] Cystatin C is a small protein (14 kDa) made by the pancreas and also found in the pancreatic juice and in granules in endocrine glands.[42,43] For this reason, it is listed in Table 2 as a good candidate for a ZG protein although only one unique peptide was detected. Clusterin, another protein that was identified, has been localized to apical region of pancreatic acinar cells undergoing regeneration,[44] which would be consistent with a ZG localization in these cells. It is reasonable to propose that a small minority of acinar cells are being renewed at any given time and therefore contain this protein in their granules. Transcobalamin 2 has been previously localized in rat pancreas where it was found primarily in pancreatic duct cells.[45] However, the investigators reported “irregular” staining for transcobalamin 2 over acinar cells, which would be consistent with a low level presence in ZGs.

Several proteins associated with the apical plasma membranes of epithelial cells were identified. It has already been established that some apical plasma membrane proteins, such as dipeptidase 1 and γ-glutamyl transpeptidase, are also found in ZGs.[32,33] Secretory granules are most likely the transport vehicle by which these proteins, but not all apical membrane proteins, are escorted to the plasma membrane.[46] ENPP3, which we have shown to be on the apical membrane of acinar cells (Figure 3) and at lower levels in granules, also would be in this category as would aminopeptidase N, which was enriched in granule membranes (Figure 5). Among the other proteins identified, aquaporin-8 is also found at the lumen of pancreatic acinar cells[47] and was identified in our ZG proteomic analysis. 5’-nucleotidase is potentially in this category as well.[48]

Very few Golgi apparatus-specific proteins were found in the preparation. Two proteins concentrated in the trans Golgi network (TGN), CPD[49] and syntaxin 16[50] were identified. Another Golgi protein, MG-160, is predominantly found in the medial Golgi but as a sialoglycoprotein is likely to be present as well in the trans-Golgi where sialylation is known to occur.[51] It was also identified in a proteomic analysis of adrenal chromaffin granules.[52] No other proteins specific for the cis and medial portions of the Golgi apparatus were detected. Granules form at the TGN and as they pinch off, it is believed that some membrane proteins of the TGN enter them. These TGN proteins are removed from the so-called immature granules (IZG) during their maturation.[53] This mechanism has been demonstrated to occur for TGN proteins, such as CPD and CALNUC (nucleobindin-1) in pituitary cells[22,54] and the cation-independent mannose-6-phosphate receptor (M6PR) in pancreatic acinar cells.[22,55] Indeed, M6PR was also identified in our preparations (Table 1) as was nucleobindin-2, a close relative of CALNUC known to be secreted from cells.[56,57] Since IZG’s would be expected to co-purify with the mature granules, it is reasonable to propose that CPD, and possibly nucleobindin-2, syntaxin 16 and MG-160 are in IZGs in acinar cells as well. This would account for the staining we observed for CPD, which was primarily in regions near the Golgi apparatus but did show low level labeling of some granules as revealed by immunofluorescence microscopy (Figure 4).

A large cohort of ion and amino acid transporters were identified including the potassium channel protein TWIK-2/rKCNK6, a two-pore weak inward rectifying channel found in many epithelial cells.[58] This protein was shown to be present in granule membranes. The only cation channel previously reported to be present in granule membranes was the ATP-sensitive potassium channel subunit Kir6.1/IRK-8,[59] which was not detected in the current study. The

amino acid transporter Slc3a2 and its subunits (L-type amino acid transporters 1 and 2) were also found. While such amino acid transporters are generally associated with the plasma membrane, it has been shown that Slc3a1, the closest homolog, is localized in secretory granules of neurons.[60] Thus Slc3a2 would be a good candidate for a ZG membrane amino acid transporter.

Small GTP binding proteins and their binding partners were found in the ZG membrane preparations. In particular, rab proteins, such as rab3 and rab27 family members are known to be regulators of secretory granule exocytosis.[61] A number of small GTPases had already been shown to be in ZGs (see Table 1). Rab26 is a candidate for a new ZG membrane protein. It is expressed in pancreas and it localizes to granules in parotid gland.[62] As mentioned previously, noc2, a rab-binding protein found in granule membranes of salivary gland cells,[5] plays a critical role in ZG release from pancreatic acinar cells.[3] It was identified by LC/MS-MS in the ZG membrane preparations as well.

In addition to the rab proteins, other proteins involved in exocytosis and vesicle trafficking were also detected. These included the SNARE proteins syntaxins 3, 6, and 7, vamps 2, 3 and 8, and SNAPS 23 and 29, which had already been detected in zymogen granules (Table 1). Syntaxins 12 and 16b were also identified, although it is not known if these are granule-specific, while syntaxins 3 and 8, which had been reported to be in zymogen granules, were not identified (see Supplementary Table 3). Other proteins in this category but never before identified in granules included synaptotagmin-like protein 1, which interacts with rab27 and is thought to be involved in granule exocytosis,[63] and the related synaptotagmin-like protein 4, which regulates amylase secretion in parotid cells.[64] Annexin A13, part of the annexin family of calcium and phospholipid binding proteins that regulates vesicle fusion at the apical membrane of polarized epithelial cells, was also identified.[65] Phosphatidylinositol lipids, including PtdIns4P, are important regulators of vesicle generation and vesicle fusion.[66] We identified phosphatidylinositol 4-kinase type-II beta (PtdIns 4-kinase). In chromaffin granules, PtdIns 4-kinase is present on secretory granules and in neuroendocrine PC12 cells, the type II form has been shown to be present in immature granules.[67,68] In addition, PtdIns 4,5-kinase regulates granule exocytosis in PC12 cells.[69] Thus, PtdIns 4-kinase is a potential regulator of granule biogenesis and exocytosis in pancreatic acinar cells as well.

As was anticipated, lysosomal membrane proteins were identified in the preparation by LC-MS/ MS. These included the major glycoproteins, LAMP-1 and LAMP-2. However, it should be noted that proteins primarily associated with lysosomes have been identified previously in secretory granules, including CD63 and cathepsin B, both of which were identified in this study as well. It is therefore an open question as to whether these and other proteins identified that have been in part localized to lysosomes and endosomes are present as well in pancreatic ZGs.

As shown in Supplemental Table 2, at least 19 proteins reported in the literature to be present in granules were not identified, in addition to the four subunits of the H^+-ATPase that were not found, as noted above. Two of the other proteins, SPINK and hsp10 are very small (<11 kDa). Small proteins have a limited tryptic peptide repertoire and do not fix well after SDS/ PAGE, making them more difficult to detect. Caveolin was reported to be released from acinar cells in a complex with apolipoproteins A1 and E.[70] This particle is large and would be removed from the content preparations during centrifugation after granule lysis. In addition, the serglycin core protein has no internal tryptic peptides. We speculate that the other proteins such as muclin, which is a major protein in mouse ZG membranes,[10] were not detected either because they are expressed at low levels in rat pancreas or because their peptides did not separate adequately from those of abundant proteins. Nevertheless, in our experiments, we did identify 79% of the ZG proteins that had been reported in the literature. This is comparable to the 80% coverage recently reported for the MS/MS analysis of synaptic vesicle proteins

separated using PAGE.[71] Admittedly, the criteria we used to eliminate false positives may also result in our failing to identify some new granule proteins.

In a recent study using mass spectrometry, Chen et al[72] reported identifying 73 new ZG proteins (along with 28 known proteins). Importantly, myosin Vc and SNAP-29, which had not been previously reported to be present in granules, were verified by immunocytochemistry to be new granule proteins. Rap1, a granule membrane protein in parotid gland secretory granules,[73] was also shown to be in ZG membranes. In addition, these investigators confirmed the localization of three proteins (rab6, rab11, and rab27B) previously identified as ZG proteins.[74–76] All of these proteins were identified in our study as well (see Table 1). Of the other 67 proteins from the Chen et al[72] study, one protein had been previously identified in granules, one is no longer in the rat database, 35 were also present in our preparations, and 31 were not detected by us. In summary, we identified with high confidence more than 300 proteins in our preparations that were not reported by Chen et al.[72] We confirmed the identity and location of a subset of these by immunocytochemistry and Western blotting, increasing the probability that many or most of the proteins found by us in our experiments are indeed novel zymogen granule proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

We thank Drs. David Colman, Lloyd Fricker, Ann Hubbard, Gert Kreibich, Rudolf Leube, Michele Maurice, Anant Mhatre, Melvin Rosenfeld, and David Sabatini for gifts of antibodies. This work was supported by grant DK067283 from the National Institutes of Health to M.J.R. and NIH Shared Instrumentation Grant S10 RR017990 to T.A.N.

