“Subcellular Proteomics” of Neuromelanin Granules Isolated from the Human Brain*

Florian Tribl‡§¶¶, Manfred Gerlach¶¶¶, Katrin Marcus***, Esther Asan‡‡, Thomas Tatschner§§, Thomas Arzberger¶¶¶¶, Helmut E. Meyer**, Gerhard Bringmann§, and Peter Riederer‡

“Subcellular proteomics” is currently the most effective approach to characterize subcellular compartments. Based on the powerful combination of subcellular fractionation and protein identification by LC-MS/MS we were able for the first time to 1) isolate intact neuromelanin granules from the human brain and 2) establish the first protein profile of these granules. This compartment containing neuromelanin (NM) is primarily located in the primate’s substantia nigra, one of the main brain regions that severely degenerates in Parkinson disease. We used mechanical tissue dissociation, discontinuous sucrose gradient centrifugation, cell disruption, and organelle separation to isolate NM granules from human substantia nigra. Using transmission electron microscopy we demonstrated that the morphological characteristics of the isolated NM granules are similar to those described in human brain tissue. Fundamentally we found numerous proteins definitely demonstrating a close relationship of NM-containing granules with lysosomes or lysosome-related organelles originating from the endosome-lysosome lineage. Intriguingly we further revealed the presence of endoplasmic reticulum-derived chaperones, especially the transmembrane protein calnexin, which recently has been located in lysosome-related melanosomes and has been suggested to be a melanogenic chaperone. *Molecular & Cellular Proteomics 4:945–957, 2005.

From the ‡Department of Clinical Neurochemistry, Clinic and Polyclinic for Psychiatry and Psychotherapy, and “The National Parkinson Foundation Research Laboratories,” Miami, Florida, at the Bayerische Julius-Maximilians-Universität Würzburg, Füchslinstraße 15, 97080 Würzburg, †Department of Clinical Neurochemistry, Clinic and Polyclinic for Child and Adolescent Psychiatry and Psychotherapy, Bayerische Julius-Maximilians-Universität Würzburg, Füchslinstraße 15, 97080 Würzburg, **Medical Proteome Center, Ruhr-Universität Bochum, Universitätsstrasse 150, 44780 Bochum, §§Institute of Anatomy and Cell Biology, Bayerische Julius-Maximilians-Universität Würzburg, Koellikerstrasse 6, 97970 Würzburg, ¶¶¶Institute of Forensic Medicine, Bayerische Julius-Maximilians-Universität Würzburg, Versbacher Strasse 3, 97078 Würzburg, ¶¶Institute of Pathology, Bayerische Julius-Maximilians-Universität Würzburg, Josef-Schneider-Strasse 2, 97080 Würzburg, and §Institute of Organic Chemistry, Bayerische Julius-Maximilians-Universität Würzburg, Am Hubland, 97074 Würzburg, Germany

Received, August 31, 2005, and in revised form, February 10, 2005

Molecular & Cellular Proteomics 4:945–957, 2005.

© 2005 by The American Society for Biochemistry and Molecular Biology, Inc.
This paper is available on line at http://www.mcponline.org

Melanins are widely distributed throughout the plant and animal kingdoms. In humans, these heterogeneous, complex polymer pigments occur naturally in the hair, the skin, the inner ear, the iris, and the choroid of the eye (1). Melanin in the brain has an appearance and structure similar to cutaneous melamins and has thus been named neuromelanin (NM)1 (2). NM is found inter alia in dopaminergic neurons of a small area in the human midbrain important for the control of movement that is known as the substantia nigra pars compacta (SN; from the Latin meaning “black body”). The loss of this dark pigment and the resulting pallor of the SN is one of the most striking features of the common movement disorder Parkinson disease. A relationship between the loss of the dopaminergic SN cells and their NM content (3), a specific affinity to iron (4), and a significant binding of α-synuclein to NM in the diseased state (5) suggest a functional role for NM in neurodegeneration in Parkinson disease (6).

Although much is known about the peripheral melamins to which NM is thought to be related, many basic questions remain to be answered about NM in the brain. Thus it is unclear why only some human dopamine neurons produce NM within their cytoplasm (7). Little is also known about the structure of NM, and the understanding of its genesis and function within the cell remains speculative.

