research articles Journal of proteome research

Proteomics of Dense Core Secretory Vesicles Reveal Distinct Protein Categories for Secretion of Neuroeffectors for Cell−Cell Communication

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Received April 3, 2010

Regulated secretion of neurotransmitters and neurohumoral factors from dense core secretory vesicles provides essential neuroeffectors for cell−cell communication in the nervous and endocrine systems. This study provides comprehensive proteomic characterization of the categories of proteins in chromaffin dense core secretory vesicles that participate in cell−cell communication from the adrenal medulla. Proteomic studies were conducted by nano-HPLC Chip MS/MS tandem mass spectrometry. Results demonstrate that these secretory vesicles contain proteins of distinct functional categories consisting of neuropeptides and neurohumoral factors, protease systems, neurotransmitter enzymes and transporters, receptors, enzymes for biochemical processes, reduction/oxidation regulation, ATPases, protein folding, lipid biochemistry, signal transduction, exocytosis, calcium regulation, as well as structural and cell adhesion proteins. The secretory vesicle proteomic data identified 371 proteins in the soluble fraction and 384 membrane proteins, for a total of 686 distinct secretory vesicle proteins. Notably, these proteomic analyses illustrate the presence of several neurological disease-related proteins in these secretory vesicles, including huntingtin interacting protein, cystatin C, ataxin 7, and prion protein. Overall, these findings demonstrate that multiple protein categories participate in dense core secretory vesicles for production, storage, and secretion of bioactive neuroeffectors for cell−cell communication in health and disease.

Keywords: secretory vesicles • human • proteomics • mass spectrometry • proteins • neuropeptides • cell−cell communication • neuron • neurological disease

Introduction

The nervous system utilizes dense core secretory vesicles for regulated secretion of chemical neurotransmitters and neurohumoral factors that are represented by neuropeptides, catecholamines, and related neuroeffector molecules for cell−cell communication.[1−5] These secretory vesicles represent the primary subcellular site for the biosynthesis, storage, and secretion of neurotransmitters and hormones utilized for cell−cell communication in the nervous and endocrine systems for health and disease.

The dense core secretory vesicles of chromaffin cells of the peripheral sympathetic nervous system are a representative model for neurochemical enzymes utilized in brain for the biosynthesis of neuroeffectors composed of neuropeptides and catecholamines (dopamine, norepinephrine, and epinephrine).[5−7] The majority of prior studies have studied individual proteins of these dense core secretory vesicles.[8−13] However, a more global understanding of secretory vesicle components is essential to gain knowledge of the repertoire of protein systems that function in this organelle. Elucidation of the proteome characteristics of dense core secretory vesicles can provide valuable insight into the functional protein processes for production and secretion of neuroeffectors, the goal of this study.

The high sensitivity of current mass spectrometry (MS) instrumentation, coupled with efficient HPLC (high-pressure liquid chromatography) separation of peptides, allows proteomic investigations to identify hundreds of proteins from small amounts of samples. Furthermore, enrichment of moderate to low abundant proteins in chromaffin secretory vesicles

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10.1021/pr1003104
Published on Web 08/09/2010

for this study was achieved by removal of the abundant chromogranin A protein. Peptide identifications from mass spectrometry data were obtained using two independent search algorithms for database searching, combined with searches against a shuffled decoy database for estimation of false discovery rate (FDR) for tryptic peptide identifications. The overall proteomic data resulted in identification of 371 soluble and 384 membrane proteins from dense core secretory vesicles, for a total of 686 distinct secretory vesicle proteins.

Significantly, proteomic data illustrated distinct biochemical functions in dense core secretory vesicles composed of proteins for neuropeptides and neurohumoral factors, protease systems, neurotransmitter enzymes, receptors, biochemical enzymes, regulation of redox status, protein folding, ATPases, lipid and carbohydrate functions, signal transduction and GTP-binding proteins, and proteins for exocytosis. Interestingly, several proteins known to participate in neurological diseases were indicated consisting of the amyloid precursor protein (APP), huntingtin-interacting protein, ataxin 7, and prion protein that represent key elements involved in the mechanisms of Alzheimer's disease,[14−18] Huntington's disease,[19−22] spinocerebellar ataxia,[23−25] and prion disease.[26−28] These secretory vesicles also contain the CLN8 protein involved in neurodegeneration and mental retardation of EPMR (epilepsy and mental retardation),[29−32] and the P20-CGGBP protein involved in the fragile X syndrome of mental retardation.[33] Furthermore, these vesicles also contain regulatory factor X4 involved in bipolar disorder[34] and KIAA0319 that is involved in dyslexia.[35]

Overall, proteomic investigation of dense core secretory vesicles revealed functionally distinct categories of protein systems in this organelle, with several involved in neurological disease. These proteomic data illustrate a view of the secretory vesicle "system" for secretion of neuroeffectors mediating neuronal and endocrine cell−cell communication in health and disease.

Materials and Methods

Purification of Chromaffin Secretory Vesicles from Bovine Adrenal Medulla and Preparation of Soluble and Membrane Components. Dense core secretory vesicles, represented by chromaffin secretory vesicles (also known as chromaffin granules), were purified from fresh bovine adrenal medulla by differential sucrose density gradient centrifugation, as described previously,[37,38] involving extensive wash steps to obtain purified chromaffin granules. We have documented the high purity of this preparation of isolated secretory vesicles by electron microscopy and biochemical markers.[36−38] Sucrose gradient purification results in a preparation of purified, intact chromaffin secretory vesicles that lack markers for the subcellular organelles of lysosomes (acid phosphatase marker),[38] cytoplasm (lactate dehydrogenase marker),[37] mitochondria (fumarase and glutamate dehydrogenase markers),[36,37] and endoplasmic reticulum (glucose-6-phosphatase marker).[37] Enzyme markers have been measured in the purified chromaffin secretory vesicle preparation as 1% or less of total homogenate markers, which, thus, indicate the high purity of these isolated secretory vesicles.[36−38]

In addition, this study further assessed the removal of the lysosomal enzyme marker acid phosphatase from the purified preparation of chromaffin granules compared to an unpurified sample of chromaffin granules obtained at an early step in the purification procedure (illustrated in Figure 1a). Purified and unpurified granules were analyzed on a multistep sucrose gradient of 2.2 to 1.2 M sucrose (2.2, 2.1, 2.0, 1.9, 1.8, 1.7, 1.6, 1.5, 1.4, and 1.2 M sucrose steps each consisting of 2.5 mL) by ultracentrifugation at 120 000× *g* in a SW28 rotor (25 000 rpm) at 4 °C for 100 min. Gradient fractions of 0.5 mL were collected from the bottom of the tube (2.2 M sucrose), and fractions were assayed for (Met)enkephalin by RIA as previously described[5] as a marker for chromaffin granules, and acid phosphatase activity as a marker for lysosomes as described previously.[38] Results show that the purified chromaffin granules lack acid phosphatase activity, indicating effective removal of lysosomes of density near that of chromaffin granules (explained in Figure 1 of results). These new data and established purity in the literature[36−38] document the purity of these chromaffin secretory vesicles for this study.

Soluble and membrane components of the purified chromaffin granules were prepared by lysing (by freeze−thawing) purified chromaffin granules in isotonic buffer conditions consisting of 150 mM NaCl in 50 mM Na-acetate, pH 6.0, with a cocktail of protease inhibitors (10 μM pepstatin A, 10 μM leupeptin, 10 μM chymostatin, 10 μM E64c, and 1 mM AEBSF). The lysed granules were centrifuged at 100 000× *g* (SW60 rotor) at 4 °C for 30 min. The resultant supernatant was collected as the soluble fraction. The pellet was collected as the membrane fraction, and washed two times by resuspending in the lysis buffer and centrifugation (100 000× *g*, 30 min). The final pellet was resuspended in the lysis buffer and designated as the membrane fraction.

The soluble and membrane fractions were each subjected to removal of the abundant chromogranin A (CgA) protein, by its binding to calmodulin-Sepharose (GE Healthcare, formerly Amersham Biosciences, Piscataway, NJ).[39] The soluble fraction and membrane fraction (solubilized in 50 mM CHAPS) were each incubated with a slurry of calmodulin-Sepharose at 4 °C overnight in equilibration buffer (50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 2 mM $CaCl_2$, and protease inhibitors consisting of 5 μM E64c, 5 μM leupeptin, 5 μM chymostatin, 5 μM pepstatin A, 5 μM bestatin, 1 μM GEMSA, and 50 mM PMSF). The mixture was centrifuged and the supernatant collected as the soluble fraction without CgA. This step removed approximately 90−95% of CgA, based on assessment by anti-CgA Western blots.

Proteins in the membrane fraction were concentrated by chloroform−methanol precipitation. To the membrane fraction (400 μg in 300 μL) was added MeOH (400 μL), chloroform (100 μL), and deionized water (300 μL) with mixing between each step, followed by centrifugation (14 000× *g* for 1 min). The top aqueous layer was removed, while retaining the protein precipitate at the top of the chloroform layer; after addition of MeOH (400 μL), mixing, and centrifugation (14 000× *g* for 2 min), the pelleted protein was collected for trypsin digestion.[40]

Reduction/Alkylation and Trypsin Digestion of Samples. Reduction and alkylation were performed using Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP) (Pierce, Rockford, IL) reduction followed by cysteine alkylation with iodoacetamide (Sigma-Aldrich, St. Louis, MO). Briefly, a sample of the CG soluble fraction (8.6 μg) was lyophilized, redissolved in 12 μL 20% acetonitrile, followed by addition of 4 μL 100 mM TCEP in 20% acetonitrile (28.7 mg/mL TCEP) and reduction at 55 °C for 20 min. The reduced sample was cooled to room temperature, 4 μL of 100 mM iodoacetamide in 20% acetonitrile was added and incubated for 30 min at room temperature (in the dark) for alkylation of free cysteine residues. For proteins of the membrane fraction (400 μg), the proteins precipitated by chloroform−methanol (described above) were subjected to the