References

1. Greene LJ, Hirs CH, Palade GE. On the protein composition of bovine pancreatic zymogen granules. J Biol Chem 1963;238:2054–2070. [PubMed: 13950163]
2. Hansen NJ, Antonin W, Edwardson JM. Identification of SNAREs involved in regulated exocytosis in the pancreatic acinar cell. J Biol Chem 1999;274:22871–22876. [PubMed: 10428873]
3. Wang CC, Ng CP, Lu L, Atlashkin V, Zhang W, Seet LF, Hong W. A role of VAMP8/endobrevin in regulated exocytosis of pancreatic acinar cells. Dev Cell 2004;7:359–371. [PubMed: 15363411]
4. Matsumoto M, Miki T, Shibasaki T, Kawaguchi M, Shinozaki H, Nio J, Saraya A, Koseki H, Miyazaki M, Iwanaga T, Seino S. Noc2 is essential in normal regulation of exocytosis in endocrine and exocrine cells. Proc Natl Acad Sci U S A 2004;101:8313–8318. [PubMed: 15159548]
5. Teramae H, Fujimoto W, Seino S, Iwanaga T. Cellular expression of Noc2, a Rab effector protein, in endocrine and exocrine tissues in the mouse. Histochem Cell Biol. 2006
6. Jennens ML, Lowe ME. Rat GP-3 is a pancreatic lipase with kinetic properties that differ from colipase-dependent pancreatic lipase. J Lipid Res 1995;36:2374–2382. [PubMed: 8656075]
7. Wagner AC, Wishart MJ, Mulders SM, Blevins PM, Andrews PC, Lowe AW, Williams JA. GP-3, a newly characterized glycoprotein on the inner surface of the zymogen granule membrane, undergoes regulated secretion. J Biol Chem 1994;269:9099–9104. [PubMed: 8132647]
8. Rindler MJ, Hoops TC. The pancreatic membrane protein GP-2 localizes specifically to secretory granules and is shed into the pancreatic juice as a protein aggregate. Eur. J. Cell Biol 1990;53:154–163. [PubMed: 2076702]
9. Imamura T, Asada M, Vogt SK, Rudnick DA, Lowe ME, Muglia LJ. Protection from pancreatitis by the zymogen granule membrane protein integral membrane-associated protein-1. J Biol Chem 2002;277:50725–50733. [PubMed: 12401800]

10. De Lisle RC, Norkina O, Roach E, Ziemer D. Expression of pro-Muclin in pancreatic AR42J cells induces functional regulated secretory granules. Am J Physiol Cell Physiol 2005;289:C1169–C1178. [PubMed: 15987769]
11. Kleene R, Dartsch H, Kern HF. The secretory lectin ZG16p mediates sorting of enzyme proteins to the zymogen granule membrane in pancreatic acinar cells. Eur J Cell Biol 1999;78:79–90. [PubMed: 10099930]
12. Edwardson JM, An S, Jahn R. The secretory granule protein syncollin binds to syntaxin in a Ca2(+)-sensitive manner. Cell 1997;90:325–333. [PubMed: 9244306]
13. Antonin W, Wagner M, Riedel D, Brose N, Jahn R. Loss of the zymogen granule protein syncollin affects pancreatic protein synthesis and transport but not secretion. Mol Cell Biol 2002;22:1545–1554. [PubMed: 11839820]
14. Thevenod F. Ion channels in secretory granules of the pancreas and their role in exocytosis and release of secretory proteins. Am J Physiol Cell Physiol 2002;283:C651–C672. [PubMed: 12176723]
15. Tartakoff A, Greene LJ, Palade GE. Studies on the guinea pig pancreas. Fractionation and partial characterization of exocrine proteins. J Biol Chem 1974;249:7420–7431. [PubMed: 4436317]
16. Whitcomb DC, Gorry MC, Preston RA, Furey W, Sossenheimer MJ, Ulrich CD, Martin SP, Gates LK Jr, Amann ST, Toskes PP, Liddle R, McGrath K, Uomo G, Post JC, Ehrlich GD. Hereditary pancreatitis is caused by a mutation in the cationic trypsinogen gene. Nat Genet 1996;14:141–145. [PubMed: 8841182]
17. Rindler, MJ. Isolation of zymogen granules from rat pancreas. In: Bonifacino, JS.; Basso, M.; Harford, JB.; Lippincott-Schwartz, J.; Yamada, KM., editors. Current Protocols in Cell Biology. Vol. 1. New York: John Wiley & Sons, Inc.; 2006. p. 3.18.1-3.18.15.
18. Wray W, Boulikas T, Wray VP, Hancock R. Silver staining of proteins in polyacrylamide gels. Anal Biochem 1981;118:197–203. [PubMed: 6175245]
19. Jordan BA, Fernholz BD, Boussac M, Xu C, Grigorean G, Ziff EB, Neubert TA. Identification and verification of novel rodent postsynaptic density proteins. Mol Cell Proteomics 2004;3:857–871. [PubMed: 15169875]
20. Roman LM, Hubbard AL. A domain-specific marker for the hepatocyte plasma membrane: localization of leucine aminopeptidase to the bile canalicular domain. J Cell Biol 1983;96:1548–1558. [PubMed: 6304108]
21. Maurice M, Durand-Schneider AM, Garbarz M, Feldmann G. Characterization of rat hepatocyte plasma membrane domains by monoclonal antibodies. Eur J Cell Biol 1985;39:122–129. [PubMed: 2417841]
22. Varlamov O, Eng FJ, Novikova EG, Fricker LD. Localization of metallocarboxypeptidase D in AtT-20 cells. Potential role in prohormone processing. J Biol Chem 1999;274:14759–14767. [PubMed: 10329672]
23. Trapp BD, Bernier L, Andrews SB, Colman DR. Cellular and subcellular distribution of 2',3'-cyclic nucleotide 3'-phosphodiesterase and its mRNA in the rat central nervous system. J Neurochem 1988;51:859–868. [PubMed: 2842456]
24. Mhatre AN, Li J, Chen AF, Yost CS, Smith RJ, Kindler CH, Lalwani AK. Genomic structure, cochlear expression, and mutation screening of KCNK6, a candidate gene for DFNA4. J Neurosci Res 2004;75:25–31. [PubMed: 14689445]
25. Kreibich G, Ulrich BL, Sabatini DD. Proteins of rough microsomal membranes related to ribosome binding. I. Identification of ribophorins I and II, membrane proteins characteristics of rough microsomes. J Cell Biol 1978;77:464–487. [PubMed: 649658]
26. Croze E, Ivanov IE, Kreibich G, Adesnik M, Sabatini DD, Rosenfeld MG. Endolyn-78, a membrane glycoprotein present in morphologically diverse components of the endosomal and lysosomal compartments: implications for lysosome biogenesis. J Cell Biol 1989;108:1597–1613. [PubMed: 2654137]
27. Paquet MR, St-Jean P, Roberge M, Beaudoin AR. Isolation of zymogen granules from rat pancreas and characterization of their membrane proteins. Eur J Cell Biol 1982;28:20–26. [PubMed: 6290220]
28. Cameron RS, Cameron PL, Castle JD. A common spectrum of polypeptides occurs in secretion granule membranes of different exocrine glands. J Cell Biol 1986;103:1299–1313. [PubMed: 3533952]

29. Meldolesi J. Membranes of pancreatic zymogen granules. Methods Enzymol 1983;98:67–75. [PubMed: 6669071]
30. Bruneau N, Lombardo D, Levy E, Bendayan M. Roles of molecular chaperones in pancreatic secretion and their involvement in intestinal absorption. Microsc Res Tech 2000;49:329–345. [PubMed: 10820517]
31. Ronzio RA, Kronquist KE, Lewis DS, MacDonald RJ, Mohrlok SH, O'Donnell JJ Jr. Glycoprotein synthesis in the adult rat pancreas. IV. Subcellular distribution of membrane glycoproteins. Biochim Biophys Acta 1978;508:65–84. [PubMed: 629968]
32. Beaudoin AR, Grondin G, Laperche Y. Immunocytochemical localization of gamma-glutamyltranspeptidase, GP-2 and amylase in the rat exocrine pancreas: the concept of zymogen granule membrane recycling after exocytosis. J Histochem Cytochem 1993;41:225–233. [PubMed: 7678269]
33. Hooper NM, Cook S, Laine J, Lebel D. Identification of membrane dipeptidase as a major glycosyl-phosphatidylinositol-anchored protein of the pancreatic zymogen granule membrane, and evidence for its release by phospholipase A. Biochem J 1997;324:151–157. [PubMed: 9164851]
34. Roussa E, Shmukler BE, Wilhelm S, Casula S, Stuart-Tilley AK, Thevenod F, Alper SL. Immunolocalization of potassium-chloride cotransporter polypeptides in rat exocrine glands. Histochem Cell Biol 2002;117:335–344. [PubMed: 11976906]
35. Beyenbach KW, Wieczorek H. The V-type H+ ATPase: molecular structure and function, physiological roles and regulation. J Exp Biol 2006;209:577–589. [PubMed: 16449553]
36. Ben-Zeev O, Doolittle MH, Davis RC, Elovson J, Schotz MC. Maturation of lipoprotein lipase. Expression of full catalytic activity requires glucose trimming but not translocation to the cis-Golgi compartment. J Biol Chem 1992;267:6219–6227. [PubMed: 1556130]
37. Haass NK, Kartenbeck MA, Leube RE. Pantophysin is a ubiquitously expressed synaptophysin homologue and defines constitutive transport vesicles. J Cell Biol 1996;134:731–746. [PubMed: 8707851]
38. Gorvel JP, Rigal A, Sarles J, Maroux S. Aminopeptidase N- and human blood group A-antigenicity along the digestive tract and associated glands in the rabbit. Cell Tissue Res 1985;239:241–248. [PubMed: 2857112]
39. Bhandari SD, Gregory JF 3rd, Renuart DR, Merritt AM. Properties of pteroylpolyglutamate hydrolase in pancreatic juice of the pig. J Nutr 1990;120:467–475. [PubMed: 2341911]
40. Kesavan V, Noronha JM. Folate malabsorption in aged rats related to low levels of pancreatic folyl conjugase. Am J Clin Nutr 1983;37:262–267. [PubMed: 6218750]
41. Sogame Y, Kataoka K, Kato M, Sakagami J, Osawa S, Takatera A, Mitsuyoshi M, Usui N, Mitsui S, Yamaguchi N. Molecular cloning and characterization of chymopasin, a novel serine protease from rat pancreas. Pancreas 2002;25:378–386. [PubMed: 12409833]
42. Gronborg M, Bunkenborg J, Kristiansen TZ, Jensen ON, Yeo CJ, Hruban RH, Maitra A, Goggins MG, Pandey A. Comprehensive proteomic analysis of human pancreatic juice. J Proteome Res 2004;3:1042–1055. [PubMed: 15473694]
43. Moller CA, Lofberg H, Grubb AO, Olsson SO, Davies ME, Barrett AJ. Distribution of cystatin C (gamma-trace), an inhibitor of lysosomal cysteine proteinases, in the anterior lobe of simian and human pituitary glands. Neuroendocrinology 1985;41:400–404. [PubMed: 2997651]
44. Min BH, Jeong SY, Kang SW, Crabo BG, Foster DN, Chun BG, Bendayan M, Park IS. Transient expression of clusterin (sulfated glycoprotein-2) during development of rat pancreas. J Endocrinol 1998;158:43–52. [PubMed: 9713325]
45. Lee EY, Seetharam B, Alpers DH, DeSchryver-Kecskemeti K. Immunohistochemical survey of cobalamin-binding proteins. Gastroenterology 1989;97:1171–1180. [PubMed: 2676682]
46. Colomer V, Rindler MJ, Lowe AL. Apical plasma membrane proteins of exocrine cells do not obligatorily transit through secretory granules en route to the cell surface. J. Cell Science 1994;107:2271–2277. [PubMed: 7983185]
47. Tani T, Koyama Y, Nihei K, Hatakeyama S, Ohshiro K, Yoshida Y, Yaoita E, Sakai Y, Hatakeyama K, Yamamoto T. Immunolocalization of aquaporin-8 in rat digestive organs and testis. Arch Histol Cytol 2001;64:159–168. [PubMed: 11436986]