Nuclear magnetic resonance spectroscopic studies have shown that NM resembles synthetic cysteyl dopamine melanin more closely than the more simple dopamine melanin; however, human NM appears to be a structurally more complex chemical structure than any of the synthetic models (8). In addition to the melanin backbone, nuclear magnetic resonance spectroscopic studies have demonstrated that cholesterol and other uncharacterized high molecular mass lipid components are closely associated with NM (8–10). Dolichol was identified as the major lipid component of NM (11). A proteinaceous component making up ~5–15% of the isolated

1 The abbreviations used are: NM, neuromelanin; SN, substantia nigra pars compacta; 1-D, one-dimensional; LAMP, lysosome-associated membrane glycoprotein; GNA, Galanthus nivalis agglutinin; LIMP, lysosome membrane protein; ER, endoplasmic reticulum; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; EEA1, early endosomal antigen 1; SNAP, soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein; AP, adaptor-related protein complex; BIP, polypeptide-binding protein.
molecule is also present that has been suggested to represent an integral component of the polymer (8, 10).

A general understanding of neuromelanogenesis could be provided by investigation of the synthetic pathway of peripheral melamins and comparison with what is known about NM. Genetic and enzymatic regulation of melanin production in the periphery has been primarily characterized by the study of fur pigmentation in the mouse. Similar experiments, however, cannot be used to elucidate the pathway of NM synthesis as NM does not occur in rodents. It has long been debated whether the NM synthesis is enzymatically controlled, like all melamins in the periphery, or whether NM arises from a simple autoxidation process (for reviews, see Refs. 12 and 13). In the apparent absence of a role for tyrosinase in neuromelanogenesis, the search for an enzyme associated with NM production has yielded no likely candidates to date (12, 13). It is noteworthy that Parkinson disease patients treated with large doses of L-DOPA, levodopa) do not exhibit increased quantities, and the NM granules, as they are called, exhibit a

**EXPERIMENTAL PROCEDURES**

**Isolation of NM Granules Using Subcellular Fractionation**—We used a sequential top-down approach that simultaneously allows the reduction of the complexity of the sample and the enrichment of the target structures at each isolation stage. Fig. 1 schematically summarizes the approach chosen. Brains were provided from the Austro-German Brain Bank in Würzburg. The use of postmortem human brain tissue was approved by the Ethics Committee of the University Clinics of Würzburg. The SN was dissected from postmortem brains of subjects with no history of neurological or neurodegenerative diseases within 36 h of death on a cool plate (−15 °C). 1.0 g of frozen SN tissue was thawed in “Separation Buffer” (10 mM HEPES, 10% glucose, pH 6) on ice and carefully passed through a polypyrrole mesh into a Petri dish. The whole procedure was performed on a plate cooler set at 4 °C. The resulting cell suspension was layered on top of a discontinuous sucrose gradient (1, 1.2, 1.4, and 1.6 m) and separated by centrifugation at 4000 × g at 4 °C for 15 min. The pelleted dark cell bodies were recovered and washed with “Isolation Buffer” (10 mM HEPES, 1 mM EDTA, 100 mM KCl, 10% sucrose, pH 7.5) containing a protease inhibitor mixture (0.01% (v/v), Sigma). Subsequently the cell disruption was carried out by 10 passages through a 26-gauge needle to yield a suspension of cellular organelles that was layered on top of an 80% Percoll cushion (Fluka, Buchs, Switzerland) and centrifuged at 4000 × g at 4 °C for 10 min. The pellet dark granules were washed once with Isolation Buffer and twice with “Washing Buffer” (10 mM HEPES, 250 mM NaCl, 0.01% (v/v) Triton X-100, pH 7.5) to remove unspecifically associated proteins. The isolated NM granules were stored at −80 °C until analyzed.

**Transmission Electron Microscopy**—The quality of the granule isolation and the preservation of the ultrastructural features were monitored by transmission electron microscopy. The aspect of cell homogenates and isolated NM granules were compared as a control to monitor the enrichment of the granules. The samples were fixed overnight in 2% (v/v) glutaraldehyde in 0.1 M phosphate-buffered saline, pH 7.4, at 4 °C and incubated in 2% OsO4, 1.5% (v/v) glutaraldehyde followed by dehydration with increasing concentrations of ethanol. After incubation in 1,2-epoxypropane (Sigma) (2 × 15 min) the samples were embedded in Epon™ epoxy resin (Sigma). Following polymerization at 65 °C (48 h) thin sections were prepared that were contrasted with lead citrate and uranyl acetate (21) before being monitored under the transmission electron microscope (LEO 912 AB, LEO Elektronenmikroskopie, Oberkochen, Germany).