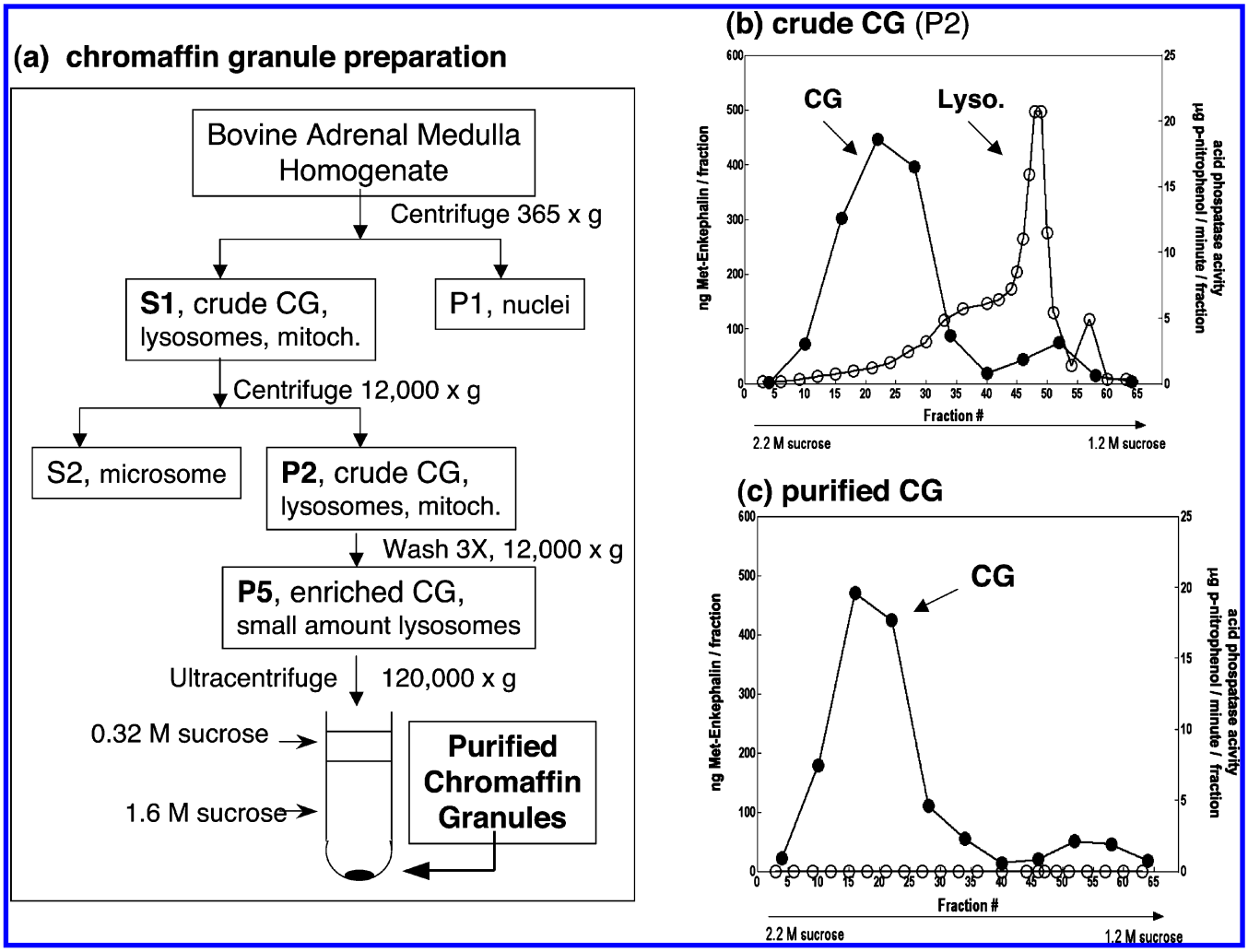

Figure 1. Purity of isolated chromaffin granules (secretory vesicles) was evaluated with (Met)enkephalin as a marker for chromaffin granules and with acid phosphatase as a marker for lysosomes. (a) Preparation of purified chromaffin granules by differential density centrifugation. The flowchart illustrates the purification scheme for chromaffin granules from bovine adrenal medulla homogenate, achieved by differential centrifugation. The homogenate (in 0.32 M sucrose buffer) is centrifuged at 365× *g* to remove nuclei (P1, pellet 1) from the supernatant (S1, soluble fraction 1) that represents a crude fraction of chromaffin granules. The granules were pelleted by centrifugation at 12 000× *g* and washed three times in 0.32 M sucrose buffer to obtain enriched chromaffin granules (P5 fraction) that undergoes purification on a 1.6/0.32 M sucrose gradient subjected to ultracentrifugation (120 000× *g*) to obtained a pellet of purified chromaffin granules. (b) Analyses of crude fraction of chromaffin granules on multistep sucrose gradient. The crude chromaffin granule fraction (P2) was analyzed on a multistep sucrose gradient of 2.2 to 1.2 M sucrose as described in the methods. Gradient fractions were assayed for (Met)enkephalin (●) that is present in chromaffin granules, and for the lysosomal enzyme marker acid phosphatase (○). The crude P2 fraction of chromaffin granules contains enkephalin and acid phosphatase. (c) Analyses of purified chromaffin granules on multistep sucrose gradient. The purified chromaffin granules were analyzed on the multistep sucrose gradient of 2.2 to 1.2 M sucrose as described in the methods. Gradient fractions were assayed for (Met)enkephalin and acid phosphatase. The presence of the purified chromaffin granules is indicated by the peak of (Met)enkephalin. The multistep gradient showed no peak of acid phosphatase, indicating effective removal of lysosomes. These data document the purity of the chromaffin granule preparation. (It is noted that cytosolic proteins may possibly associate with the outside of the granule membrane during homogenization, and after freeze−thawing, such cytosolic proteins may become present in the soluble fraction giving the interpretation that they might be luminal proteins of the granules. Nonetheless, cytosolic proteins are likely to have importance because cellular function of the chromaffin granule must involve cytoplasmic proteins for regulated movement to achieve exocytosis.)

same procedure for reduction and alkylation using 20 µL of TCEP solution and 5 µL of iodoacetamide solution.

The reduced and alkylated proteins of the chromaffin granule soluble (CGS) and membrane (CGM) fractions were subjected to trypsin digestion. The CGS protein sample (8.67 µg in approximately 1 µL) was diluted by addition of 20 µL 25 mM ammonium bicarbonate (pH 7.0) and 1 µL 100 mM $CaCl_2$, and 10 µL trypsin stock solution was added (200 ng total trypsin, using stock solution consisting of 20 ng/µL sequencing grade trypsin in 25 mM ammonium bicarbonate, pH 7.0, trypsin was from Promega #V5111). Final trypsin digestion conditions for CGS (31.5 µL total volume) were 0.275 µg/mL CGS protein, 6.5 ng/µL trypsin, 25 mM ammonium bicarbonate, pH 7.0, and 3.2 mM $CaCl_2$. For the CGM sample (100 µg protein in ~10 µL), it was prepared by addition of 90 µL trypsin (1800 ng total trypsin, using stock solution consisting of 20 ng/µL trypsin in 25 mM ammonium biocarbonate, pH 7.0), 95 µL ammonium bicarbonate, pH 7.0, and 5 µL 100 mM $CaCl_2$. The trypsin digestion conditions for CGM (200 µL total volume) were 0.5 µg/µL protein, 9.0 ng/µL trypsin, 25 mM ammonium bicarbon-

ate, pH 7.0, and 2.5 mM $CaCl_2$. Trypsin digestion of CGS and CGM samples was conducted by incubation at 37 °C for 18 h.

Nanoliquid Chromatography Tandem Mass Spectrometry (nano-HPLC Chip MS/MS). Soluble and membrane chromaffin granule sample digests were subjected to nano-LC−MS/MS, loaded at 2.9 μg and 1.0 μg total protein for each LC−MS/MS analysis. All LC−MS/MS analyses were performed in triplicate on an Agilent XCT Ultra ion trap mass spectrometer coupled to an Agilent 1100 nano-HPLC system fitted with a HPLC Chip system. The LC separation was performed on an Agilent C18 analytical HPLC chip (Agilent Zorbax C18 Chip, 150 mm × 75 μm, 40 nL trap) and utilized a gradient of solvent B (acetonitrile with 0.25% formic acid) in solvent A (water with 0.25% formic acid). The gradient progressed from 3% B to 45% B in 40 min followed by an increase to 75% B in 10 min. The mass spectrometer was set for data dependent scanning in MS/MS mode on the three most abundant ions present in the MS scan. The exclusion time was set to 0.1 min, isolation window set to 4 amu, and voltages set to −1850 V (capillary), −500 V (counterelectrode) and 1.30 V (fragmentation). Smart ion target was set to 500 000 to correct for background ions. The maximum injection time was set to 100 ms. All other default settings were used and left unaltered in all experiments.

Analyses of MS/MS Data and Database Search Parameters Using Spectrum Mill Bioinformatics Platform (Agilent Technologies, Santa Clara, CA). The Spectrum Mill database search platform used 1.4 amu for precursor mass tolerance and 0.8 amu fragment mass tolerance with all other default parameters retained. The search was unrestricted and included nontryptic peptide identifications to prevent forced identifications for nontryptic peptides. Protein identifications resulted from searches against a bovine protein database (extracted from the NCBI-nr protein sequence database consisting of 38,197 protein entries). We also employed the OMSSA MS/MS search engine using a 1.0 amu precursor mass tolerance and 0.3 amu fragment mass tolerance against the IPI bovine database. The search was restricted to tryptic and semitryptic peptides. In all searches, carbamidomethylation was included as a variable modification to compensate for the possibility of incomplete alkylation. Spectrum Hill and OMSSA were comparable in their scoring thresholds resulting in identification of approximately the same total number of proteins.

Validation for MS/MS Identifications. Protein identifications were evaluated using analysis against proteins known to be present in these samples and by Spectrum Mill database search against a scrambled decoy database.[41] Based on these analyses, protein identifications utilized a two-tiered system for peptide identifications. The first tier consisted of proteins with high confidence, peptide scores of ≥10 and Scored Peak Intensity Percent (%SPI) ≥70. When implemented, the False Discovery Rates (FDR) for these protein identifications using tryptic peptides were ∼1%, even when single peptides were considered. Protein identification with single tryptic peptides can be confidently achieved as illustrated by previous reports on confident protein identifications based on single peptides.[42] The second tier targeted proteins showed confidence levels corresponding to peptide score ≥8 and %SPI >60, combined with the requirement that at least two tryptic peptides of the parent protein were utilized for identification. The levels of these scores were considered to represent identification of proteins based on prior biochemical studies documenting the presence of low abundance proteins in the chromaffin secretory vesicles, which include endopin,[43] cathepsin D,[44] cathepsin B,[45] and prohormone convertase.[46] The FDR for this second tier was estimated at 1−2% by decoy database analysis. It is important to note that the FDR determined by decoy database analysis is an estimate and is dependent on the quality of spectra and the randomized database.[41] Therefore, we manually evaluated our scoring thresholds in a manner similar to reported by Wang, et al.[41]

Protein Organization and Clustering. Tryptic peptides shared between several proteins are only counted for the protein that has overall the most matching, unique peptides. Batch Entrez (http://ncbi.nlm.nih.gov/entrez/batchentrez.cgi?db=Protein) was used to generate FASTA formatted protein sequence databases for each GenInfo Identifier (GI) number for proteins identified by the MS experiment. BLASTCLUST was used to perform pairwise comparisons followed by single-linkage clustering of the statistically significant matches (>95% sequence similarity over 90% of the sequence length) (http://www.ncbi.nlm.nih.gov/blast/). The protein list is thus the smallest set of proteins explaining the identified proteins present. Following this analysis, an annotated, nonredundant table of soluble and membrane proteins was compiled (Supplemental Tables A and B, Supporting Information).

The functional categories of identified proteins were defined by the gene ontology (GO) resource (http://www.geneontology.org). Further information on the function of proteins was obtained through KEGG and Interact pathway databases, as well as through the MEROPS database to provide additional information relevant to proteases. A series of GO terms in each category was acquired though text searching of specific keywords relating to function and localization. In addition to gene ontologies, both identified and unidentified protein sequences were queried against the InterPro (http://www.ebi.ac.uk/interpro/) database, SignalP resource (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM resource (http://www.cbs.dtu.dk/services/TMHMM/) to assess protein family. All automated searches were enhanced with PubMed searches to assess recent literature where proteins are known to serve multiple functions.

Several peptide sequences were identified that were not functionally annotated in initial database searches. Based on the identified tryptic peptide sequences, predicted mouse and human sequences were aligned back to bovine sequences using the TIGR gene indices (http://tigrblast.tigr.org/tgi/). Proteins demonstrating strong homology to existing bovine sequences were included in the nonredundant assembly of identified chromaffin granule proteins.

Proteomic data (Table 1) combines proteins identified in the sample after the calmodulin affinity step from experiments of this study with proteins identified before the calmodulin affinity step from our previous more limited proteomic study of bovine chromaffin granules.[47] Thus, proteins that may bind to the calmodulin affinity column are included in this complete proteomic data set of proteins identified from this study and our previous, smaller proteomic study of bovine chromaffin granules; the combined proteomic data set is illustrated in Table 1.