48. Uchiyama Y. A histochemical study of variations in the localization of 5'-nucleotidase activity in the acinar cell of the rat exocrine pancreas over the twenty-four hour period. Cell Tissue Res 1983;230:411–420. [PubMed: 6303596]

49. Varlamov O, Fricker LD. Intracellular trafficking of metallocarboxypeptidase D in AtT-20 cells: localization to the trans-Golgi network and recycling from the cell surface. J Cell Sci 1998;111(Pt 7):877–885. [PubMed: 9490632]

50. Mallard F, Tang BL, Galli T, Tenza D, Saint-Pol A, Yue X, Antony C, Hong W, Goud B, Johannes L. Early/recycling endosomes-to-TGN transport involves two SNARE complexes and a Rab6 isoform. J Cell Biol 2002;156:653–664. [PubMed: 11839770]

51. Gonatas JO, Mezitis SG, Stieber A, Fleischer B, Gonatas NK. MG-160. A novel sialoglycoprotein of the medial cisternae of the Golgi apparatus [published eeratum appears in J Biol Chem 1989 Mar 5;264(7):4264]. J Biol Chem 1989;264:646–653. [PubMed: 2909545]

52. Wegrzyn J, Lee J, Neveu JM, Lane WS, Hook V. Proteomics of Neuroendocrine Secretory Vesicles Reveal Distinct Functional Systems for Biosynthesis and Exocytosis of Peptide Hormones and Neurotransmitters. J Proteome Res. 2007

53. Arvan P, Castle D. Sorting and storage during secretory granule biogenesis: looking backward and looking forward. Biochem J 1998;332(Pt 3):593–610. [PubMed: 9620860]

54. Lavoie C, Meerloo T, Lin P, Farquhar MG. Calnuc, an EF-hand Ca(2+)-binding protein, is stored and processed in the Golgi and secreted by the constitutive-like pathway in AtT20 cells. Mol Endocrinol 2002;16:2462–2474. [PubMed: 12403836]

55. Klumperman J, Kuliawat R, Griffith JM, Geuze HJ, Arvan P. Mannose 6-phosphate receptors are sorted from immature secretory granules via adaptor protein AP-1, clathrin, and syntaxin 6-positive vesicles. J Cell Biol 1998;141:359–371. [PubMed: 9548715]

56. Islam A, Adamik B, Hawari FI, Ma G, Rouhani FN, Zhang J, Levine SJ. Extracellular TNFR1 release requires the calcium-dependent formation of a nucleobindin 2-ARTS-1 complex. J Biol Chem 2006;281:6860–6873. [PubMed: 16407280]

57. Oh IS, Shimizu H, Satoh T, Okada S, Adachi S, Inoue K, Eguchi H, Yamamoto M, Imaki T, Hashimoto K, Tsuchiya T, Monden T, Horiguchi K, Yamada M, Mori M. Identification of nesfatin-1 as a satiety molecule in the hypothalamus. Nature 2006;443:709–712. [PubMed: 17036007]

58. Lesage F, Lazdunski M. Molecular and functional properties of two-pore-domain potassium channels. Am J Physiol Renal Physiol 2000;279:F793–F801. [PubMed: 11053038]

59. Abu-Hamdah R, Cho WJ, Cho SJ, Jeremic A, Kelly M, Ilie AE, Jena BP. Regulation of the water channel aquaporin-1: isolation and reconstitution of the regulatory complex. Cell Biol Int 2004;28:7–17. [PubMed: 14759764]

60. Nirenberg MJ, Tate SS, Mosckovitz R, Udenfriend S, Pickel VM. Immunocytochemical localization of the renal neutral and basic amino acid transporter in rat adrenal gland, brainstem, and spinal cord. J Comp Neurol 1995;356:505–522. [PubMed: 7560263]

61. Seabra MC, Mules EH, Hume AN. Rab GTPases, intracellular traffic and disease. Trends Mol Med 2002;8:23–30. [PubMed: 11796263]

62. Yoshie S, Imai A, Nashida T, Shimomura H. Expression, characterization, and localization of Rab26, a low molecular weight GTP-binding protein, in the rat parotid gland. Histochem Cell Biol 2000;113:259–263. [PubMed: 10857477]

63. Fukuda M. Rab27 and its effectors in secretory granule exocytosis: a novel docking machinery composed of a Rab27.effector complex. Biochem Soc Trans 2006;34:691–695. [PubMed: 17052176]

64. Fukuda M, Imai A, Nashida T, Shimomura H. Slp4-a/granuphilin-a interacts with syntaxin-2/3 in a Munc18-2-dependent manner. J Biol Chem 2005;280:39175–39184. [PubMed: 16186111]

65. Lafont F, Lecat S, Verkade P, Simons K. Annexin XIIIb associates with lipid microdomains to function in apical delivery. J Cell Biol 1998;142:1413–1427. [PubMed: 9744874]

66. De Matteis MA, Godi A. PI-loting membrane traffic. Nat Cell Biol 2004;6:487–492. [PubMed: 15170460]

67. Panaretou C, Tooze SA. Regulation and recruitment of phosphatidylinositol 4-kinase on immature secretory granules is independent of ADP-ribosylation factor 1. Biochem J 2002;363:289–295. [PubMed: 11931656]