**Sample Preparation for 1-D SDS-PAGE**—Proteins of isolated NM granules were extracted with 16 mM 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate and SDS, mixed with reducing sample buffer containing 0.12 M dithiothreitol, and heated for 10 min at 95 °C.

1-D SDS-PAGE—The protein samples were separated electrophoretically on 10–20% Tricine gels (Novex, San Diego, CA) in an XCell II™ Mini-Cell (Invitrogen) using Tricine-SDS running buffer. Following electrophoresis the gel was either stained with colloidal Coomassie Brilliant Blue G-250 (22) or further processed for Western blotting.

**Antibodies and Materials**—For Western blot analysis anti-human monoclonal antibodies to cis-Golgi matrix protein (GM130), induced myeloid leukemia cell differentiation protein (Mcl-1), early endosomal antigen 1 (EEA1), integrin α2 (VLA-2α), nucleoporin p62, lysosome-associated membrane glycoprotein 1 (LAMP-1), clathrin, and 78-kDa glucose-regulated protein (BiP/grp78) were used (BD Biosciences). Anti-human monoclonal antibody to dynamin was purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Anti-human antibodies to calnexin and cathepsin B were purchased from Calbiochem. Biotinylated Galanthus nivalis agglutinin (GNA) and horseradish peroxidase-linked streptavidin were obtained from Vector Laboratories Inc. (Burlingame, CA).
Subcellular Proteomics of Neuromelanin Granules

**FIG. 1.** Outline of isolation of neuromelanin from human SN. The isolation procedure includes two consecutive steps. In the first step the tissue is disaggregated leading to the enrichment of pigmented neuronal cell bodies after centrifugation through a discontinuous sucrose gradient. In the second step, these pigmented cell bodies are disrupted and subjected to subcellular fractionation to yield NM granules.

**FIG. 2.** Comparative morphological analysis of the organelle preparations to monitor the enrichment of the NM granules. The organelle preparations were fixed in glutaraldehyde and OsO4 and were counterstained with lead citrate and uranyl acetate. Compared with the homogenate of pigmented cell bodies (A and B), according to the second step of the isolation strategy (Fig. 1), NM granules are highly enriched after isolation (C and D).

**Tissue Homogenate**—To provide a positive control for the Western blot analysis, 0.5 g of SN tissue was disrupted using a Potter-Elvehjem homogenizer in Lysis Buffer containing protease inhibitor mixture (0.01%, w/v), and proteins were extracted with 16 mM 3-[3-cholamidopropyl]dimethylamino-1-propanesulfonate (Calbiochem).

**Western Blot Analysis**—The separated proteins were transferred electrophoretically onto nitrocellulose membranes (Invitrogen) using the XCell II blot module. Nonspecific binding was blocked with 5% (w/v) nonfat dried milk, 0.5% (v/v) Tween 20 in Tris-buffered saline, pH 7.3, for 1 h at 20 °C. Immunoblots were probed with primary antibodies at the appropriate dilutions at 4 °C overnight or at room temperature for 1 h. Membranes were washed in Tris-buffered saline containing 0.1% (v/v) Tween 20 (3 × 10 min) followed by incubation with the secondary antibody at 20 °C for 1 h. Additional washing was performed with Tris-buffered saline containing 0.1% (v/v) Tween 20 (3 × 10 min), and the immunocomplexes were visualized by enhanced chemiluminescence (ECL system, Boehringer Ingelheim). Stripping of immunoblots for repeated probing was performed by incubating the membranes at 50 °C for 15 min in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7.