Analyses of Selected Chromaffin Granule Proteins by Western Blots and Immunofluorescence Confocal Microscopy. Western blot analyses of chromaffin granules were utilized to assess the presence of several proteins related to neurological diseases. Western blots of cystatin C, huntingtin interacting protein, ataxin 7, and prion protein were conducted using SDS-PAGE gel electrophoresis and Western blots methods as we have

Table 1. Functional Categories of Soluble and Membrane Proteins in Chromaffin Secretory Vesicles[a]

Protein Function	Genbank	Soluble	Membrane
Production of Neurotransmitters and Neurohumoural Factors			
Neuropeptides (Proproteins) and Neurohumoural Factors	Genbank	Soluble	Membrane
Adrenomedullin*	27806927		
Amyloid beta A4 precursor#	76613693		
Angiopoietin-4 precursor (ANG-4)	76633205		
Cathelicidin 1+	27807341		
Chromogranin A*	116548		
Chromogranin B*	12		
Chromogranin C*	27806421		
Decorin (bone proteoglycan II)*	54660107		
Epithelium-derived growth factor (EGF)	76637576		
Glycoprotein II+	1809215		
Glycoprotein III (clusterin)*	27806907		
Interleukin 27	76655433		
Neuroendocrine secretory protein 55 (NESP-55)*	2262205		
Osteocrin	76671357		
Osteogenin (BMP3)*	76620194		
Platelet basic protein precursor (PBP)	76619991		
Proenkephalin*	83405428		
Pro-Neuropeptide Y*	40022234		
Secretogranin III*	76663325		
Transforming growth factor-beta binding protein*	135671		
Ubiquitin/ribosomal fusion protein	28189665		
VEGF (vascularendothelial growth factor)+	27806821		
VGF nerve growth factor inducible*	76654056		
Protease Systems	Genbank	Soluble	Membrane
ADAM metallopeptidase with thrombospondin type 1	76648708		
AFG3-like protein 1	76677962		
Alpha-1-antichymotrypsin precursor (ACT)	76694546		
Alpha-2-plasmin inhibitor	76643654		
Autophagy 4 homolog D (APG4)	76621759		
Bleomycin hydrolase	76643627		
Carboxypeptidase D*	76643631		
Carboxypeptidase E*	76675819		
Carboxypeptidase G2	76647796		
Cathepsin B+	9955277		
Cathepsin D*	76658398		
Cathepsin L#	1542853		
COP9 constitutive photomorphogenic	74267826		
COP9 signalosome	76654132		
Cystatin C+	27806675		
Cystatin E/M	61097917		
Disintegrin and metalloprotease domain 4	76684774		
Endopin 2C#	62126072		
HECT, UBA and WWE domain containing 1	76659533		
IAP, Inhibitor of Apoptosis	76638039		
Leukotriene A4 hydrolase	74355010		
Meltrin	47564064		
Metalloprotease 1	76672659		
Neuroendocrine protein 7B2*	88682959		
Polyubiquitin	89994036		
PP11 serine protease	76618319		
Prohormone convertase 1*	61817689		
Prohormone convertase 2+	13878928		
Proprotein convertase subtilisin/kexin type 4	76622897		
Reelin precursor	76677746		
Serine (or cysteine) proteinase inhibitor	74268410		
Tissue inhibitor of metalloproteinase 1 (TIMP-1)+	27806161		
Transmembrane protease, serine 3	76608355		
Ubiquitin*	76638698		
Ubiquitin A-52 (residue ribosomal protein fusion product 1)	76620757		

Table 1. Continued

Ubiquitin associated protein 2	76624820		
Ubiquitin protein ligase E3B	76639147		
Vpr-binding protein	76649016		
XIAP associated factor-1	78045549		
YME1-like, metalloprotease	76632200		
Neurotransmitter Enzymes/Transporters	**Genbank**	**Soluble**	**Membrane**
4-aminobutyrate aminotransferase precursor	76678944		
Bestrophin anion channel	76629024		
Calcium channel, voltage-dependent, alpha	76615073		
Dopamine beta-monooxygenase*	1083022		
Glutamate decarboxylase 1	76609605		
Glutaminase	86438072		
Neuronal pentraxin I*	76669694		
PNMTase (phenylethanolamine N-methyltransferase)+	130374		
Protein tyrosine phosphatase, receptor	76614769		
Rod photoreceptor cng-channel	1050441		
Sodium channel protein type IV alpha subunit	76645224		
Sodium/potassium/calcium exchanger 1	76684545		
Synaptic vesicle monoamine transporter (VAT2)*	457486		
Transient receptor potential cation channel	76625397		
Tyrosine 3-monooxygenase (Tyrosine hydroxylase)	27807401		
Vesicle amine transport protein 1 (VAT1)	76671278		
Vesicular inhibitory amino acid transporter	76646508		
Receptors	**Genbank**	**Soluble**	**Membrane**
Adiponectin receptor 2	76679749		
Bone morphogenetic protein receptor	76619536		
Bradykinin receptor B1	76647810		
Cholinergic receptor, nicotinic, alpha polypeptide 3	27807295		
EC2-V2R pheromone receptor	76675084		
EPH receptor A7	76679477		
Estrogen-related receptor gamma	76636898		
Fc receptor-like 3 precursor	76670706		
Fibroblast growth factor receptor 4 precursor (FGFR-4)	76622759		
Gamma-aminobutyric acid receptor	76637789		
Glutamate [NMDA] receptor	76631132		
Insulin receptor substrate 4	76659092		
Interleukin-1 receptor-like 2 precursor (IL-1Rrp2)	76628778		
Muscarinic acetylcholine receptor M5	76627274		
Olfactory receptor 212	76683959		
Olfactory receptor 5A1	76636626		
Olfactory receptor 833	76693698		
Peripheral-type benzodiazepine receptor-associated protein 1	76639758		
Progesterone receptor (PR)	76678665		
T-cell receptor alpha chain C region	76626982		
Thyroid hormone receptor associated protein 2	76639110		
TNF receptor associated protein	81673141		
Vascular endothelial growth factor receptor 3 (VEGFR-3)	76663199		
Biochemical Processes			
Enzymes	**Genbank**	**Soluble**	**Membrane**
1-aminocyclopropane-1-carboxylate synthase	76636325		
Ash1 (absent, small, or homeotic)	76612493		
Arylacetamide deacetylase (AADAC)	76607894		
Aspartate aminotransferase 1	29135295		
Bisphosphoglycerate mutase	61839453		
C-1-tetrahydrofolate synthase, cytoplasmic	76628072		
CG7544-PA	76643676		
Cyclophilin B (PPIB)	74268324		
Dihydropyrimidinase-related protein 4 (DRP-4)	61878819		
Enolase 2	88682888		
Enolase 3	88954201		
Folate receptor 1 precursor	76635818		
Glycerol-3-phosphate acyltransferase	76628542		
Malate dehydrogenase (MADH2)*	81674781		
Metallothionein-like 5 (testis-specific, tesmin)	76658427		
Methionine adenosyltransferase II, beta	81673843		
NAD synthetase 1	73587273		
Paraoxonase 2	61888862		
Phosphoribosylglycinamide formyltransferase	61966468		
Reticulon 4 interacting protein 1	76649171		

Table 1. Continued

Ribonucleoside-diphosphate reductase	76630263		
Splicing factor, arginine/serine-rich 15	76645770		
Thioredoxin domain containing protein 5	76676581		
Carbohydrate Functions	**Genbank**	**Soluble**	**Membrane**
Alpha-(1,3)-fucosyltransferase (Galactoside 3-L-fucosyltransferase)	76631232		
Alpha-1,6-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase	61864843		
Amylo-1,6-glucosidase, 4-alpha-glucanotransferase	76613348		
Bactericidal/permeability-increasing protein-like 3	76633471		
Beta 1,4-N-acetylgalactosaminyltransferase-transferase-3	76663169		
Beta-1,3-N-acetylglucosaminyltransferase bGnT-6	76645514		
Beta-1,4-galactosyltransferase 2	76614325		
Chondroitin sulfate glucuronyltransferase	76616133		
Galactose-1-phosphate uridylyltransferase isoform 1	61555177		
Galactose-3-O-sulfotransferase 3 isoform 1	76658175		
Glucosidase II*	76657688		
Golgi sialoglycoprotein MG-160+	76639850		
Maltase-glucoamylase	76678173		
Mannosidase*	76624022		
N-acetylglucosaminyltransferase V+	76610006		
Ribophorin II	76638094		
UDP-glucose:glycoprotein glucosyltransferase 1	76609241		
Lipid Functions	**Genbank**	**Soluble**	**Membrane**
3,2-trans-enoyl-CoA isomerase, mitochondrial precursor	76652799		
Acetyl-Coenzyme A carboxylase beta	76639133		
Acyl-Coenzyme A dehydrogenase	27806205		
Acyl-Coenzyme A synthetase	76676487		
Aminophospholipid transporter (APLT)+	27807317		
Arachidonate 12-lipoxygenase	76643357		
Carnitine O-palmitoyltransferase I	76658339		
Cerebroside sulfate activator (SAP-1)+	115502446		
Diacylglycerol kinase, beta	76681581		
Fatty acid binding protein 11	89994084		
Glucocerebrosidase precursor	76612148		
High density lipoprotein-binding protein	76634040		
Lipin-2	76651852		
Low-density lipoprotein receptor-related protein	76609709		
N-acylsphingosine amidohydrolase+	76655702		
Patatin-like phospholipase domain containing 2	76658479		
Phospholipase A1 member A (Pla1a)	83405374		
Transport-secretion protein	76658477		
Internal Conditions of Secretory Vesicles			
Reduction-Oxidation	**Genbank**	**Soluble**	**Membrane**
Biliverdin reductase A precursor	76615466		
Catalase	78369302		
Cytochrome b561*	27807323		
Cytochrome C oxidase+	117102		
Cytochrome P450*	76669152		
Dimethylaniline monooxygenase [N-oxide-forming] 2	76637349		
Endoplasmic reticulum oxidoreductin 1-Lbeta	76656155		
Glutathione peroxidase 3*	585223		
Myeloperoxidase precursor (MPO)	76642663		
NAD(P) transhydrogenase, mitochondrial precursor	128400		
Ubiquinol-cytochrome C reductase complex+	136691		
ATPases and Nucleotide Metabolism	**Genbank**	**Soluble**	**Membrane**
ADP-ribosylation factor-like 10C	76648606		
ANT 1 (adenine nucleotide translocator 1)+	32189340		
ATP H+ transporting VI+	4502315		
ATP Synthase (gamma chain)+	2493093		
ATP synthase alpha chain, mitochondrial precursor	76652040		
ATP6IP1 protein	28461231		
ATPase type 13A2	74267862		
ATPase, aminophospholipid transporter (APLT), Class I, type 8A*	27807317		
ATPase, H+ transporting*	102		
ATPase, H+ transporting, lysosomal, V1 subunit C	28603816		