68. Wiedemann C, Schafer T, Burger MM. Chromaffin granule-associated phosphatidylinositol 4-kinase activity is required for stimulated secretion. Embo J 1996;15:2094–2101. [PubMed: 8641275]
69. Aikawa Y, Martin TF. ARF6 regulates a plasma membrane pool of phosphatidylinositol(4,5) bisphosphate required for regulated exocytosis. J Cell Biol 2003;162:647–659. [PubMed: 12925709]
70. Liu P, Li WP, Machleidt T, Anderson RG. Identification of caveolin-1 in lipoprotein particles secreted by exocrine cells. Nat Cell Biol 1999;1:369–375. [PubMed: 10559965]
71. Takamori S, Holt M, Stenius K, Lemke EA, Gronborg M, Riedel D, Urlaub H, Schenck S, Brugger B, Ringler P, Muller SA, Rammner B, Grater F, Hub JS, De Groot BL, Mieskes G, Moriyama Y, Klingauf J, Grubmuller H, Heuser J, Wieland F, Jahn R. Molecular anatomy of a trafficking organelle. Cell 2006;127:831–846. [PubMed: 17110340]
72. Chen X, Walker AK, Strahler JR, Simon ES, Tomanicek-Volk SL, Nelson BB, Hurley MC, Ernst SA, Williams JA, Andrews PC. Organellar proteomics: analysis of pancreatic zymogen granule membranes. Mol Cell Proteomics 2006;5:306–312. [PubMed: 16278343]
73. D'Silva NJ, DiJulio DH, Belton CM, Jacobson KL, Watson EL. Immunolocalization of rap1 in the rat parotid gland: detection on secretory granule membranes. J Histochem Cytochem 1997;45:965–973. [PubMed: 9212822]
74. Iida H, Tanaka S, Shibata Y. Small GTP-binding protein, Rab6, is associated with secretory granules in atrial myocytes. Am J Physiol 1997;272:C1594–C1601. [PubMed: 9176151]
75. Hori Y, Takeyama Y, Hiroyoshi M, Ueda T, Maeda A, Ohyanagi H, Saitoh Y, Kaibuchi K, Takai Y. Possible involvement of Rab11 p24, a Ras-like small GTP-binding protein, in intracellular vesicular transport of isolated pancreatic acini. Dig Dis Sci 1996;41:133–138. [PubMed: 8565746]
76. Chen X, Li C, Izumi T, Ernst SA, Andrews PC, Williams JA. Rab27b localizes to zymogen granules and regulates pancreatic acinar exocytosis. Biochem Biophys Res Commun 2004;323:1157–1162. [PubMed: 15451418]
77. Tooze J, Hollinshead M, Hensel G, Kern HF, Hoflack B. Regulated secretion of mature cathepsin B from rat exocrine pancreatic cells. Eur J Cell Biol 1991;56:187–200. [PubMed: 1802707]
78. Lechene de la Porte P, Amouric M, Figarella C. Immunolocalization of kallikrein in porcine pancreas at the ultrastructural level. Hoppe Seylers Z Physiol Chem 1981;362:439–443. [PubMed: 6909109]
79. Wang S, Lukinius A, Zhou Y, Stalberg P, Gobl A, Oberg K, Skogseid B. Subcellular distribution of phospholipase C isoforms in rodent pancreas and gastric mucosa. Endocrinology 2000;141:2589–2593. [PubMed: 10875262]
80. Lechene de la Porte P, de Caro A, Lafont H, Sarles H. Immunocytochemical localization of pancreatic stone protein in the human digestive tract. Pancreas 1986;1:301–308. [PubMed: 3550781]
81. Bendayan M. Ultrastructural localization of cytoskeletal proteins in pancreatic secretory cells. Can J Biochem Cell Biol 1985;63:680–690. [PubMed: 2412674]
82. Massey-Harroche D, Mayran N, Maroux S. Polarized localizations of annexins I, II, VI and XIII in epithelial cells of intestinal, hepatic and pancreatic tissues. J Cell Sci 1998;111(Pt 20):3007–3015. [PubMed: 9739074]
83. Fukuoka S, Kern H, Kazuki-Sugino R, Ikeda Y. Cloning and characterization of ZAP36, an annexin-like, zymogen granule membrane associated protein, in exocrine pancreas. Biochim Biophys Acta 2002;1575:148–152. [PubMed: 12020832]
84. Hatskelzon L, Dalal BI, Shalev A, Robertson C, Gerrard JM. Wide distribution of granulophysin epitopes in granules of human tissues. Lab Invest 1993;68:509–519. [PubMed: 7684475]
85. Kelly ML, Abu-Hamdah R, Jeremic A, Cho SJ, Ilie AE, Jena BP. Patch clamped single pancreatic zymogen granules: direct measurements of ion channel activities at the granule membrane. Pancreatology 2005;5:443–449. [PubMed: 15985770]
86. Braun JE, Scheller RH. Cysteine string protein, a DnaJ family member, is present on diverse secretory vesicles. Neuropharmacology 1995;34:1361–1369. [PubMed: 8606785]
87. Kraemer J, Schmitz F, Drenckhahn D. Cytoplasmic dynein and dynactin as likely candidates for microtubule-dependent apical targeting of pancreatic zymogen granules. Eur J Cell Biol 1999;78:265–277. [PubMed: 10350215]
88. Poucell-Hatton S, Perkins PS, Deerinck TJ, Ellisman MH, Hardison WG, Pandol SJ. Myosin I is associated with zymogen granule membranes in the rat pancreatic acinar cell. Gastroenterology 1997;113:649–658. [PubMed: 9247487]

89. Wishart MJ, Andrews PC, Nichols R, Blevins GT Jr, Logsdon CD, Williams JA. Identification and cloning of GP-3 from rat pancreatic acinar zymogen granules as a glycosylated membrane-associated lipase. J Biol Chem 1993;268:10303–10311. [PubMed: 8486693]

90. von Zastrow M, Tritton TR, Castle JD. Exocrine secretion granules contain peptide amidation activity. Proc Natl Acad Sci U S A 1986;83:3297–3301. [PubMed: 3458183]

91. Brand SH, Laurie SM, Mixon MB, Castle JD. Secretory carrier membrane proteins 31–35 define a common protein composition among secretory carrier membranes. J Biol Chem 1991;266:18949–18957. [PubMed: 1717458]

92. Blum R, Feick P, Puype M, Vandekerckhove J, Klengel R, Nastainczyk W, Schulz I. Tmp21 and p24A, two type I proteins enriched in pancreatic microsomal membranes, are members of a protein family involved in vesicular trafficking. J Biol Chem 1996;271:17183–17189. [PubMed: 8663407]

93. Gaisano HY, Sheu L, Grondin G, Ghai M, Bouquillon A, Lowe A, Beaudoin A, Trimble WS. The vesicle-associated membrane protein family of proteins in rat pancreatic and parotid acinar cells. Gastroenterology 1996;111:1661–1669. [PubMed: 8942747]

94. Shimada O, Hara-Kuge S, Yamashita K, Tosaka-Shimada H, Yanchao L, Einan L, Atsumi S, Ishikawa H. Localization of VIP36 in the post-Golgi secretory pathway also of rat parotid acinar cells. J Histochem Cytochem 2003;51:1057–1063. [PubMed: 12871987]

95. Cronshagen U, Voland P, Kern HF. cDNA cloning and characterization of a novel 16 kDa protein located in zymogen granules of rat pancreas and goblet cells of the gut. Eur J Cell Biol 1994;65:366–377. [PubMed: 7720729]

96. Chen CY, Cronshagen U, Kern HF. A novel pancreas-specific serpin (ZG-46p) localizes to the soluble and membrane fraction of the Golgi complex and the zymogen granules of acinar cells. Eur J Cell Biol 1997;73:205–214. [PubMed: 9243181]

97. Padfield PJ, Panesar N. Identification of Goalpha, Gqalpha, and Gsalpha immunoreactivity associated with the rat pancreatic zymogen granule membrane. Biochem Biophys Res Commun 1997;237:235–238. [PubMed: 9268692]

98. Sattar AA, Boinpally R, Stromer MH, Jena BP. G(alpha)(i3) in pancreatic zymogen granules participates in vesicular fusion. J Biochem (Tokyo) 2002;131:815–820. [PubMed: 12038977]

99. Ohnishi H, Ernst SA, Yule DI, Baker CW, Williams JA. Heterotrimeric G-protein Gq/11 localized on pancreatic zymogen granules is involved in calcium-regulated amylase secretion. J Biol Chem 1997;272:16056–16061. [PubMed: 9188511]

100. Valentijn JA, Sengupta D, Gumkowski FD, Tang LH, Konieczko EM, Jamieson JD. Rab3D localizes to secretory granules in rat pancreatic acinar cells. Eur J Cell Biol 1996;70:33–41. [PubMed: 8738417]

101. Wagner AC, Strowski MZ, Williams JA. Identification of Rab 5 but not Rab 3A in rat pancreatic zymogen granule membranes. Biochem Biophys Res Commun 1994;200:542–548. [PubMed: 8166729]

102. Roussa E, Alper SL, Thevenod F. Immunolocalization of anion exchanger AE2, Na(+)/H(+) exchangers NHE1 and NHE4, and vacuolar type H(+)-ATPase in rat pancreas. J Histochem Cytochem 2001;49:463–474. [PubMed: 11259449]

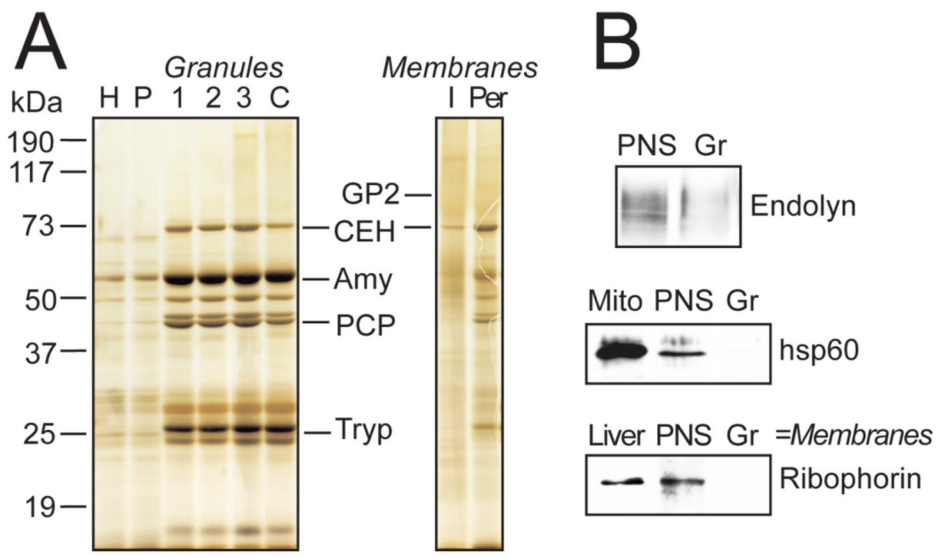

Figure 1.

Preparation of zymogen granules from rat pancreas. A) Granules were prepared as described in the Methods. 5 μg of the initial homogenate (H), the post-nuclear supernatant (PNS), the first granule pellet (1), the Percoll gradient fraction (2), and the final purified granules recovered from the sucrose gradient (3) were subjected to SDS/PAGE on 10% gels. The granule content fraction (C) as well as the integral (I) and peripheral (Per) membrane fractions isolated after treatment of the membranes with 0.1 M sodium carbonate are also depicted. Amylase and other content protein bands were enriched ~ 5 fold. B) 5 μg of protein from the indicated fractions was used in immunoblotting experiments after SDS/PAGE and transfer to nitrocellulose. Antibodies specific for the endosome/lyososome membrane marker endolyn, the mitochondrial protein hsp60 and the endoplasmic reticulum membrane protein ribophorin 1 were used as probes and the blots developed using the enhanced chemiluminescence method. Anti-endolyn samples were membranes from the homogenate, PNS, and granule fractions. Anti-hsp60 samples were a mitochondrial fraction prepared from rat pancreas (Mito.), PNS and purified granules (Gr). Anti-ribophorin samples consisted of total rat liver membranes, pancreas PNS membranes, and granule membranes. The data show that lysosomes are present in the granule preparation but not enriched while mitochondria and endoplasmic reticulum were not detected by this method, indicating that they were selectively depleted during zymogen granule purification. The intensity of the bands using these methods was linear over ~8 fold range (not shown).