**Detection of Mannosylated Proteins by GNA Lectin**—The biotinylated GNA lectin was applied to visualize mannosylated proteins (23, 24) of isolated NM granules and total SN tissue homogenate. The proteins were transferred electrophoretically onto polyvinylidene difluoride membranes (Invitrogen) using the XCell II blot module. Nonspecific binding was blocked with 5% (w/v) nonfat dried milk, 0.5% (v/v) Tween 20 in Tris-buffered saline, pH 7.3, for 1 h at 20 °C. Immunoblots were probed with primary antibodies at the appropriate dilutions at 4 °C overnight or at room temperature for 1 h. Membranes were washed in Tris-buffered saline containing 0.1% (v/v) Tween 20 (3 × 10 min) followed by incubation with the secondary antibody at 20 °C for 1 h. Additional washing was performed with Tris-buffered saline containing 0.1% (v/v) Tween 20 (3 × 10 min), and the immunocomplexes were visualized by enhanced chemiluminescence (ECL system, Boehringer Ingelheim). Stripping of immunoblots for repeated probing was performed by incubating the membranes at 50 °C for 15 min in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7.

**In-gel Digestion with Trypsin**—An entire lane of a gel previously stained with colloidal Coomassie Brilliant Blue G-250 was sliced into 4-mm cubes, and each of these was placed into a separate quartz reaction tube (Sigma) (25). The gel cubes were washed three times with 10 mM NH4HCO3, pH 7.8, and 10 mM NH4HCO3, pH 7.8, acetonitrile (1:1, v/v) each for 10 min. The gel cubes were subsequently reswollen by addition of 2 μl of modified trypsin (Promega, Madison, WI; 0.05 μg/μl in 10 mM NH4HCO3, pH 7.8). The digestion was performed overnight at 37 °C.

**Sample Preparation for LC Separation**—10 μl of 0.1% (v/v) trifluoroacetic acid/acetonitrile (1:1, v/v) were added to each gel slice followed by sonication for 10 min. This step was repeated twice, and the supernatants containing the extracted peptides were combined in separate quartz tubes.

**Mass Spectrometric Analysis—Nano-HPLC-ESI-MS/MS analysis** of the tryptically generated peptides was carried out as described previously (26). Spectra were recorded on a Finnigan LCQ Classic (Thermo Electron, San Jose, CA) ion trap mass spectrometer equipped with a nanoelectrospray ion source (PicoViewTM 100, New Objective Inc., Woburn, MA). The peptides were preconcentrated by loading onto a μ-precolumn (0.3-mm inner diameter × 5 mm, PepMap™, LC Packings Dionex, Idstein, Germany) before being separated by reverse-phase nano-LC (75-μm inner diameter × 250 mm, PepMap, LC Packings Dionex) using a precolumn split.

**Mass Spectrometry Data Analysis**—The analysis of the raw MS/MS data occurred automatically based on the SequestTM algorithm (27, 28). The data were searched against the human NCBI_nr data base (www.ncbi.nlm.nih.gov) using the following parameters: average masses, partial oxidation of methionine (+16 Da), a mass tolerance of ±1.5 Da, trypsin was used as a specific protease, and a maximum of two missed cleavage sites was tolerated. Furthermore the analysis was restricted to ions in the mass range of 500–5000 Da and a total ion current greater than 3 × 10^5. In general, a cross correlation value (Xcorr) of greater than 2.0 and a D correlation score (ΔCn) greater than 0.1 was accepted for confident identification; inspection of the spectra was performed to confirm the Sequest results.
RESULTS

Subcellular Fractionation to Isolate NM-containing Granules—we developed a mild procedure for the isolation of intact and pure NM granules from human SN to enable subcellular protein analysis. Fig. 1 shows a schematic summary of the approach used. As a first step, the tissue was disaggregated by mechanical sieving into a cell suspension that was subsequently fractionated by centrifugation through a discontinuous sucrose gradient. This step allowed the enrichment of dark cell bodies as a pellet at the bottom sucrose layer. In the second step, these dark cell bodies were disrupted and subjected to an additional centrifugation step for finally isolating the NM granules by subcellular fractionation.

Fig. 3. The ultrastructural features of the NM granules are well preserved, showing the lobulated form of the granules. Electron-dense regions (arrows) are regarded as the pigment NM; lipid bulbs (arrowheads) are still attached to the granule.