Table 1. Continued

ATPase, H+ transporting, subunit A	27807453		
ATPase, H+ transporting, V1 subunit B*	28603772		
ATP-binding cassette sub-family D	61863306		
ATP-binding cassette, sub-family A	76652817		
Concentrative Na+-nucleoside cotransporter	76661614		
Ectonucleoside triphosphate diphosphohydrolase 1 protein	76654745		
HT028 (ATPase)*	61823467		
MSTP042+	75832069		
Proton-associated sugar transporter A	76671385		
TER ATPase (transitional endoplasmic reticulum)+	73586667		
V-ATPase (vacuolar ATPase accessory subunit B)+	549205		
V-ATPase (vacuolar ATPase accessory subunit D)	62460538		
V-ATPase (vacuolar ATPase accessory subunit E1)	27807375		
V-ATPase (vacuolar ATPase accessory subunit F1)*	94574271		
V-ATPase (vacuolar ATPase accessory subunit SFD alpha isoform)	2895578		
V-ATPase (vacuolar ATPase polypeptide IV)	89602		
V-ATPase (vacuolar ATPase synthase subunit H)+	12643366		
Protein Folding	**Genbank**	**Soluble**	**Membrane**
C1GALT1-specific chaperone 1	74356336		
Chaperonin 10+	1167		
Cysteine string protein (CSP)*	1232163		
Glucose-regulated protein precursor (GRP 78)	76630569		
Heat Shock Protein 27*	71037405		
Heat Shock Protein 40*	76619510		
Heat shock protein 60	76648520		
Heat shock protein 70	73586960		
Peptidylprolyl isomerase B+	27806469		
Prion protein	21666990		
Wiskott-Aldrich syndrome protein interacting protein (WIP)	76609571		
Transporters (solute)	**Genbank**	**Soluble**	**Membrane**
Organic anion transporter 3	42538740		
P87	259174		
Sodium-dependent glucose transporter SGLT-I	14486596		
Solute carrier family 2	76661712		
Solute carrier family 22, anion/cation transporter	76657700		
Solute carrier family 25, mitochondrial carrier glutamate	27807185		
Solute carrier family 26, anion transporter	76668427		
Solute carrier family 38, carrier protein	94966787		
Solute carrier family 39, zinc transporter	76651620		
Solute carrier family 4, sodum borate transporter	30794360		
Solute carrier family 6	76693476		
Regulated Secretion Mechanisms			
Signal Transduction and GTP-Binding Proteins	**Genbank**	**Soluble**	**Membrane**
1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase	76648298		
1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase-like 4	76637596		
Activator of S phase kinase	76671561		
Adenylate cyclase-inhibiting G alpha protein	27805887		
A-kinase anchor protein 4	41386786		
AHNAK-related protein*	76657680		
Ankyrin repeat domain 37	76668139		
AXL receptor tyrosine kinase	76684583		
Breast cancer membrane protein 11	76614919		
Calcium/calmodulin-dependent protein kinase IIA	76623502		
Calcium/calmodulin-dependent protein kinase IV	76679349		
CAMP-dependent protein kinase inhibitor beta	76625537		
Catenin	76656278		
CDK5 and ABL1 enzyme substrate 2 (Interactor with CDK3 2)	76632977		
Centaurin-gamma 1 GTPase	76618822		
Cyclic nucleotide-gated channel beta subunit 1e	3309626		
DIRAS family, GTP-binding RAS	73587391		
Doublecortin kinase 2	76668033		
Dual specificity protein phosphatase 2	76628632		
FK506 binding protein+	25066280		
Frizzled 9 precursor (Frizzled-9)	76653703		
FYVE, RhoGEF and PH domain containing 2	76650063		
FYVE, RhoGEF and PH domain containing protein 5	76661294		
Guanine nucleotide binding protein (G protein), alpha*	30794332		

Table 1. Continued

Guanine nucleotide binding protein (G protein), beta*	1085447		
Guanine nucleotide binding protein (G protein), type B	399711		
Guanine nucleotide binding protein (G protein), gamma*	27807509		
Guanine nucleotide exchange factor	77362757		
Guanylate kinase-associated protein	76647874		
Heart alpha-kinase	76662373		
Immunity-related GTPase family, Q1	76641801		
Inositol polyphosphate-4-phosphatase, type II	76660578		
Intestinal cell kinase	76650734		
IP3 receptor associated cGMP kinase	7341097		
Leucine-rich repeat kinase 1	76646793		
Lin-7	76641963		
Mitogen-activated protein kinase 1+	28461209		
Mitogen-activated protein kinase 5	76660373		
Mitogen-activated protein kinase 8	76656852		
Mitogen-interacting protein kinase I*	77736562		
Myomegalin	76670843		
N-ethylmaleimide-sensitive factor	76645272		
Phosphatase and tensin homolog	76655292		
Phosphatidylinositol 4-kinase beta	38372429		
Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase PTEN	76671072		
Phosphoinositide-3-kinase adaptor protein 1	76608515		
PKCI-1-related HIT protein+	14211923		
Plexin B2 precursor (MM1)	76617505		
Polo-like kinase 2	76660620		
Preferentially expressed antigen in melanoma like 5	92096623		
Protein kinase C, epsilon type (nPKC-epsilon)	76629287		
Protein kinase, DNA-activated, catalytic polypeptide	76634333		
Protein phosphatase 1, regulatory (inhibitor)	76645130		
Protein phosphatase 1J (PP2C domain containing)	76613182		
PYRIN-containing APAF1-like protein 7	76614247		
Rab-1	76629651		
Rab-12	76651878		
Rab-14*	76668547		
Rab-15	76628095		
Rab-2	76686605		
Rab-21+	2500067		
Rab-27A	61875226		
Rab-27B	24459171		
Rab-2B	76627105		
Rab-33B	76638452		
Rab-34	73587147		
Rab-35+	76639295		
Rab-37	76645964		
Rab-39A	76611110		
Rab-3A	27806127		
Rab-3B	27806113		
Rab-3C+	86438380		
Rab-4B	76641702		
Rab-5B	76618738		
Rab-6B	76608256		
Rab-7+	74354082		
Rab-8B	76627752		
Receptor-type tyrosine-protein phosphatase N2	76676438		
Regulator of G-protein signalling 2, 24kDa	76636875		
Retinitis pigmentosa	21303187		
Rho guanine nucleotide exchange factor 12	76635393		
Rho guanine nucleotide exchange factor 4	76609133		
Serine/threonine-protein kinase 38	76650116		
Serine/threonine-protein kinase PLK2	76660624		
SET binding factor 1	76617331		
Signal-induced proliferation-associated 1	76628293		
Son of sevenless homolog 1	76629071		
Src homology 3 domain-containing guanine nucleotide exchange factor	76676576		
Sterile alpha and TIR motif containing 1	76643315		
Synaptojanin 2 binding protein	74354054		
Testis-specific serine kinase 6	76621013		
Tousled-like kinase 1	76609603		
Transducin protein 4	76666047		
Tuberin (Tuberous sclerosis 2 homolog protein)	76652568		
Tyrosine-protein kinase JAK2	76661504		
Tyrosine-protein phosphatase-like N precursor (R-PTP-N)*	76610663		
Very large G-protein coupled receptor 1	76660074		
Virus-induced signaling protein	86438388		
WD-repeat protein	76612957		
Wingless-type MMTV integration site family, member 10B	76618188		
WNK lysine deficient protein kinase 2	76664151		
Wnt inhibitory factor 1 precursor (WIF-1)	76618509		

Table 1. Continued

Vesicular Trafficking and Exocytosis	Genbank	Soluble	Membrane
Clathrin+	27806689		
Coatomer alpha subunit (Alpha-coat protein)	76615759		
Dynamin 2	76660288		
Epsin-2	76644169		
Formin binding protein 4	76636519		
Golgi autoantigen*	76641568		
Huntingtin interacting protein 1	76649404		
Islet cell autoantigen 512	5305476		
Kinesin	76648026		
Piccolo (presynaptic cytomatrix protein)	76615069		
Pleckstrin	76608906		
SEC31-like 2	76654762		
Sec5 protein	76661880		
Sorting nexin 4	76640294		
Synapsin Ia	108935		
Synaptophysin*	33112658		
Synaptotagmin 1*	27806387		
Synaptotagmin II	76687864		
Synaptotagmin VI	76613150		
Synaptotagmin VII*	76657575		
Synaptotagmin-4	61809317		
Syntaxin-1A	417841		
THUMP domain containing 1	76653091		
Tomosyn	76626187		
Unc-18 protein	631583		
VAMP 3 (cellubrevin)*	61845787		
Vesicular membrane protein p24	76651226		
Calcium Regulation	**Genbank**	**Soluble**	**Membrane**
Annexin A1	74		
Annexin A2*	27807289		
Annexin A4*	1063258		
Annexin A6*	76623595		
Annexin A7	76656523		
Annexin A11*	113969		
Bestrophin isoform 1	61842255		
Calcium binding protein P22	76688289		
Calnuc (Nucleobindin)+	189308		
Mucolipin 3	81673761		
SPARC-like 1	74354032		
Voltage-dependent T-type calcium channel alpha-1	76674163		
Morphological Functions of Secretory Vesicles			
Structural Proteins	**Genbank**	**Soluble**	**Membrane**
Bone proteoglycan II	28189579		
Brevican	88682949		
Cartilage acidic protein 1	76654689		
Centromere protein I (CENP1)	86821813		
Centromeric protein E (CENP-E)	76676943		
CG15021-PA	76648468		
CG2843-PA	76644813		
Collagen, type I	76686475		
Collagen, type VI	76667061		
Collagen, type XVIII	76608417		
Collagen, type XXVII	76625290		
Collogen cyanogen bromide, type II	5354051		
Colonic and hepatic tumor over-expressed protein	76636503		
Cytokinesis 8	76624391		
Desmoplakin	76651410		
Diaphanous 3	76661030		
Drebrin 1	76622581		
Dynein	76648966		
Echinoderm microtubule associated protein like 5	76673417		
Elastin microfibril interfacer 1*	61845535		
Fibrinogen	75812954		
Fibronectin type III	76613259		
Galectin-related inter-fiber protein	76654374		
Gap junction protein, (connexin 31.9)	76644686		
Hydrocephalus inducing	76673899		
KIAA1914 protein	76655117		

Table 1. Continued

Microtubule associated serine/threonine kinase 2	76614276		
Microtubule-associated protein 4	27806553		
Myosin, heavy polypeptide 9+	27807325		
Myosin, light polypeptide kinase	76609105		
Nebulin	76609853		
Nesprin-2	76628088		
Obscurin	76620689		
Pericentrin 2	76607770		
Periphilin 1	76618432		
Proline arginine rich coiled coil 1	76614586		
Proteoglycan 3	76705880		
Proteolipid protein 1 (PLP1)	74354814		
Radial spokehead-like 3	76625577		
Spectrin domain with coiled-coils 1	76644035		
Sperm associated antigen 4-like	76633467		
Symplekin	76641008		
Talin 1+	76627770		
Testican 2+	76656478		
Tubulin*	76628240		
Cell Adhesion/Cell-Cell Interactions	**Genbank**	**Soluble**	**Membrane**
Ankyrin 3	76656250		
Cadherin*	76640658		
CD18 antigen	2407809		
CD63 antigen*	45439308		
CD81 antigen (target of antiproliferative antibody 1)	73586978		
Cell adhesion molecule JCAM	76608323		
Contactin 1+	1060861		
Dishevelled-associated activator of morphogenesis 1	76665330		
Dystonin	76649774		
Epithelial V-like antigen 1	62751654		
Integrin alpha-8 precursor	76632354		
KIAA0319 (dyslexia)	76674660		
Laminin beta-1 chain precursor	76615137		
Leucine rich repeat and fibronectin type III domain containing	76658000		
Nidogen-2 precursor (NID-2) (Osteonidogen)	76627657		
Swan isoform 4 (neurexin)	76634905		
Thrombospondin-3 precursor	76612130		
UCC1+	76664807		
Vinexin (SH3-containing adapter molecule-1)	76624656		
Other Protein Categories			
Cell growth and development	**Genbank**	**Soluble**	**Membrane**
Gametogenetin	76641302		
Mitotic-specific cyclin B1	76615016		
Tectonic	76639025		
Immune	**Genbank**	**Soluble**	**Membrane**
Anti-testosterone antibody*	440		
Cardiotrophin-1 (CT-1)	76687585		
Collectin-43	50355694		
Complement component 6	88954145		
Complement component 7	88954301		
Ig gamma-1 chain C region, membrane-bound form	76678688		
Ig heavy chain*	1575493		
Ig lambda chain V-I region BL2 precursor	76690231		
IgG Fc receptor FcRN	7339746		
Immunoglobulin superfamily, member 2	76612971		
Killer cell immunoglobulin-like receptor 3DL1	76687295		
Large proline-rich protein (BAT2)	76650909		
Major histocompatibility complex, class I-related	76660744		
Myeloid/lymphoid or mixed-lineage leukemia	76644552		
Stromal cell derived factor 4	73586919		
TOLL-like receptor 7	76151015		
UL16 binding protein 3	76693242		
Transcription and Translation	**Genbank**	**Soluble**	**Membrane**
Ataxin 7	76613103		
BCOR protein (BCL-6 corepressor)	76670256		