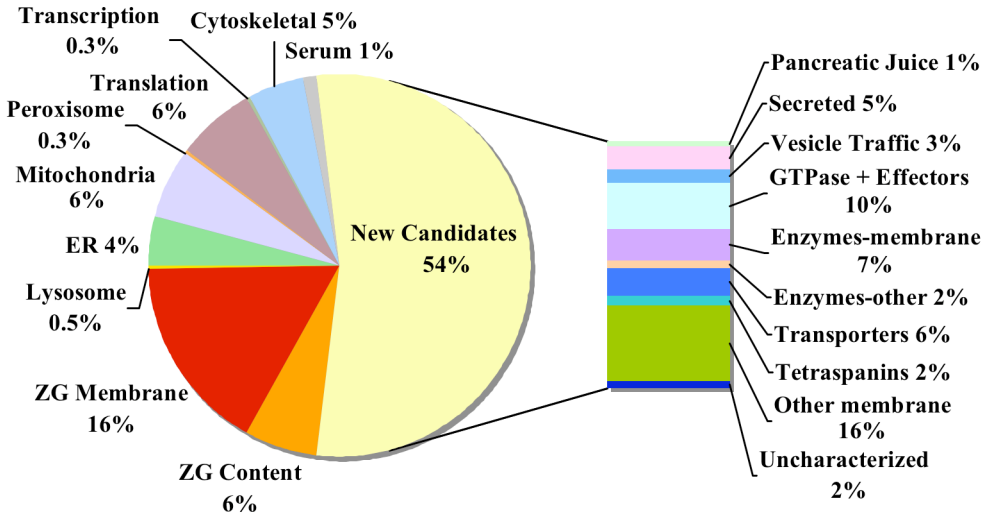

Figure 2.

Diagram of the functional categories of the soluble and membrane proteins identified by LC-MS/MS. Based on annotations in the ProteinCenter, Uniprot and Genbank databases or predictions based on similarity to related proteins in the same databases, the 371 proteins identified with high confidence (see text) are grouped in the pie chart according to their functional and subcellular distribution. 23% were known zymogen granule proteins and 54% were candidates for new granule proteins. At right, the new candidates are broken out by category together with the percent of the total proteins identified (as listed in Table 3; also see Supplemental Table 2 for detailed annotation).

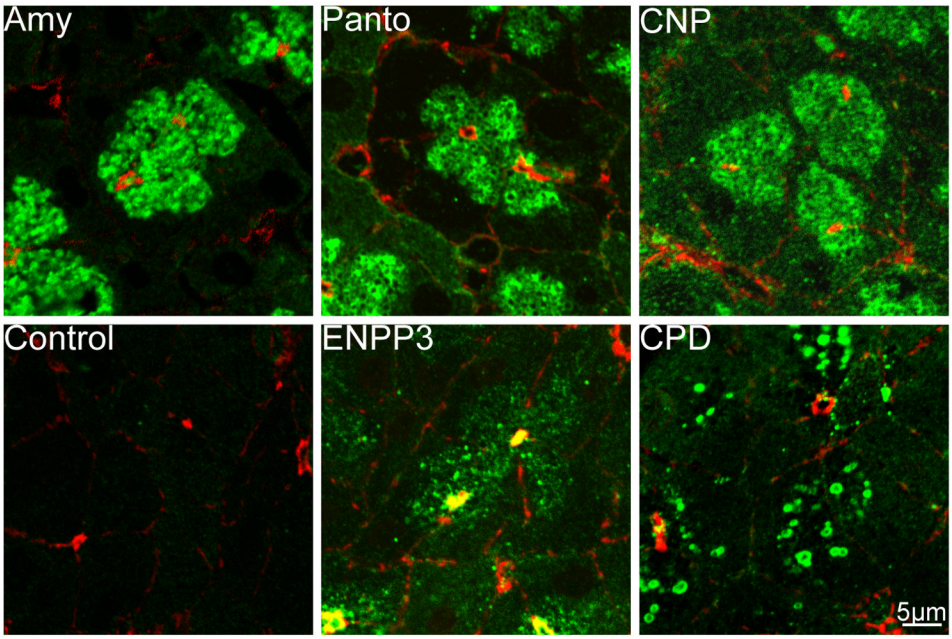

Figure 3.

Immunofluorescence microscopy of pancreatic sections. Frozen thin sections of rat pancreas were incubated with rabbit antibodies to amylase (Amy), pantophysin (Panto), carboxypeptidase D (CPD), and cyclic nucleotide phosphodiesterase (CNP) as well as a monoclonal antibody to ecto-nucleoside pyrophosphatase 3 (ENPP3). The negative control was a nonimmune rabbit serum used at the same dilution (1:200). Controls performed using irrelevant mouse monoclonal antibodies gave even lower nonspecific background (data not shown). Alexa 488 - conjugated anti-mouse or anti-rabbit IgG was used in a second step along with TX-Red conjugated phalloidin, which binds strongly to actin at the acinar lumen. Pantophysin and CNP labeling was over the granule region and to some extent over other membranes of the cell. CPD labeling was primarily in regions deeper than the bulk of the granules and is consistent with its presence in the trans Golgi network. ENPP3 was localized predominantly over the lumenal membranes of the acinar cells but also at a low level over the granule region beneath it. By comparison, amylase labeling, as expected, was over the ZG's surrounding the acinar lumens.

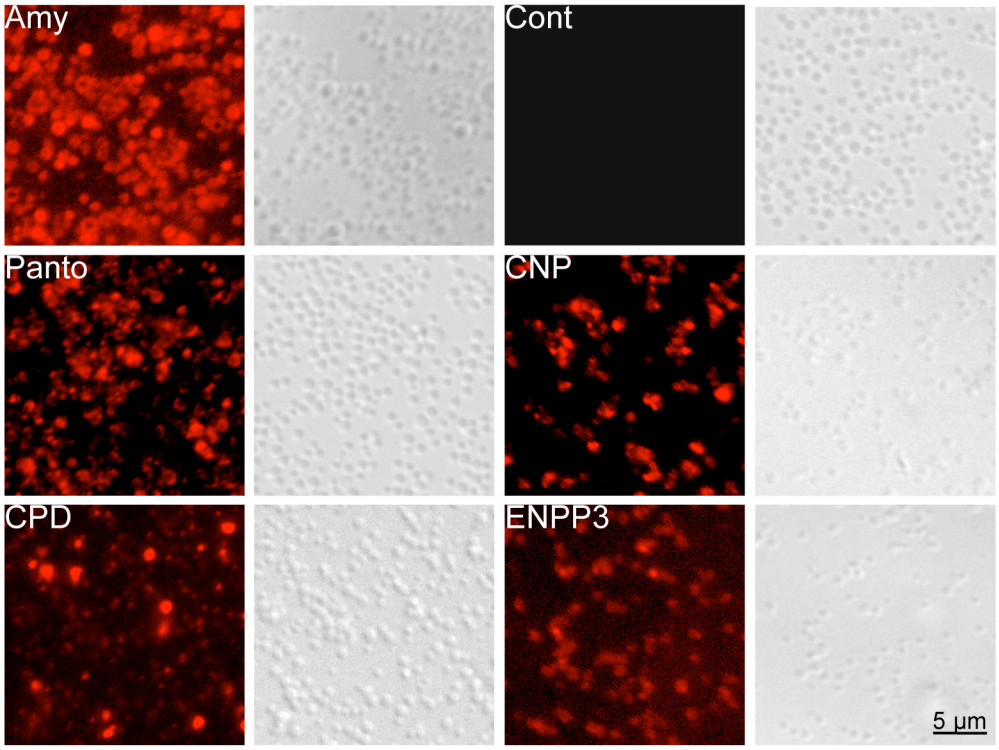

Figure 4.
Immunofluorescence microscopy on isolated granules. Zymogen granules on coverslips were stained for immunofluorescence microscopy using antibodies against amylase (Amy), pantophysin (Panto), CPD, CNP, and ENPP3. Rhodamine-conjugated anti-mouse or anti-rabbit IgG was used in a second step. Depicted in parallel are representative fields using fluorescent and differential interference contrast filters. All of the antibodies gave specific signal although anti-CPD labeled some large vesicles very intensely with low level labeling over the bulk. ENPP3 staining was weak compared to pantophysin or CNP, as expected (see Figure 3). Control coverslips were incubated in a nonimmune rabbit serum and rhodamine-conjugated anti-rabbit IgG. They were photographed at the longest exposure used for the antibody-stained coverslips and processed similarly using Adobe Photoshop.