Fig. 4. Quality control by Western immunoblot analysis of proteins extracted from NM granules isolated from human SN compared with SN tissue homogenate. A, the Western immunoblot analysis shows the absence of organelle marker proteins of the Golgi complex (a, cis-Golgi matrix protein 130 (GM130)), mitochondria (a, induced myeloid leukemia cell differentiation protein (Mcl-1)), early endosomal compartments (b, early endosomal antigen 1 (EEA1)), plasma membrane (b, integrin α2 (VLA-2α)), and nucleus (c, d, nucleoporin p62 (np62)). The protein extract of isolated NM granules compared with SN homogenate shows the presence of cathepsin B (d), a lysosomal proteinase, and LAMP-1 (e), a marker for late endosomes, lysosomes, and lysosome-related organelles. Dynamin (f) and clathrin (g), which are involved in vesicular traffic and are suggested to be associated to endosomal compartments, are detected. The melanogenic chaperone calnexin (h) is present in NM granules, although the marker for the endoplasmic reticulum (i, 78-kDa glucose-regulated protein (BiP/GRP78)) is absent after stripping. B, blotted proteins were probed with GNA lectin, which specifically binds to mannosylated proteins found in lysosomes or lysosome-related organelles.
Quality Control of Isolated Specimens by Transmission Electron Microscopy—The purity and quality of the granule isolation were monitored by transmission electron microscopy to evaluate the level of enrichment achieved by this approach as well as the structural and morphological appearance of the isolated granules (Fig. 2). Compared with the homogenates of pigmented cell bodies prior to the NM granule isolation, the isolated NM granules were virtually free from contaminating organelles; this was attributed to the exceptional density of NM granules. Up to now the essential density to penetrate an 80% (v/v) Percoll cushion has only been reported for highly melanized stage IV melanosomes isolated from *Xenopus laevis* melanophores (29, 30).

Purified granules (Fig. 3) displayed all the morphological structures described previously for primate brain tissue by electron microscopy studies (16, 31) showing 1) the highly electron-dense patches attributable to the iron-rich NM, 2) the medium electron-dense protein matrix, and 3) vacuolar lipid bulbs. These characteristics were well preserved during isolation underscoring the potential of this strategy for isolation of NM granules.

Quality Control by Western Immunoblotting—The level of enrichment was additionally assessed by Western immunoblotting applying antibodies against “marker proteins” specific for cell organelles (Fig. 4A) such as the Golgi network (GM130), mitochondria (Mcl-1), early endosomal compartments (EEA1), the plasma membrane (VLA-2α), the nucleus (nucleoporin p62), lysosomes (cathepsin B and LAMP-1), and the endoplasmic reticulum (BiP/grp78). The proteins extracted from NM granules were compared with control tissue homogenate and show the presence of lysosomal markers, whereas the other “organelle marker proteins” are absent (Fig. 4A, a–e and i).

Protein Identification by Mass Spectrometry and Western Immunoblotting—As depicted in Fig. 5, the proteins extracted
TABLE I
Proteins identified in NM granules by LC-MS/MS analysis