Table 1. Continued

Bromodomain and PHD finger containing, 1 isoform 6	76648396		
CAMP responsive element binding protein 5	76615443		
Cbp/p300-interacting transactivator	76668278		
CCAAT/enhancer binding protein zeta	76629167		
Cell division cycle 16	76634504		
Cell division cycle 42	76611597		
Centrosomal protein 2	76633389		
C-Fos	32481980		
Chromodomain helicase DNA binding protein 7	76634422		
Chromosome condensation (RCC1) and BTB (POZ) domain	76667931		
Cleavage and polyadenylation specific factor 1	27807297		
Clock isoform 4	76619910		
Core-binding factor, beta subunit	76640732		
CPEB3 (cytoplasmic polyadenylation element binding protein 3)	76661464		
DEAD (Asp-Glu-Ala-Asp) box polypeptide 48	76645558		
DNA cross-link repair 1A protein	76655155		
DNA methyltransferase 1 associated protein 1	89994078		
DNA replication complex GINS protein PSF2	76639980		
DNA-binding protein SATB1	76608636		
DRE1 protein	76636127		
Eukaryotic translation initiation factor 2	76629540		
Eukaryotic translation initiation factor 3	74353982		
Exosome component 10	76637212		
Fidipidine	76610089		
Forkhead box K1	76654354		
Fusilli	76634878		
General transcription factor II H	76650520		
High mobility group protein 4	61878473		
High-mobility group protein 3	76658822		
Histone acetyltransferase GCN5	76644862		
Histone H2B 291B	76625998		
HIV TAT specific factor 1	76658704		
HP1-BP74	76611630		
Hypothetical zinc finger protein KIAA1196	76632891		
Hypoxia-inducible factor-3 alpha	76642167		
Ladybird homeobox homolog 1	76654810		
Luc7	76652552		
Methyl-CpG binding domain protein 1	86437962		
Myoneurin	77567823		
Neuro-oncological ventral antigen	76667375		
Nibrin+	76634945		
Nuclear autoantigen Sp-100	76610804		
Nuclear RNA export factor 3	76659002		
Nucleolar complex associated 3	76640610		
Nucleoporin	76646535		
P20-CGGBP	76608705		
PGC-1 related co-activator	76654860		
PHD finger protein 22	76684907		
Pinin, desmosome associated protein	27807293		
Polyhomeotic 1	76616376		
Pre-B-cell leukemia transcription factor interacting protein 1	76612171		
Regulatory factor X4 isoform c	76619030		
Replication initiator 1	76616173		
Retinoic acid induced 16	76624642		
Ribosomal protein L23a	76613395		
Ribosomal protein L29	74268009		
Ribosomal protein L4	62460480		
Ribosomal protein S27a	76620759		
Ribosomal protein S6 kinase polypeptide 3	76659718		
RNA-binding protein 11	94966923		
RNA-binding protein 28	76615708		
RNA-binding protein EWS 8	76639325		
RPS27A protein	73586974		
Schlafen 10	76642925		
Serologically defined colon cancer antigen 33	76651519		
Sex comb on midleg-like protein 2	76676174		
SFRS5 splicing factor	76662260		
Shugoshin-like 2	76610144		
Sp4 transcription factor	76673507		
Spermatogenic leucine zipper 1	94966881		
Target of myb1, Tom-1 protein	78042494		
TATA box binding protein like 2	76660220		
Transcription elongation factor B polypeptide 3 binding protein 1	76622840		
Transcription factor PU.1	76636497		
Transcription factor RAM2	76614939		
Transcription factor Sp1	76617820		
Transcriptional intermediary factor 1-gamma (TIF1-gamma)	76613148		
Treacle protein	76683158		
Woc	76672108		

Table 1. Continued

Valosin-containing protein (VCP)	77735541		
Zinc finger CCCH-type containing 12A	76614519		
Zinc finger protein 111	76616182		
Zinc finger protein 262	76614570		
Zinc finger protein 385	76617575		
Zinc finger protein 398	76616180		
Zinc finger protein 469	76640087		
Zinc finger protein 608	76667970		
Zinc finger protein 623	76634062		
Zinc finger protein 644	76613525		
Zinc finger protein 694	76610151		
Zinc finger protein 8	76685031		
Zinc finger protein 84	76642204		
Zinc finger protein 85	76641574		
Miscellaneous			
Miscellaneous	**Genbank**	**Soluble**	**Membrane**
Absent in melanoma 1 protein	76608722		
Albumin	162648		
Apical-like protein (APXL protein)	76659839		
Apoptotic chromatin condensation inducer	76626863		
BCL2-like 14	82571795		
Beta2-Microglobulin*	41386683		
Breast cancer antiestrogen resistance 3	66792756		
Bromodomain and WD repeat domain containing 2	76655253		
C9orf55	76659997		
Cas-Br-M ecotropic retroviral transforming sequence-like 1	76615140		
CG7593-PA	76657521		
Chromosome 17 open reading frame 27	76670292		
Chromosome 9 open reading frame 19	61812939		
Desmocollin 2	76651700		
ELG protein	76643730		
ESCO2 (establishment of cohesion)	76624097		
F33H2.2	76638347		
Golgi phosphoprotein 3	76650110		
HBx-Hepatitis B virus X interacting+	74268261		
Hemoglobin*	12655818		
Kelch protein 3	76623241		
KIAA1862	76616193		
KIAA1900	76663072		
Lysosome membrane protein II (LIMP II)	76620042		
Membrane-associated ring finger 3 (C3HC4)	76660388		
Membrane glycoprotein 1 precursor (LAMP-1)	1683365		
MGC133986 protein	86437964		
MICAL-2	76676434		
Mitochondrial carrier homolog 1	76650069		
Mitochondrial import inner membrane translocase subunit Tim17 A	76680711		
Odd Oz/ten-m homolog 2	76623795		
Otolin-1	76607840		
Partitioning defective-6 homolog alpha (PAR-6 alpha)	76633889		
PC4 and SFRS1 interacting protein 1	76624197		
Phosphorylated CTD interacting factor 1	76633669		
PRBP (plasma retinol-binding protein)+	132403		
Pyrin domain containing 4	82571578		
R119.5	76665822		
RAD52	66792838		
Ran-binding protein 3 (RanBP3) isoform 2	76622053		
Ring finger protein 111	76627819		
Ryanodine receptor 1, skeletal muscle	76641296		
SCD6 homolog A	77736251		
SID1 transmembrane family member 1 precursor	76684359		
Spastin isoform 1	76682638		
Spermatogenesis associated 13	76631579		
Spermatogenesis associated 5	76687571		
Storkhead box 2	76655606		
Tankyrase 1	76655843		
Tetraspanin 7*	76665086		
Transcobalamin II+	27806385		
Transmembrane protein 16B	76673511		
Transmembrane protein 63C	76628460		
Transmembrane protein 79	59858019		
Tripartite motif protein 7	76622789		
Tumor necrosis factor, alpha-induced protein 3	76626126		
WW domain binding protein 11	76633774		
Vacuolar protein sorting factor 4B	76652249		
Y37D8A.2	76669076		

Table 1. Continued

Unknown	Genbank	Soluble	Membrane
CG11617-PA	76632406		
CG6379-PA	76650147		
CG13957-PA	76609707		
CG17569-PB	76665263		
CG32045-PC	76631513		
CG3338-PA	76643561		
CG4751-PA	76614067		
CG5987-PA	76644502		
CG7709-PA	76642510		
FLJ44048 protein	76668361		
H43E16.1	76680309		
Hypothetical gene supported by AK075558; BC021286	76637226		
Hypothetical protein FLJ13868	61554841		
Hypothetical protein LOC539970	74267976		
Hypothetical protein XP_583091	76659995		
Hypothetical protein XP_584302	61823940		
Hypothetical protein XP_585938	76658228		
Hypothetical protein XP_590236	76616022		
Hypothetical protein XP_592499	76672076		
Hypothetical protein XP_594162	76637663		
Hypothetical protein XP_598213	76619718		
Hypothetical protein XP_600405	76690587		
Hypothetical protein XP_600782	76678727		
Hypothetical protein XP_868808	76649193		
Hypothetical protein XP_869427	76635764		
Hypothetical protein XP_873356	76769235		
Hypothetical protein XP_580444	76634839		
Hypothetical protein XP_596183	76638981		
Hypothetical protein XP_608396	76678883		
Hypothetical protein XP_876844	76638096		
Hypothetical protein XP_876914	76638098		
Hypothetical protein XP_881202	76639161		
Hypothetical protein XP_882313	76632294		
Hypothetical protein XP_882414	76662668		
Hypothetical protein XP_882587	76630059		
Hypothetical protein XP_883381	76631223		
Hypothetical protein XP_883390	76640533		
LPR protein	619		
Neuroblastoma-amplified protein	76666100		
Protein for IMAGE:8054235	83406129		
Protein for MGC:140076	92098401		
RIKEN cDNA 0610040D20	61870202		
RIKEN cDNA 9130210N20	76634018		
Sushi repeat-containing protein	94966791		
TAG-278	76685297		
THAP domain containing 4	83759162		
Transmembrane 9 superfamily protein member 4	76633198		
Unknown (protein for MGC:127406)	73587279		
Unknown (protein for MGC:140139)	92096913		
YEATS domain containing 2	76607509		
ZK1067.4	76650437		
ZK742.2	76620462		

[a] The functional categories of proteins identified as soluble and membrane components of chromaffin secretory vesicles are illustrated in this table. The main functional categories (large bolded titles) are divided into subcategories. This table combines the extensive group of proteins identified by nano-HPLC Chip MS/MS tandem mass spectrometry in this study with other proteins in these vesicles identified by 1-D gel separation and MS/MS analyses.[47] Proteins indicated by "+" were found in the Wegrzyn et al. 2007 study[47] but not in this study. Proteins indicated by "*" are proteins identified in both the Wegryzn et al. 2007 study[47] and this study. In addition, protein indicated by "#" were identified by previous focused studies on the amyloid precursor protein,[55,143] cathepsin L,[8] and endopin 2C.[56] Thus, this table represents the overall proteomic characterization of chromaffin secretory vesicle proteins.

described previously.[48−51] Cystatin C in Western blots was detected with anticystatin C (at 1:1000 dilution, from US Biological, Swampscott, MA). Huntingtin interacting protein was analyzed in Western blots of chromaffin granules with anti-SET2 antisera by immunoprecipitation prior to Western blots (at 1:1000 dilution, from Chemicon, Temecula, CA) as described previously.[51,52] Ataxin 7 was detected by antiataxin 7 generated by the La Spada laboratory (antisera K[49] utilized at 1:2000, after immunoprecipitation of ataxin 7 performed as we have described previously.[51,52] Detection of prion in Western blots utilized monoclonal antibody SAF-84 (1 μL/mL, from Cayman Chemical, Ann Arbor, MI).