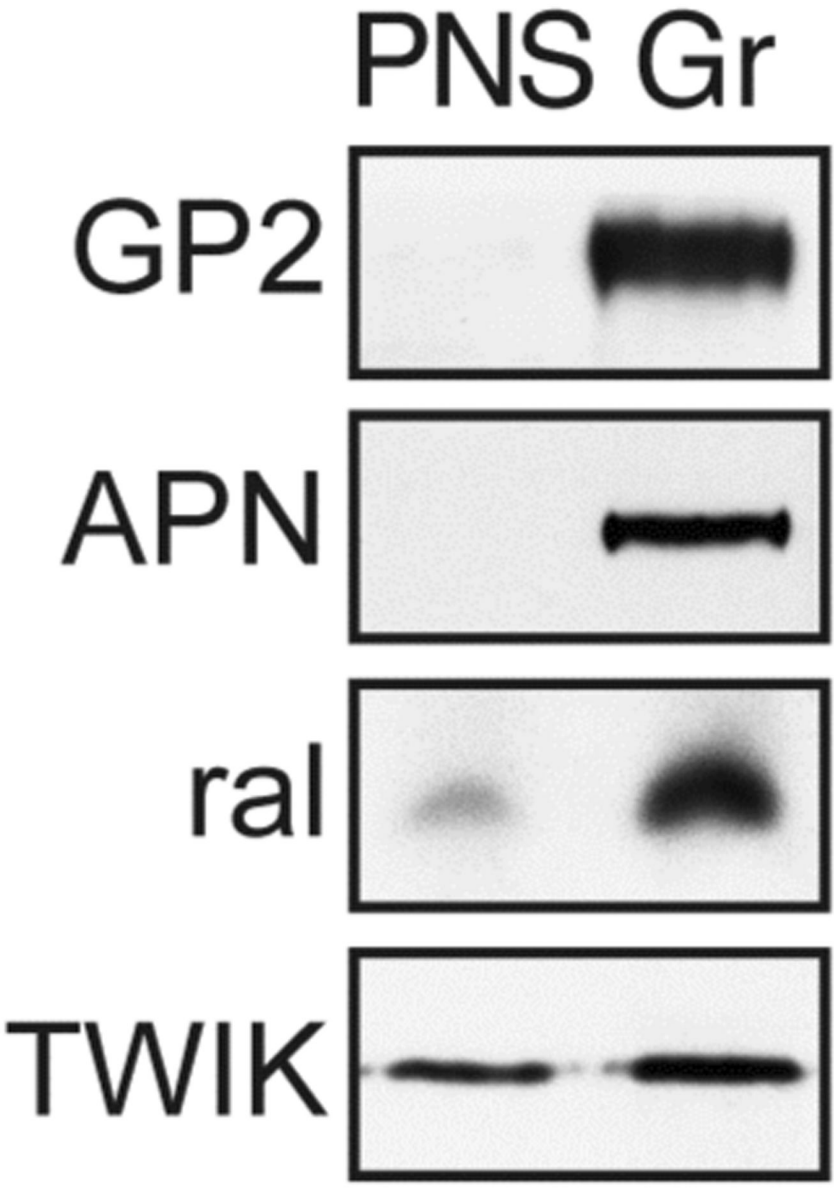

Figure 5.
Immunoblotting of purified zymogen granule fractions. Granules and their membranes were prepared from bovine pancreas (top 2 panels) or rat pancreas (bottom 2 panels). 3 μg of membranes from the PNS or purified granules were subjected to SDS/PAGE and transferred to nitrocellulose. Immunoblotting was conducted using enhanced chemiluminescence and antibodies to GP2, aminopeptidase N, ral and TWIK-2/rKCNK6. All of these proteins were enriched in granule membranes (Gr) as compared to total PNS membranes.

Table 1

List of Known Zymogen Granule Proteins Identified by LC-MS/MS. Proteins listed were identified in our LC-MS/MS experiments (see Methods for criteria) and had been previously reported in the literature (Ref) to be in zymogen granules (ZG). The ZG preparations where the protein was identified by us are listed and the preparation with the most unique peptides and highest score is in bold [= **C**ontent**1** or **2 or both (C)**, **I**ntegral or **P**eripheral membranes]. The database (Swiss or NCBI) that yielded the Mascot score is also given. Proteins of salivary gland granules, which are related to pancreatic ZG are also included. Numbers of identified total, unique (Uni) and shared (Sh) peptides are listed, as are the previously reported major subcellular locations in addition to granules (Other Location).

Protein Name	NCBI Access #	Mass	Mascot Score	Peptides Total	Uni(Sh)	Prep	Ref
Content Proteins							
alpha-amylase, pancreatic	67373	56522	30828	1198	15	I,P,**C**	
caldecrin (chymotrypsin C/elastase IV)	1246029	29355	1298	80	6	I,P,**C**	
carboxypeptidase A1	1345702	47168	5614	277	14(2)	I,P,**C**	
carboxypeptidase A2	61556903	46883	6600	245	14	I,P,**C**	
carboxypeptidase B1	6978697	47485	7618	263	12	I,P,**C**	
cathepsin B	1705630	37470	65	2	2	**C2**	77
cholesterol esterase	55943	66997	27724	2288	24	I,**P**,C	
chymotrypsinogen B	6978717	27831	9996	317	5	I,P,**C**	
colipase	203503	12272	139	32	2	P,**C**	
elastase 1	6978801	28979	3184	103	3	I,P,**C**	
elastase 2	6978803	28866	9192	258	7	I,P,**C**	
elastase 3B	62649890	28767	1042	53	7	I,P,**C**	
heat shock protein (hsp60)	56383	60927	102	3	1	**C2**	30
kallikrein, tissue	818030	23515	419	20	3	**C2**	78
pancreatic lipase	1865646	51407	5931	430	17	I,**P**,C	
pancreatic lipase-related protein 1	14091772	52345	1904	120	14	I,**P**,C	
phospholipase A2	129416	16413	760	32	3	**C**	
phospholipase c, beta1	117647200	133309	47	6	1	**P**	79
protein disulfide-isomerase A3	1352384	56588	196	21	10	I,**P**	30
reg-1(pancreatic stone protein)	6981470	18660	77	3	2	**C**	80
trypsinogen I	6981420	25943	3632	180	3	I,P,**C**	
trypsinogen II, anionic	6981422	26211	1091	56	2	P,**C**	
trypsinogen III, cationic	27465583	26252	2747	110	5	I,P,**C**	
Membrane Proteins							
beta-actin	13592133	41724	46	6	5	I,**P**,C2	81
annexin II; calpactin 1	9247200	38951	99	5	3	I,**P**	82
annexin A4/ZAP36	37999910	35826	187	8	4	**I**,P,C2	83
cation-independent man-6-PO4 receptor	6981078	273222	60	2	2	**P**	55
CD63/granulophysin	5929904	13830	213	24	2	I,**P**	84
chloride channel protein 3 (CLC-3)	4762023	90796	75	9	4(1)	**I**	85
cysteine string protein	1095322	22086	576	35	4	**I**,P	86
dipeptidase 1	400240	45477	1852	204	18	**I**,P,C2	33
dynactin 2	50926127	44121	70	5	3	**P**	87
dynein cytoplasmic heavy chain	294543	53174	174	26	7	**I,C2**	87
gamma-glutamyltranspeptidase 1	121150	61517	5772	491	17	**I,P,C**	32
GP2/ZG membrane glycoprotein 2	19705557	58707	16175	1005	14	**I,P,C**	8,31
integral membrane-associated protein ITMAP	2460316	68596	1070	99	10	I,**P**,C2	9
myosin Ic heavy chain	400429	118017	52	9	2	I,**P**,C2	88
myosin Vc	62653910	234186	2171	132	34(4)	**I**,P	72
pancreatic lipase-related protein 2 (GP-3)	1708841	52501	10973	1010	20	I,**P**,C	89
peptidylglycine α-amidating monooxygenase	56841	94745	116	9	4	**I**	90
secretory carrier membrane protein 1	34853043	37974	1024	50	5	**I**,P	28,91
secretory carrier membrane protein 2	10764633	34318	499	26	3	I,**P**	28,91
secretory carrier membrane protein 3	10764631	38344	453	30	4	**I**,P	28,91