| Protein no. and name | Swiss-Prot accession number | Molecular mass (kDa) | [Peptides] sequenced; sequence coverage | Location | Comments |
|----------------------|-----------------------------|----------------------|-------------------------------|----------|----------|
| **Lysosomal membrane** |                            |                       |                               |          |          |
| 1 CD63 antigen (LAMP-3) | P08962                     | 25.6                 | 1; 4.2%                      | Ly       | Integral membrane protein; ocular melanoma-associated antigen |
| 2 LIMP II | Q14108                        | 54.3                 | 3; 20.9%                     | Ly       | Type II membrane protein; may act as a lysosomal receptor |
| 3 LAMP-1 (CD107a) | P11279                        | 41.6                 | 2; 7.1%                      | Ly, E    | Lysosomal and endosomal membrane |
| 4 V-ATPase, subunit A1 | P38606                     | 68.2                 | 2; 5.2%                      | Ly       | Acidification of intracellular compartments |
| 5 V-ATPase, subunit B2 | P21281                     | 58.4                 | 3; 14.8%                     | Ly       | Acidification of intracellular compartments |
| 6 V-ATPase, subunit E | P36543                     | 26.2                 | 2; 12.0%                     | Ly       | Acidification of intracellular compartments |
| **Proteases** |                            |                       |                               |          |          |
| 7 Cathepsin B | P07858                      | 28.8                 | 10; 43.3%                   | Ly       | Thiol protease; peptidase family C1 |
| 8 Cathepsin D | P07339                      | 44.6                 | 10; 34.2%                   | Ly       | Aspartyl protease; peptidase A1 |
| 9 Cathepsin Z (contains cathepsins X and Z) | Q9UBR | 33.8 | 2; 7.9% | Ly | Peptidase family C1 |
| 10 Tripeptidyl-peptidase I (TPP-I; LPIC) | O14773 | 61.2 | 5; 16.3% | Ly | Lysosomal serine protease; peptidase family S53; ceroid lipofuscinosis, neuronal 2, late infantile (CLN2) |
| **(Glyco)lipid metabolism** |                            |                       |                               |          |          |
| 11 γ-Glutamyl hydrolase | Q09280                     | 35.9                 | 11; 52.1%                  | Ly       | Lysosomal or secreted; peptidase family C26 |
| 12 Lysosomal Pro-X carboxypeptidase (angiotensinase C) | P42785 | 55.8 | 3; 9.0% | ? | Serine carboxypeptidase; peptidase family S28 |
| 13 Ubiquitin carboxyl-terminal hydrolase 5 (isopeptidase T) | Q96J22 | 93.2 | 2; 5.3% | Ly? | Zinc-containing ubiquitin specific protease; peptidase family C19 |
| 14 Dipeptidyl-peptidase II (DPP-II) | Q9UHL4 | 54.3 | 4; 17.8% | Ly | Lysosomal and intracellular vesicles; peptidase family S28 |
| **Glycoprotein metabolism** |                            |                       |                               |          |          |
| 15 Acid ceramidase (acylsphingosine deacylase) | Q13510 | 44.6 | 14; 46.2% | Ly | Hydrolyzes the sphingolipid ceramide into sphingosine and free fatty acid; Farber lipogranulomatosis |
| 16 Phospholipase D3 | Q81V08                     | 54.7                 | 7; 19.7%                   | ?        | Removal of thioester-linked fatty acyl groups from modified cysteine residues in proteins; ceroid lipofuscinosis, neuronal 1, infantile (CLN1) and neuronal 4, adult type (CLN4) |
| 17 Palmitoyl protein thioesterase 1 | P50897 | 34.2 | 5; 34.2% | Ly | Removal of thioester-linked fatty acyl groups from modified cysteine residues in proteins; ceroid lipofuscinosis, neuronal 1, infantile (CLN1) and neuronal 4, adult type (CLN4) |
| 18 β-Hexosaminidase (N-acetyl-β-glucosaminidase) | P07686 | 63.1 | 4; 17.7% | Ly | Degradation of GM2 gangliosides; Sandhoff disease |
| 19 Galactosylceramidase | P54803 | 75.1 | 2; 4.9% | Ly | Lysosomal catabolism of galactosylceramide, a major lipid in myelin; Krabbe disease |
| 20 Proactivator polypeptide (cerebroside sulfate activator protein) | P07602 | 58.5 | 3; 6.1% | Ly | Contains saposins A, B, C, D; lipid binding and transport; glycosphingolipid metabolism; lysosome organization and biogenesis |