The presence of a neurological disease protein, cystatin C, as an example, in chromaffin cells was assessed by immunofluorescence confocal microscopy to confirm its localization in secretory vesicles. Chromaffin cells in primary culture were prepared from fresh bovine adrenal medulla tissue as previously described.[53] Cells were subjected to colocalization studies of enkephalin-containing chromaffin secretory vesicles for the presence of cystatin C (anticystatin C rabbit, 1:50 dilution, from

US Biological, Swampscott, MA) in enkephalin-containing secretory vesicles (detected by anti-(Met)enkephalin mouse, from Abcam company, Cambridge, MA or from Chemicon-Millipore company, Billerica, MA) by immunofluorescence confocal microscopy, conducted as we have previously described.[8,50] Cystatin C was detected with antirabbit IgG-Alexa Fluor 568 (goat) (1:50 dilution, red fluorescence, Molecular Probes, Eugene, Oregon) with comparison to localization of (Met)enkephalin (ME) in secretory vesicles detected with antimouse IgG Alexa Fluor 488 (goat) (1:50 dilution, green fluorescence). Immunofluorescent images were obtained with the Delta Vision Spectris Image Deconvolution Systems on an Olympus IX70 confocal microscope using the software Softwrox Explorer from Applied Precision.

Results and Discussion

Identification of an Extensive Number of Chromaffin Granules (CG, also Known As Secretory Vesicles) Proteins by Nano-HPLC Chip MS/MS Tandem Mass Spectrometry. Differential centrifugation was utilized to obtain purified chromaffin granules (CG) from bovine adrenal medulla homogenate, illustrated in Figure 1a. This purification scheme is well-established in the field.[36−38] CG were isolated by a series of centrifugation steps that remove other organelles consisting of nuclei (P1 fraction), microsomes (S2 fraction), and mitochondria and lysosomes (S3, S4, and S5 fractions, not shown in Figure 1a). The enriched fraction of CG (P5 fraction) is purified by a 0.32/1.6 M sucrose gradient, resulting in a pellet of purified CG. Comparison on a multistep sucrose gradient of 2.2 to 1.2 M sucrose shows that both the crude and purified CG samples contain peaks of (Met)enkephalin at about 1.7−2.0 M sucrose. While the crude CG contains a peak of acid phosphatase (Figure 1b), a marker for lysosomes, such a peak for lysosomes is absent in the purified CG (Figure 1c). The purity of these CG have been confirmed by electron microscopy[47] that shows the homogeneity of the preparation. These data and those from other studies[36−38] establish the purity of chromaffin granules obtained by density gradient.

The soluble and membrane fractions of these purified secretory vesicles were separated to provide functional predictions of identified proteins in the soluble secreted pool, or as membrane-related proteins that participate in maintaining integrity of the organelle. Soluble and membrane fractions were obtained by lysis and centrifugation of chromaffin granules in isotonic salt conditions in buffer of pH 6.0 that represents the internal vesicle environment.[54]

Furthermore, to enhance nano-LC−MS/MS analyses of moderate to lower abundance proteins, the highly abundant chromogranin A (CgA) protein was removed by affinity chromatography (as described in methods), which removes the major 66−70 kDa CgA protein band (Figure 2). Each of the soluble and membrane fractions were subjected to trypsin digestion and nano-HPLC Chip MS/MS tandem mass spectrometry with the XCT Ultra ion trap mass spectrometer (Agilent) for sensitive analyses of peptides (estimated down to the attomole range). Evaluation of MS/MS spectra by the Spectrum Mill search program yielded identification of proteins in soluble and membrane fractions with ~1% FDR (determined by shuffled decoy database analysis). Subsequently the entire data set was also searched using an alternate search algorithm, OMSSA, to confirm and validate peptide scoring thresholds.

Proteins found in the soluble and membrane fractions of the secretory vesicles by the nano-HPLC Chip MS/MS approach

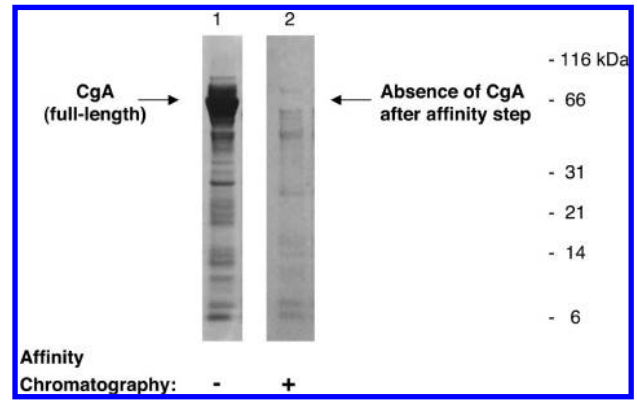

Figure 2. Removal of abundant chromogranin A protein from soluble and membrane fractions of purified chromaffin granules. Chromaffin granule soluble and membrane fractions were each subjected to calmodulin affinity chromatography to remove the most abundant protein consisting of full-length CgA. The soluble fraction of these chromaffin granules is illustrated (lane 1), showing full-length CgA of ~66−70 kDa that has been identified by mass spectrometry in previous studies (10, 137). After affinity chromatography on calmodulin-Sepharose conducted two times, removal of the full-length CgA is illustrated (lane 2). Equal relative volumes (5 μL) of soluble chromaffin granule sample was applied to lanes 1 and 2 (corresponding to ~2 μg and 0.65 μg protein, respectively). The CgA depletion step recovered ~30−35% of the original proteins of the soluble chromaffin granule sample. CgA also exists as cleaved proteolytic fragments in the chromaffin granules which presumably are largely removed by the calmodulin-Sepharose affinity step. After the affinity step, the overall pattern of protein bands (lane 2) resembles that of the soluble granule sample before the affinity step, with the exception of removal of CgA protein(s).

are illustrated in Supplemental Tables A and B, respectively. Nano-HPLC Chip MS/MS identified more than 600 proteins in both the soluble and membrane fractions. These results demonstrate the high efficiency of the nano-HPLC Chip MS/MS tandem mass spectrometry system to identify hundreds of proteins from several micrograms of sample per analysis.

Total Proteome of Chromaffin Secretory Vesicles. To obtain an overall proteomic view of the purified adrenal medullary secretory vesicles (chromaffin granules) the extensive nano-HPLC Chip MS/MS identification of more than 600 proteins obtained in this study was combined with our prior data of proteins from these secretory vesicles subjected to gel electrophoresis separation prior to MS/MS analyses,[47] and several purified proteins.[8,55−57] These results have identified 371 distinct soluble proteins and 384 distinct membrane proteins (Figure 3, Venn diagram), with 69 proteins present in both soluble and membrane fractions. These data illustrate the presence of a total of 686 distinct proteins identified in the soluble and membrane fractions of chromaffin secretory vesicles.

Distinct Functional Categories of Proteins in Soluble and Membrane Components of Chromaffin Secretory Vesicles. The categorization of the chromaffin secretory vesicle proteins indicates the presence of distinct functional categories of biochemical systems (Figure 4, and Table 1). Protein categories were identified for neurotransmitter and neurohumoral mechanisms, as well as diverse biochemical processes that include maintenance of the internal environment of these vesicles. A large portion of proteins participate in regulated secretion via signal transduction and exocytosis. These functions are com-

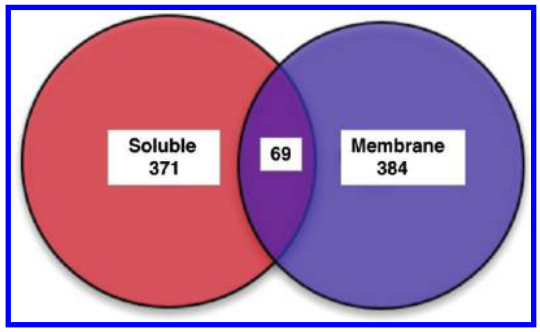

Figure 3. Venn diagram of common and different proteins in soluble and membrane fractions of chromaffin granules. This Venn diagram illustrates the majority of the chromaffin secretory vesicle proteins identified in this study using nano-HPLC Chip MS/MS, combined with several proteins identified in in earlier proteomic studies using gel electrophoresis for protein enrichment.[43] The soluble fraction contained 371 distinct proteins and the membrane fraction contained 384 distinct proteins. Proteins common to both soluble and membrane fractions are illustrated as the intersecting area of the Venn diagram, indicating 69 proteins that were present in both soluble and membrane compartments of these secretory vesicles. The soluble and membrane fractions contained a total of 686 unique proteins in chromaffin secretory vesicles.

bined with vesicular trafficking that involves structural proteins. Protein components within these categories are described in detail below.

Production of Neurotransmitters and Neurohumoral Factors: Neuropeptides and Neurohumoral Factors, Protease Systems, Neurotransmitter Enzymes/Transporters, and Receptors. Proteins involved in secretory vesicle-mediated cell−cell communication were identified as proneuropeptides and processing proteases, neurohumoral agents, enzymes and transporters for classical small molecule neurotransmitters, and receptors. Numerous proneuropeptide and prohormone precursors were identified such as proenkephalin, proNPY, chromogranins, and others that undergo proteolytic processing[5,8−10,46,50,58−63] to generate active neuropeptides that function as neurotransmitters and hormones. These vesicles also contain several neurohumoral factors such as VGF nerve growth factor and vascular endothelial growth factor.[64−66]

Numerous proteases of the serine, aspartyl, cysteine, and metalloprotease classes were identified. Several subtilisin-like prohormone convertases (PC1/3, PC2, PACE 4) were identified, which participate in proneuropeptide processing.[5,67−69] The cysteine protease cathepsin L has been identified by MS/MS tandem mass spectrometry utilizing enrichment prior to MS/MS;[5,8] cathepsin L participates in secretory vesicles for processing proneuropeptides for the production of enkephalin,[5,8] NPY,[62] and POMC-derived peptide hormones consisting of β-endorphin, ACTH, and α-MSH.[46] The cysteine protease cathepsin B was identified, which was recently discovered to participate in the production of neurotoxic beta-amyloid related to Alzheimer's disease.[45,70] The aspartyl protease cathepsin D was also present.[38] Numerous metalloproteases were found including the ADAM metallopeptidase, and carboxypeptidases (D, E, and G2).[71−73] Components of the ubiquitin system for protein degradation were identified.[74−76] In addition, endogenous protease inhibitors were indicated that included TIMP-1,[77] inhibitor of PC1/3,[78] and cystatins.[79] These results indicate the presence of numerous protease and protease inhibitors in these secretory vesicles.