Protein Name	NCBI Access #	Mass	Mascot Score	Peptides Total	Peptides Uni(Sh)	Prep	Ref
secretory carrier membrane protein 4	13929018	25495	127	7	1	I,**P**	28,91
synaptosomal-associated protein SNAP23	12083641	23220	168	26	6	**I**,P	3
synaptosomal-associated protein SNAP29	62751974	29082	226	11	5(1)	**I**,P	72
syncollin	3366638	15026	638	79	3	I,**P**,C	12
syntaxin 3	13592097	33236	477	16	5	**I**,P	2
syntaxin 6	13928922	29039	170	7	7	**I**	55
syntaxin 7	12229954	29643	163	7	6	**I**,P	3
transmembrane trafficking protein 21	16758214	24842	68	2	2	**P**,C2	92
vesicle associated membrane protein 2	6981614	12683	98	8	1(1)	**I**,P,C	93
vesicle associated membrane protein 3	16923936	11473	98	8	1(1)	**I**,P,C	93
vesicle-associated membrane protein 8	13929182	11313	85	9	2	**I**	3
VIP36/vesicular integral-membrane protein	27682691	40367	54	3	1	**I**,P	94
ZG16	25006249	18201	2058	158	7	I,**P**,C	95
ZG46/ZG21/serpin I2	34857087	45890	535	43	12	I,**P**,C1	96
GTP binding proteins							
G(o)α1/2	8394152	40043	176	11	3(2)	I,**P**	97
G(i)α3	6980964	40496	261	33	7(1)	**I**,P	98
G(q)α	9296968	41443	28	5	4	**I**	97
G(q)α 11	13591951	42000	62	6	2	**P**	99
G(s)α	9506737	45635	371	33	10	I,**P**	97
Gβ2	71089941	37307	187	9	5	**I**,P	99
rab3D	18034781	24275	1271	123	9	**I**,P	100
rab5A	12083645	23600	66	5	2	I,**P**	101
rab5B	34862219	24873	44	5	1(1)	**I**	101
rab5C	27689505	23411	73	4	2	**I**	101
rab6A	5020094	15763	74	4	3	**I**	74
rab11A	55741722	24247	510	50	1(6)	**I**	75
rab11B	38303832	24342	543	51	3(6)	**I**,P	75
rab27B	16758202	24473	1486	131	9(3)	**I**,P,C2	76
rap1A	51859268	20974	605	51	1(4)	**I**,P	72
rap1B	595280	20916	457	54	2(5)	I,**P**	72
Vacuolar H+ATPase subunits							
H+ATPase V0 subunit a1	139352	96265	3248	280	23		
H+ATPase V0 subunit a2	62990119	142777	129	12	2		
H+ATPase V0 subunit c	18677757	15798	45	21	2	**I**,P	
H+ATPase V0 subunit d	58865424	40275	755	66	11	**I,P**	
H+ATPase V1 subunit A1	34869154	68222	388	30	9	**I**	102
H+ATPase V1 subunit B2	19705578	56515	535	65	13	I,**P**	
H+ATPase V1 subunit C1	58865560	50827	350	26	8	I,**P**	
H+ATPase V1 subunit D	38648872	28291	143	9	4	**I**,P	
H+ATPase V1 subunit E1	37589624	26112	150	22	7	I,**P**	102
H+ATPase V1 subunit H	62078587	43873	170	23	6	I,**P**	

Table 2

List of Putative Novel Zymogen Granule Content Proteins Identified by LC-MS/MS. Secreted proteins identified but not previously reported in zymogen granules are listed. The protein identification and classification criteria were as described in the Methods section. ** also reported by Chen et al.[72]

Protein Name	NCBI Access #	Mass	Mascot score	Peptides Total	Peptides Uni(Sh)	Prep
Pancreatic Juice Proteins						
chymopasin	16758930	28098	1077	52	5	I,P,**C**
clusterin**	57241	51342	220	18	7	I,**P**,C2
cystatin C	83301921	14012	335	8	1	**C**
gamma-glutamyl hydrolase	6978890	35807	287	7	3	I,P,**C**
Other Secreted Proteins						
17-beta hydroxysteroid dehydrogenase 13	77416416	33473	142	9	7	I,**P**
17-beta-hydroxysteroid dehydrogenase 11**	51948390	32917	140	13	7	**I**,P
amylase, salivary	58293772	58792	5110	215	1(2)	**C2**
C10orf58 homolog	73620083	25746	28	3	2	**P**
cgi67 serine protease precursor (predicted)	50927378	32180	116	9	4	**I**
eosinophil peroxidase (predicted)	62656751	98656	63	10	3	I,**P**
epidermal growth factor precursor	38303863	123870	195	10	8	**I**,P
granzyme A	23618883	28545	74	7	2	**I**
lipoprotein lipase	6981168	53049	201	23	7	**I**,P
metalloproteinase inhibitor 3 (TIMP-3)	1351251	24210	55	2	2	**P**
nucleobindin-2	17367404	50059	29	3	2	**C2**
peroxiredoxin-4	37490233	30988	100	6	5	I,P,**C2**
prostatic acid phosphatase	576258	39620	39	3	2	**I**
semaphorin 3C (predicted)	62646703	112085	47	6	2	I,**P**
sphingomyelin phosphodiesterase, acid-like 3B	71043890	51613	218	10	7	I,**P**
transcobalamin II	5910985	47390	125	5	5	**P**,C2
trypsinogen Vb	57415	26802	58	2	1	**C**
trypsinogen 8 (shares with 9)	34855586	26249	558	27	2(1)	**C2**
trypsinogen 9 (shares with 8)	34855584	26245	458	27	2(1)	**C**

Table 3

List of Putative Novel Zymogen Granule Membrane-Associated and Soluble Proteins Identified by LC-MS/MS. The criteria for protein identification were as described in the Methods section. Abbreviations are the same as in Table 1. [$$] shown in this study to be present in ZG; [**] also reported by Chen et al.[70]

Protein Name	NCBI Access #	Mass	Mascot score	Peptides Total	Peptides Uni(Sh)	Prep
Transporters and Channels						
aquaporin 8	9506395	28036	46	8	2	**I**
ATPase, aminophospholipid transporter class I, type 8A	109499663	134677	1261	66	15	**I**,P
ATPase, aminophospholipid transporter class I, type 8B	62664531	143742	172	16	5(1)	**I**,P
cation-chloride cotransporter 6	51859114	95800	75	8	2	**I**
cation-chloride cotransporter 9	24899633	77022	138	14	4	**I**
solute carrier family 7 member 4	34869937	68335	82	6	2	I,**P**
chloride channel protein 5 (CLC-5)	1122330	83014	41	6	1(1)	**I**
choline transporter-like protein 1/CD92	73918925	78685	30	2	2	**I**
ligand gated ATP receptor P2X4	51260025	43487	111	15	5	I,**P**
L-type amino acid transporter 1(4F2 light chain)	12643400	55867	56	7	2(1)	**I**,P
L-type amino acid transporter 2	16758188	58153	61	8	3	I,**P**
Na,K-ATPase alpha-1 subunit	205632	113192	140	9	4	**I**,P
P-type ATPase class II 9b	62664679	129095	614	37	6	**I**,P
similar to R13A5.9	27683291	87947	934	51	6	**I**,P
Slc3a2/CD98hc (L-type amino acid transporter)	38303937	58036	1147	109	14	**I**,P,C2
solute carrier family 1 member 3/Slc1a3	9507115	59659	62	8	3	**I**,P
solute carrier family 16, member 1	6981542	53203	161	6	2	I,**P**
solute carrier family 35, member C2	62646377	40306	39	3	2	**I**,P
solute carrier family 36, member 1	18426842	52535	575	23	6	**I**,P
solute carrier family 38, member 5	20302002	51669	90	5	3	**I**,P
transient receptor potential cation channel, subfamily M4	109458719	144038	127	10	3(1)	**I**
TWIK-2/2P domain K+ channel (rKCNK6) [$$]	9971949	34193	293	26	3	**I**
Vesicular Trafficking						
α-soluble NSF attachment protein (α-SNAP)	2143586	33150	127	4	4	**I**,P
annexin A13	109480798	43736	221	11	5	I,**P**
charged multivesicular body protein 3/vps24	27229308	25046	87	13	2	**I**,P
charged multivesicular body protein 5/SNF7DC2	73917772	24560	121	6	2	**I**
myosin Va	11559935	211630	397	22	1(4)	**I**,P
phosphatidylinositol 4-kinase type-II beta	54400734	54430	77	10	5	**P**
synaptotagmin-like 1	71043698	59435	474	47	12	**I**,P
synaptotagmin-like 4 (granuphilin-a)	17939356	75853	154	14	6	**I**,P
syntaxin 16b	109469301	68308	96	4	3	**I**,P
syntaxin 12	77695930	31168	220	13	4	**I**
vacuolar protein sorting-associated protein 45	23396892	64853	32	4	2	**I**
Vesicular Trafficking -- GTPases and Effectors						
ADP ribosylation factor ARF-4	6680720	20384	58	6	3	I,**P**
ADP ribosylation factor-related Arl8b	8922601	21525	155	9	7	I,**P**
cdc42	24637541	21271	107	15	2	**I**,P
G(i)α1	6980962	40119	196	21	2(2)	**I**,P
G(i)α2	13591955	40473	224	11	2(3)	I,**P**
G protein alpha 13 (GNA13)	61557003	43984	142	18	2(1)	**I**
MAP-kinase activating death domain	16758360	177880	120	13	2	**I**,P
noc2/rabphilin 3A-like without C2 domains	19424292	33412	67	4	2	I,**P**
rab1a[**]	32527715	22663	788	87	7(1)	**I**,P,C2
rab1b (shares with 1a)[**]	52138628	22176	436	56	2(6)	**I**,P
rab2a	13929006	23521	586	59	9	**I**,P,C2
rab2b	83415090	24070	286	38	1(4)	**I**
rab3a	61098195	24954	865	81	1(5)	**I**

J Proteome Res. Author manuscript; available in PMC 2008 November 10.

Protein Name	NCBI Access #	Mass	Mascot score	Peptides Total	Peptides Uni(Sh)	Prep
rab4a	38303943	23877	97	12	2(1)	**P**
rab4b	21313012	23614	121	11	2(1)	I,**P**
rab7	92022	22789	153	11	7	I,**P**
rab8a[**]	49522647	23522	346	35	4(3)	**I**,P
rab8b	23463313	23588	233	26	1(5)	I,**P**
rab10	420269	22844	357	35	3(2)	**I**,P
rab13	21952483	22887	133	17	1(2)	**P**
rab14[**]	420272	23850	335	20	5(1)	**I**,P
rab18	27685547	22962	78	9	4	**I**,P
rab26	1083775	28186	227	22	3(1)	**I**,P
rab27a	8394142	25052	558	45	4(3)	**I**,P
rab35	62900797	23011	397	33	4(2)	**I**,P
rac1[**]	54607147	21436	272	35	3	**I**,P
ralA[$$]	13592039	23538	286	41	2(4)	**I**,P
ralB	47939184	23303	159	17	2(4)	I,**P**
rap2b	47117735	20491	60	2	2	I,**P**
h-ras	131873	21301	73	10	3	**I**,P
k-ras2 protein	13928698	21481	105	8	3	I,**P**
n-ras	18158431	21230	62	7	1(2)	**P**
r-ras	34856057	23894	48	2	2	**I**,P
rhoA	2225894	22109	105	11	2	**P**,C2
rhoG	79152381	21295	64	5	2	I,**P**
rho activating protein 1 (p50-rhoGAP)	62645722	53024	195	14	5	I,**P**
rho-GDI3	34870596	25295	92	10	5	**I**,P
Tetraspan Membrane Proteins						
CD151 antigen	11968106	28336	46	2	2	**I**,P
CD81 antigen	6978639	25871	288	14	2	I,**P**
claudin domain containing 1	55741538	28529	77	7	2	**I**
glycoprotein, synaptic 2	37589607	36099	98	4	2	**I**
pantophysin[$$]	62079261	28610	378	45	4	**I**,P
tetraspanin-7	62666547	32122	323	29	3	I,**P**
tetraspanin-8/transmembrane 4 member 3	38197346	25528	94	7	2	I,**P**
transmembrane 4 superfamily member 6 Tspan6	27674865	27517	228	16	4	**I**,P
Enzymes – Membrane-associated						
2',3'-cyclic nucleotide 3-phosphodiesterase[$$]	203493	47239	907	61	8	I,**P**
5'-nucleotidase	11024643	63928	112	8	6	**I**,P
aminopeptidase M/N[$$]	601865	109249	2752	140	23	**I**,P
aminopeptidase Vp165	19424264	104503	37	4	2	**P**
beta Klotho protein	109500603	86988	96	6	4	**I**
carbonic anhydrase XIV	62643907	37502	72	2	2	**I**,P
carboxypeptidase D[$$]	6978699	152520	671	32	18	**I**,P
DHHC24 zinc finger protein RGD1305755	86129558	42604	167	11	2	**I**
ectonucleoside triphosphate diphosphohydrolase/CD39	12018242	57371	244	12	4	I,**P**
ectonucleotide pyrophosphatase/phosphodiesterase 3[$$**]	1526949	99024	5554	357	27	**I**,P
glycerophosphodiester phosphodiesterase domain containing 5	62640954	68644	151	13	3	I,**P**
hepsin	57929	44898	137	6	5	I,**P**
hypothetical protein LOC304325 (putative kinase)	70794760	46848	375	37	4	**I**,P
interferon gamma induced GTPase	32527749	161531	140	6	4	**P**
itchy homolog E3 ubiquitin protein ligase	54312102	97685	126	8	3	**I**
leukocyte common antigen-related protein	249840	211427	805	53	23	**I**,P
lyn B protein tyrosine kinase	294581	58492	41	4	2(1)	**I**
metallo-beta-lactamase superfamily protein like	34853071	31170	58	3	3	**I**
nicastrin	27819651	78350	1499	59	12	**I**,P,C2
phogrin/IA2-beta	13928818	111794	229	23	10	I,**P**
phospholipid scramblase 1	17105346	36687	75	10	3	**I**,P

Protein Name	NCBI Access #	Mass	Mascot score	Peptides Total	Peptides Uni(Sh)	Prep
presenilin-1	6174931	52756	33	6	2	**I**,P
protease, serine, 8 (prostasin)	20301968	36820	131	7	2	**I**,P
protein tyrosine phosphatase non-receptor type 9	61556770	67919	300	26	5	**I**,P
protein tyrosine phosphatase receptor type D	109475037	200136	177	24	5(2)	**I**
Enzymes – Other						
aldehyde dehydrogenase 1	109480409	107333	91	5	3	**C2**
glutathione S-transferase pi	25453412	23424	119	5	3	**C2**
heat shock cognate 71 kDa protein	13242237	70884	422	22	11	I,**P**
heat shock protein hsp 90-beta	1346320	83264	106	2	2	I,**C2**
peroxiredoxin-1	2499470	22095	58	4	4	P,**C2**
pyrroline-5-carboxylate reductase 1	109489508	48113	100	3	3	**I**
vesicle amine transport protein 1 homolog	76096306	43091	312	23	6	I,**P**
Other Membrane-associated Proteins						
alpha catenin	55742755	100174	49	8	2	**I**
Alzheimer's precursor protein (APP)	55617	86649	367	15	7	**I**,P,C2
B-box and SPRY domain containing protein	38541109	49153	107	8	4	I,**P**
beta-2-microglobulin	7549746	13711	65	4	2	**I**
beta-filamin	109501396	291287	154	8	5	**I**,P
cation-dependent man-6-PO4 receptor	56090485	31075	78	6	2	**P**
CD166	47605356	64981	62	3	2	**I**
CD1d**	2118857	38616	754	74	9	I,**P**,C2
CD47 integrin-associated signal transducer**	9506469	32974	156	20	4	I,**P**
c-met/hepatocyte growth factor receptor	1771558	153843	95	7	3	**I**,P
coxsackie-adenovirus-receptor	6013133	39923	68	4	3	**I**
Fras1 related extracellular matrix protein 2	109464881	378472	108	14	5	I,**P**
glypican 4 heparan sulfate proteoglycan	62078949	62522	245	11	5	**I**,P
Golgi sialoglycoprotein MG-160	8393450	133469	38	7	2	I,**P**
hippocalcin-like protein 1	8393864	22324	52	10	4	I,**P**
hypothetical protein DKFZp566N034	109497812	55939	118	5	3	**P**
immunoglobulin superfamily member 3	109465349	159874	91	11	2	**I**
insulin receptor	8393621	156656	50	7	3	**I**,P
integral membrane protein 2B	55741681	30294	252	14	6	**I**,P
integrin alpha 5	299145	27655	138	9	4	**I**
integrin alpha 6	109468286	108731	422	24	14	**I**,P
integrin alpha FG-GAP repeat containing 3	57527508	60599	156	5	3	I,**P**
integrin beta 1	8393636	88436	238	15	8	**I**,P
integrin beta 4	6981108	200462	318	18	11	**I**,P
integrin beta-5	109494417	53410	130	4	2	**I**
interleukin 1 receptor accessory protein	6981096	65556	39	2	2	**P**
interleukin-6 receptor beta chain (IL-6R-ß)	729835	112873	379	17	8	**I**,P
KIAA0152	62900389	32398	121	8	5	**I**
LDL receptor-related protein 5	62641684	178866	56	2	2	**I**
leukemia inhibitory factor receptor	13591979	122317	306	17	12	**I**,P
low density lipoprotein receptor	28461161	96559	87	2	2	**I**
MARCKS-related protein	76363234	19704	28	2	2	**I**
membrane targeting tandem C2 domain containing	68342017	55019	195	35	7	**I**,P
mucin 1	62643778	176643	235	14	3	**I**,P
myeloid-associated differentiation marker	33590376	35125	258	28	4	**I**,P
neogenin	10720132	159959	187	10	7	**I**,P
ninjurin 1	25742745	16529	29	3	2	**I**
occludin	81885277	59148	50	7	2	**I**
osteoclast inhibitory lectin	13958626	25670	218	8	2	**I**
phosphatidylethanolamine binding protein	8393910	20788	146	3	3	**C2**
plexin-B2	109481135	215981	131	10	6	**I**
polymeric immunoglobulin receptor	27151742	84745	1553	63	10	**I**,P,C2

Protein Name	NCBI Access #	Mass	Mascot score	Peptides Total	Peptides Uni(Sh)	Prep
polyubiquitin**	1050930	8560	247	23	3	I,**P**
prostaglandin F2 receptor negative regulator	9507007	98669	141	10	9	I,**P**
similar to CG8841-PA, isoform A	109489377	88698	76	6	3	**I**,P
similar to RAS and EF-hand containing	109472665	127613	913	62	11	**I**,P
similar to Y73F8A.5	109467419	110513	72	8	2(1)	**I**,P
single Ig IL-1-related receptor	76363407	46142	90	9	3	**I**
sushi domain containing 2	109509348	95414	383	24	7	**I**,P
syndecan 4	6981522	21948	236	4	3	**I**,P
syndecan binding protein/syntenin-1	14010891	32403	92	16	2	**P**
T-cell immunomodulatory protein	19424236	67294	359	33	5	I,**P**
transmembrane protein 16A	109459694	146996	41	5	2	I,**P**
transmembrane protein 16F	62652993	138377	603	29	7	**I**,P
transmembrane protein 2	62642028	153857	66	5	2	**I**
transmembrane protein 30A	50925775	37149	63	5	4	**I**,P
transmembrane protein 63A	109498279	105429	1763	121	13	**I**
transmembrane protein 63B	109486727	123841	85	9	3	**I**,P
tumor-associated calcium signal transducer 1	49117328	35185	78	3	2	I,**P**
very low density lipoprotein receptor	6981706	96479	643	31	10	**I**,P
V-set and immunoglobulin domain containing 1	83649788	43f910	57	4	2	**P**
Uncharacterized Proteins						
hypothetical protein LOC290303	57526900	21667	125	8	2	**I**,P
hypothetical protein LOC307833	62945264	39917	64	5	2	**P**
hypothetical protein NipSnap2	62945328	32921	64	6	2	**P**
similar to CG5149-PA	109508213	73536	119	8	3	**P**
uncharacterized protein family UPF0227 member	109459017	51511	114	9	3	**I**
uncharacterized protein family UPF0227 member	55741536	33972	88	6	2	**I**,P