Proteins that participate in the biosynthesis and metabolism of small molecule neurotransmitters, as well as receptors, were identified. Catecholamine synthesizing enzymes were present that include tyrosine hydroxylase, dopamine beta-monooxygenase, and PNMT (phenylethanolamine *N*-methyltransferase).[80,81] In addition, transporters for vesicular localization of catecholamines were identified.[82−84] Interestingly, several receptor proteins were identified which may be present in secretory vesicles for transport to the plasma membrane.[85]

Biochemical Processes: Enzymes, Carbohydrate and Lipid Functions, Protein Folding, Transporters. The secretory vesicles contained enzymes for a variety of biochemical reactions. Enzymes for numerous biochemical reactions were identified including aspartate aminotransferase for amino acid modification, cofactor related tetrahydrofolate synthase, and enolase.[86−88] In addition, a number of carbohydrate and lipid metabolizing enzymes were identified.[89,90] These included

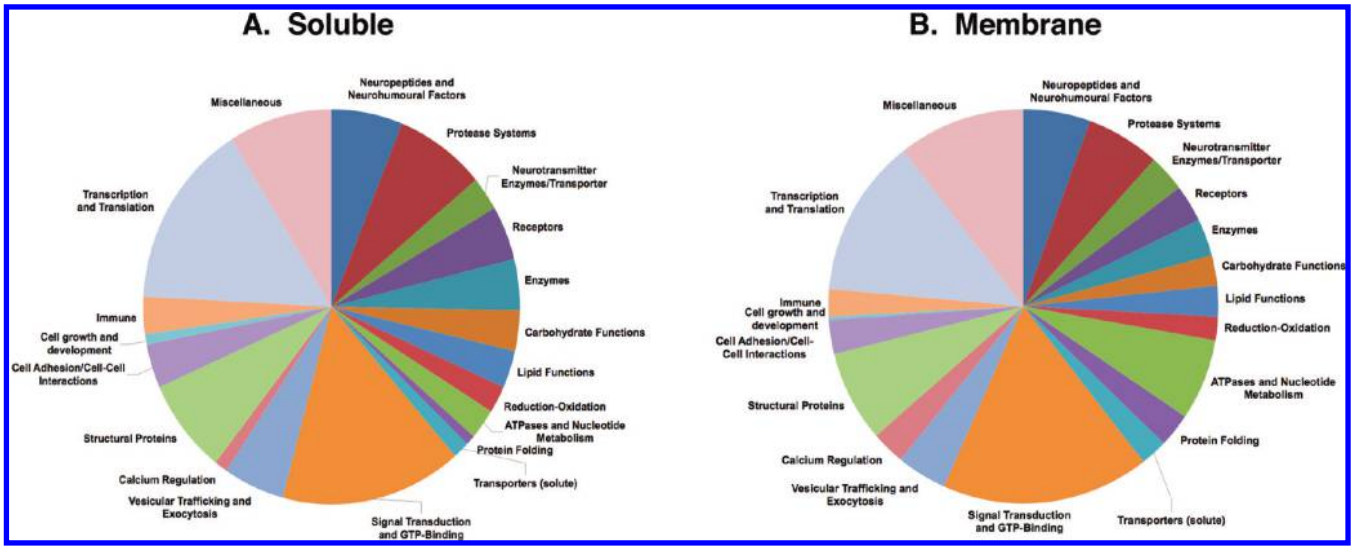

Figure 4. Comparison of soluble and membrane proteins by pie charts. The relative portion of proteins in each functional category are compared for the (A) soluble and (B) membrane fractions of chromaffin secretory vesicles. Each functional category of the pie chart is shown as a distinct color.

carbohydrate transferases, mannosidase, and glucosidase. Lipid-related enzymes included arachidonate lipoxygenase, phospholipase, and acyl-CoA synthetase.

Internal Environment of Secretory Vesicles: Reduction Oxidation, ATPases and Nucleotide Metabolism. Homeostatic mechanisms for maintaining the unique internal conditions of the secretory vesicle require reduction−oxidation regulation, ATP/nucleotide related proteins for pH regulation, and protein factors for protein folding. Regulation of reducing and oxidative conditions is evident with the presence of cytochromes, peroxidase, catalase, and related proteins. Numerous membrane-associated ATPase isoforms were present which participate in proton transport that maintains the acidic internal pH (pH 5−6) of these secretory vesicles.[54,91−95] Conditions for appropriate protein folding or protein configuration are represented by chaperone proteins that include heat shock proteins, chaperonin, and isomerase.[96−98] These regulators of the internal secretory vesicle environment are utilized for effective production of neurotransmitters, hormones, and neurohumoral factors in this organelle.

Regulated Secretion: Signal Transduction and GTP-Binding Proteins, Vesicular Trafficking and Exocytosis, Calcium Regulation. Significant representation of secretory vesicle proteins consisted of functions for regulated secretion involving signal transduction and GTP-binding proteins, proteins for exocytosis and vesicular trafficking, and calcium regulation. An extensive collection of Rab GTP-binding proteins[99−103] was present, which are critical for intracellular trafficking and transport of secretory vesicles and in vesicle exocytosis. Furthermore, numerous proteins involved in signal transduction pathways by protein kinases[104−107] and phosphatases[107−109] were identified. The presence of these proteins suggest regulation of the phosphorylation status of target proteins within secretory vesicles. The process of exocytosis of secretory vesicles for release of vesicle contents to the extracellular environment utilizes synaptotagmin isoforms and synaptophysin related proteins.[110−112] Also, the presence of kinesin suggests its utilization by dense core secretory vesicles for trafficking to the plasma membrane via interactions with cellular structural proteins.[113,114] Notably, regulated secretion is calcium-dependent, which utilizes proteins that regulate calcium metabolism in secretory vesicles. These proteins include several isoforms of annexins which function in calcium-dependent phospholipid binding during exocytosis.[105]

Morphological Functions of Secretory Vesicles: Structural Proteins, Cell Adhesion and Cell−Cell Interactions. Numerous structural proteins were identified including collagen, myosin, spectrin, proteoglycan, and tubulin which may function in morphological features of secretory vesicles.[116,117] Such cytoplasmic proteins are utilized for intracellular movement of the secretory vesicles to the plasma membrane for regulated secretion. For example, microtubule and myosin structural proteins may be involved in intracellular granule movement. Such cytoplasmic proteins may be linked to the chromaffin granule membrane through protein interactions, but would lack signal peptide sequences. In addition, several proteins involved in cell adhesion or cell−cell interactions were found, which included cadherin, integrin, laminin, and related components.

Potentially Novel Functional Proteins of Secretory Vesicles. It was of interest that several identified proteins included those with functions in the immune system, cell growth and development, and transcription and translation. Several proteins with immune system functions were found including immunoglobulins and immunoglobulin receptor. Several studies have demonstrated nervous system stimulation of immunoglobulin secretion,[118] as well as immunoglobulin receptor trafficking through regulated secretory vesicles.[119] Recent studies have also indicated transport of chemokines in large dense core vesicles as mechanisms for secretion of cytokines.[120,121] Thus, dense core secretory vesicles may be involved in both the regulated secretion of immunological factors as well as neurohumoral factors, hormones, and neurotransmitters.

Proteins involved in translation of RNAs were identified such as ribosomal proteins and RNA-binding proteins. Recent studies have demonstrated localization and translation of mRNAs in axons,[122−124] which occur in the vicinity of secretory vesicles that are transported to axons and nerve terminals. It is possible that components for mRNA translation may reside in subregions of the neuroendocrine cell where secretory vesicles undergo transport and trafficking for regulated secretion. An alternative possibility is that contaminating RNA granules may be present in the chromaffin granule preparation, but that is unlikely since RNA granule markers (FMRP, Pur alpha and beta, RACK1, S6, Staufen2, or Syncrip)[125] were not identified in this chromaffin granule proteomic study. Indeed, the purity of the isolated chromaffin granules has been established in prior studies,[36−38] as well as in this study (Figure 1).

In addition, several proteins representing transcription factors were identified. Thus far, little is known about the roles of such factors in secretory vesicles. Furthermore, several miscellaneous and unknown proteins were indicated from MS/MS data analyses.

Chromaffin Granule Proteins Related to Neurological Diseases. Several key proteins involved in neurological disease mechanisms were present in these secretory vesicles. These vesicles contain several neurodegenerative disease related proteins consisting of the amyloid precursor protein (APP), huntingtin interacting protein, ataxin 7, CLN8 protein, and prion protein (Table 2). The amyloid precursor protein (APP) undergoes proteolytic processing to generate toxic beta-amyloid peptide, a neurotoxic factor involved in the development of Alzheimer's disease.[14−18,39,45,70] Beta-amyloid peptide and proteases for its production have been demonstrated in chromaffin secretory vesicles.[39,45,70] The protease inhibitor cystatin C is involved in epilepsy.[126,127] The huntingtin interacting

Table 2. Proteins Identified in Chromaffin Secretory Vesicles with Neurological and Neurodegenerative Disease Functions[a]

protein	neurological disease
Amyloid precursor protein	Alzheimer's Disease
Ataxin 7	Spinocerebellar Ataxia type 7
CLN8 protein	Neuronal Lipofuscinosis, EPMR, epilepsy and mental retardation
Cystatin C	Epilepsy
Huntingtin interacting protein 1	Huntington's Disease
KIAA0319	Dyslexia
Nesprin-2	Muscular Dystrophy
P20-CGGBP	Fragile X Syndrome, mental retardation
Prion protein	Prion Disease
Regulatory factor X4 isoform c	Bipolar Disorder

[a] Proteins were identified from chromaffin secretory vesicles by LC−MS/MS and subjected to bioinformatic analyses as described in the methods. Several proteins were found which are known to participate in several neurological and neurodegenerative disease conditions.

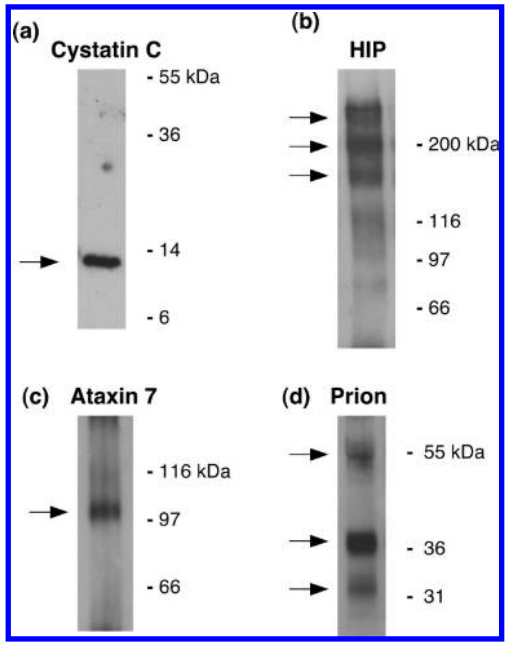

Figure 5. Presence of cystatin C, huntingtin interacting protein, ataxin 7, and prion protein in chromaffin secretory vesicles demonstrated by Western blots. Purified chromaffin secretory vesicles were subjected to Western blots for analyses of (a) cystatin C, (b) huntingtin interactin protein (HIP), (c) ataxin 7, and (d) prion protein. Cystatin C of ~12−14 kDa in these vesicles (a) is similar in MW (molecular weight) to that reported in other studies.[132,133] Huntingtin-interacting proteins (3 bands) in the area of ~150−250 kDa were observed (b). Ataxin 7 of about 98−100 kDa (c) is similar to that found in prior studies.[49] Prion protein of several apparent molecular weights of ~30 kDa, 36−40 kDa, and 50−60 kDa were observed (d).

protein[19−22] is known to bind to the mutant huntingtin (htt) protein with polyglutamine expansion of Huntington's disease.[128] The ataxin 7 protein is the product of the SCA7 gene that possesses polyGln expansions, representing a CAG triplet-repeat neurodegenerative disease.[23−25] The CLN8 protein is involved in genetic EPMR syndrome for epilepsy and mental retardation as a mutant CLN8 autosomal recessive disorder.[29−32] The mutant CLN8 gene represents one of several neuronal ceroid lipofuscinoses (NCLs) neurodegenerative disorders characterized by the accumulation of autofluorescent lipopigment in various tissues. The prion protein is a key component of prion neurodegenerative diseases based on misfolding of the prion protein.[26−28] It is notable that multiple factors known to participate in severe neurodegenerative diseases are present in chromaffin secretory vesicles.

Furthermore, proteins related to several neurological diseases were identified (Table 2) as the P20-CGGBP protein of the fragile X syndrome for mental retardation,[33] the regulatory factor X4 involved in bipolar disorder,[34] the KIAA0319 protein involved in dyslexia,[35] and nesprin-2 related to muscular dystrophy.[129−131] Thus, the dense core secretory vesicle organelle contains several key proteins that participate in neurological diseases.

The presence of selected neurological disease proteins in chromaffin secretory vesicles were assessed by Western blots of purified vesicles. Western blots (Figure 5) show the presence of cystatin C of ~14 kDa,[132,133] huntingtin (htt) interacting protein as several bands of 150−250 kDa that interact with fragments of htt present in brain,[48] ataxin 7 of ~98−100 kDa,[49,134] and prion protein of a main and of ~30−35 kDa[135,136] combined with a 55−60 kDa band (a possible multimer form). Cellular immunofluorescence microscopy indicated the localization of cystatin C (Figure 6), as example, in chromaffin secretory vesicles that contain the enkephalin peptide neurotransmitter.[8] These data show that the LC−MS/MS results can appropriately indicate the presence of such proteins in chromaffin secretory vesicles.

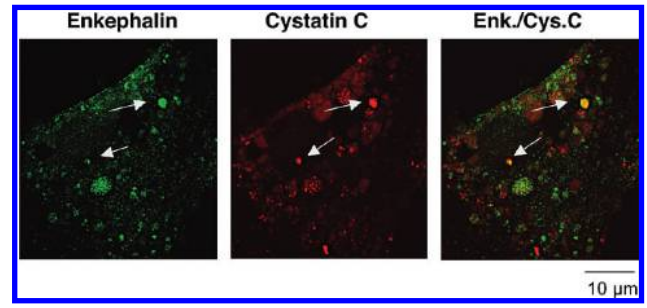

Figure 6. Cellular localization of cystatin C with enkephalin-containing secretory vesicles of chromaffin cells in primary culture. The localization of cystatin C to secretory vesicles that contain the enkephalin neuropeptide was observed by immunofluorescence confocal microscopy of chromaffin cells in primary culture. Colocalization of enkephalin (green fluorescence) and cystatin C (red fluorescence) was demonstrated by the yellow fluorescence of merged images. Examples of secretory vesicle colocalization of cystatin C and enkephalin are indicated by the arrows.

Table 3. Total Number of Proteins Identified in the Soluble and Membrane Secretory Vesicle Compartments in Designated Functional Categories[a]

protein category	soluble	membrane
Neuropeptides/Neurohumoral	18	19
Protease Systems	26	21
Neurotransmitter Enzymes/Transporters	9	11
Receptors	14	11
Enzymes	13	11
Carbohydrate Functions	11	9
Lipid Functions	10	9
Reduction−Oxidation	7	7
ATPases/Nucleotide Metabolism	8	23
Protein Folding	2	10
Transporters (solute)	4	8
Signal Transduction/GTP-binding	65	76
Vesicular Trafficking and Exocytosis	17	15
Calcium Regulation	4	10
Structural Proteins	24	26
Cell Adhesion/Cell−Cell Interactions	12	10
Cell growth and development	2	1
Immune	11	2
Transcription/Translation	49	47
Miscellaneous	28	37
Unknown	37	15

[a] Numbers of proteins found in each functional category for the soluble and membrane compartments of chromaffin secretory vesicles are indicated.

Number of Proteins Identified in Distinct Functional Categories of Chromaffin Secretory Vesicles. Comparisons of the numbers of proteins in each category showed the varying distributions of components within each category in chromaffin secretory vesicles (Table 3). The functional categories of these proteins represent the diverse biochemical systems utilized for

secretory vesicle exocytosis of active molecules for cell−cell communication.

Conclusion: Proteomic Data Reveals That Secretory Vesicles Utilize Multiple Categories of Proteins for Production and Release of Neuroeffectors for Cell−Cell Communication.

Secretory Vesicle Biosynthesis, Storage, and Secretion of Neuroeffector Molecules. This study illustrates the most comprehensive proteomic data of chromaffin secretory vesicles that provide neuroeffectors mediating cell−cell communication from the adrenal medulla. Proteins of these secretory vesicles function in the biogenesis and maturation of secretory vesicles, synthesis and storage of bioactive molecules consisting of neuropeptides and catecholamines, and secretion of bioactive molecules which involves secretory vesicle transport and docking to the plasma membrane for regulated secretion (Figure 7). These proteins provide the biochemical basis for regulated secretion of active neurotransmitter and neurohumoral agents for cell−cell communication. These data illustrate that the dense core secretory vesicles function with distinct functional protein categories. Production of peptide neurotransmitters and hormones, as well as catecholamine neuroeffectors, within the chromaffin secretory vesicles utilize proteins that control the intravesicular environment with respect to redox conditions, acidity (acid pH at 5.5−6.0), protein folding, as well as carabohydrate and lipid conditions. Transporters are needed for bringing certain neuroeffectors into the chromaffin granule. Regulated secretion of chromaffin granules requires extensive use of signal transduction and GTP-binding proteins, combined with proteins needed for secretory vesicle trafficking and exocytosis (including structural proteins for this purpose). Furthermore, because regulated secretion is calcium-dependent, calcium binding proteins are present to mediate regulated secretion. The proteomic data indicate that secretory vesicles utilize proteins of multiple functions for production, storage, and regulated secretion of neuroeffectors for cell−cell communication.

Complementary Biochemical Studies Assist in Substantiating Proteomic Data. An important criterion for proteomic studies is the subject of confidence and validation of protein identifications obtained from tandem mass spectrometry data. The unique feature of this proteomic study was consideration of protein biochemistry data from these secretory vesicles to guide and enhance appropriate confidence levels in bioinformatic analyses of MS/MS data for protein identification, including single peptide identifications.[137] Protein biochemistry studies of chromaffin secretory vesicles provide information of previously identified proteins in this organelle at moderate and low levels; this information can assist in defining reliable scoring thresholds. For example, the serpin endopin 2C is of moderate abundance since it was isolated from these secretory vesicles with a 500-fold enrichment,[43] and was observed in this study at intermediate peptide scoring thresholds for identification. Cathepsin B and cathepsin L are low abundance proteins, demonstrated by their enrichment requiring 2×10^5-fold[45] and 2×10^6-fold purification,[8] respectively; after enrichment of these proteins, they were identified with high confidence scoring MS/MS data.[8,45] Confidence levels of these MS/MS data are consistent with previous bioinformatic analyses of MS/MS data.[137,138] These types of biochemical studies assisted in guiding appropriate confidence levels for identification of proteins from tandem mass spectrometry data, including those identified by single peptide (tryptic) identification. Application of biochemical data for relative protein abundances enhances analyses of proteomic data.

Proteomic Data of Secretory Vesicle Proteins from Adrenal Medulla in This Study and from Other Tissues in Related Studies. Important secretory mechanisms for the chromaffin secretory vesicles are indicated by the expansive nature of these proteomic studies that have identified 686 distinct proteins in chromaffin secretory vesicles. Other studies of regulated secretory vesicles have been reported for the dense core secretory vesicles[144−149] and synaptic vesicles[150−154] for investigation of their proteomic features. While it is of interest to compare the identified proteins among these studies, their different methodologies for purification of organelles and protein extraction conditions, combined with different mass spectrometry instrumentation and different bioinformatic database searches should be considered in such comparisons. Nonetheless, features of some of the prior proteomic studies of various secretory vesicle systems are summarized here. A proteomic study of pancreatic zymogen granule membranes

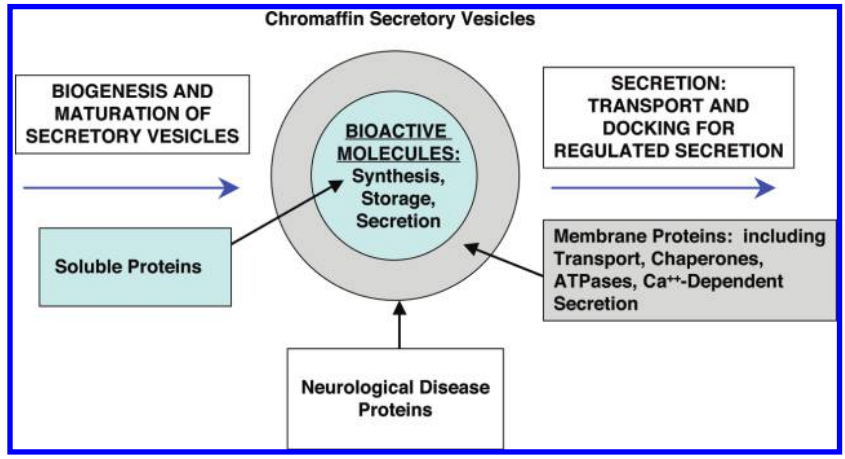

Figure 7. Multiple protein categories for biosynthesis, storage, and regulated secretion of neuroeffectors for cell−cell communication in health and disease. The chromaffin granule proteome consists of distinct functional categories of proteins utilized for secretory vesicle production, storage, and regulated secretion of neuroeffector molecules. The architecture of proteins of the soluble (blue area) and membrane (gray area) function in the initial biogenesis and subsequent maturation of secretory vesicles, which produce and store bioactive chemical molecules for secretion. The secreted neurotransmitters and hormones mediate cell−cell communication among physiological target organs.

yielded identification of 101 proteins by 2-D gel electrophoresis, in-gel trypsin digestion, followed by LC−MALDI.[139] Another proteomic study of insulin secretory granules has identified 130 different proteins by SDS-PAGE, excision of gel slices, trypsin digestion, followed by nano-LC−ESI−MS/MS.[140] Proteomic analyses of synaptic vesicles, which undergo regulated secretion, identified 410 proteins.[141] Analyses of multiple organelles isolated from abundant rat liver yielded identification of proteins of the rough and smooth microsomes, and Golgi fractions of the rat secretory pathway; this study identified 1400 proteins that was possible with multiple fractions from an abundant tissue source.[142] However, these rat liver organelle components represent the constitutive secretory pathway present in liver cells, which lack the regulated secretory pathway. It will be of interest for future joint efforts to compare different secretory vesicle systems in different cell types.

Summary

This proteomic study of chromaffin dense core secretory vesicles has provided new knowledge of the protein architecture of the regulated secretory vesicle system. The application of sensitive high throughput nano-HPLC Chip MS/MS to proteomic studies, combined with biochemical information, of the dense core secretory vesicle has revealed the presence of distinct functional protein categories for secretory vesicle production and secretion of bioactive molecules—neurotransmitters and hormones—that control physiological functions through cell−cell communication. Significantly, several proteins involved in neurodegenerative and neurological diseases were identified in these secretory vesicles, suggesting their involvement with the secretory vesicle protein systems in the regulation of cell−cell communication. Overall, this proteomic study has revealed an extensive group of functional protein systems in regulated dense core secretory vesicles for cell−cell communication in health and disease.

Abbreviations: ACTH, adrenocorticotropin hormone; ADAM, a disintegrin and metalloprotease; AEBSF, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride; APP, amyloid precursor protein; CG, chromaffin granule; CGS, chromaffin granule soluble fraction; CGM, chromaffin granule membrane fraction; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; EPMR, epilepsy and mental retardation; FDR, false discovery rate; GEMSA, guanidinoethylmercaptosuccinic acid; GTP, guanosine triphosphate; MS, mass spectrometry; MS/MS, tandem mass spectrometry; α-MSH, α-melanocyte stimulating factor; nano-HPLC, Chip MS/MS, nanohigh pressure liquid chromatography Chip tandem mass; spectrometry, NCL, neuronal ceroid lipofuscinoses; NPY, neuropeptide Y; PC, prohormone convertase; PMSF, phenylmethanesulphonylfluoride; POMC, proopiomelanocortin; PNMT, phenylethanolamine *N*-methyltransferase; TCEP, Tris-(2-carboxyethyl)-phosphine; TEM, transmission electron microscopy.

Acknowledgment. We appreciate support of this research by grants to V.H. consisting of R01DA04271, R01NS24553, and R01MH077305 from the NIH, as well as an award from the Alzheimer's Association to V.H., to D.T.O. consisting of P01HL58120 from NIH, and to A.L.S. consisting of R01EY014061 from the NIH. S.B. was supported by a NIH Mentored Scientist Award (K01DA023065). We also appreciate scientific advice by Dr. Shin-Rong Hwang and technical assistance by Mr. Thomas Toneff at the Skaggs School of Pharmacy and Pharmaceutical Sciences, Univ. of Calif., San Diego, La Jolla, CA.

Supporting Information Available: Supplementary Tables A and B. This material is available free of charge via the Internet at http://pubs.acs.org.

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PR1003104