**Subcellular Proteomics of Neuromelanin Granules**
| Protein no. and name                      | Swiss-Prot accession number | Molecular mass | Peptides sequenced; sequence coverage | Location | Comments                                                                                     |
|------------------------------------------|----------------------------|----------------|---------------------------------------|----------|---------------------------------------------------------------------------------------------|
| **Protein no. and name**                 |                            |                |                                       |          |                                                                                             |
| **25** Lysosomal α-glucosidase            | P10253                     | 105.3                      | 1; 1.9%                              | Ly       | Essential for degradation of glycogen in lysosomes; Pompe disease                           |
| **Unknown**                              |                            |                |                                       |          |                                                                                             |
| **26** High mobility group protein 1     | P09429                     | 24.7                      | 9; 49.8%                             | ?        | Secreted or nuclear (73); gastrointestinal stromal tumor marker (74)                      |
| **27** R31109_1 (25-kDa protein; similar to high mobility group 20B) | Q9Y4A2                    | 24.6                      | 3; 22.5%                             | ?        | Similar to high mobility group protein 1                                                   |
| **28** Hypothetical protein LOC196463    | Q8NHP8                     | 65.5                      | 3;                                   | ?        |                                                                                             |
| **29** 25-kDa brain-specific protein     | O94811                     | 23.7                      | 13; 57.7%                            | ?        | Brain-specific expression; glycogen synthase kinase 3 (GSK3) inhibitor p24; p25 could act as a unique MAP in vivo (75) |
| **Probable cytosolic proteins**          |                            |                |                                       |          |                                                                                             |
| **30** Mammalian ependymin-related protein-1 (MERP-1; UCC1 protein) | Q9UM22                     | 19.9                      | 5; 55.0%                             | ?        | Secreted                                                                                    |
| **31** Tyrosine 3-monooxygenase (tyrosine 3-hydroxylase) | P07101                     | 58.5                      | 3; 12.3%                             | Cytosol  | Catalyzes the first step in catecholamine biosynthesis; putatively involved in the synthesis of neuromelanin (76) |
| **32** 14-3-3 protein β                 | P29312                     | 27.7                      | 2; 12.7%                             | Cytosol  | Activates tyrosine 3-hydroxylase                                                            |
| **33** 14-3-3 Protein γ                  | P35214                     | 28.3                      | 3; 13.4%                             | Cytosol  | Activates tyrosine 3-hydroxylase                                                            |
| **34** Peroxiredoxin 1                   | Q06830                     | 22.1                      | 3; 15.5%                             | Ly, cytosol | Antioxidant protection                                                                     |
| **35** Heat shock cognate 71-kDa protein (heat shock 70-kDa protein 8) | P11142                     | 53.5                      | 4; 10.4%                             | Cytosol  | Chaperone, protein folding; ATPase activity; maybe functions as an endogenous inhibitory regulator of hsc70 |
| **36** α-Crystallin B chain              | P02511                     | 22.4                      | 11; 76.8%                            | ?        | Accumulates in the brains of patients with Alexander disease; HSP20 family                 |
| **37** Heat shock 70-kDa protein 9B (mortalin-2) | Q8N1C8                     | 73.7                      | 3; 7.4%                              | ?        | Calcium and ATP binding; modulates toxicity of α-synuclein (77)                            |
| **38** Heat shock protein 90-α            | P07900                     | 84.6                      | 5; 7.5%                              | Cytosol  | ATPase activity (by similarity)                                                            |
| **39** Glycogen phosphorylase, brain form | P11216                     | 96.7                      | 5; 9.2%                              | Cytosol  | Carbohydrate metabolism                                                                  |
| **40** 6-Phosphofructokinase              | P78457                     | 85.0                      | 5; 9.9%                              | Cytosol  | Glycolysis                                                                                  |
| **41** Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | P04406                     | 35.9                      | 2; 9.6%                              | Cytosol  | Glycolysis                                                                                  |
| **Others**                               |                            |                |                                       |          |                                                                                             |
| **42** Ubiquitin                         | P02248                     | 8.6                       |                                       |          | Proteasome- or lysosome-based protein degradation; protein transport to lysosomes           |
| **43** Similar to cytosolic sialic acid 9-O-acetylesterase homolog | Q8IUT9                     | 58.1                      | 11; 38.8%                            | Ly, E    | 9-O-Acetylation of sialic acids, localized in lysosomes and endosomes                      |
| **44** Myelin basic protein              | P02686                     | 21.5                      | 17; 61.9%                            |          |                                                                                             |
| **45** Myelin proteolipid protein (PLP)   | P60201                     | 30.1                      | 2; 8.7%                              |          | Integral membrane protein; Pelizaeus-Merzbacher disease                                    |
| **46** 2’,3’-Cyclic nucleotide 3’-phosphodiesterase | P09543                     | 47.6                      | 8; 20.7%                             |          | Bound to membrane structures of brain white matter, myelin-associated                      |
| **47** Sodium/potassium-transporting ATPase, α-3 chain | P13637                     | 111.7                     | 3; 6.0%                              | PM, E    | Sodium/potassium exchange; probably regulated by endocytosis (57)                        |
| **48** Voltage-dependent anion-selective channel P (VDAC 1) | P21796                     | 30.6                      | 3; 18.1%                             | Mt, E, Ly | Allows diffusion of small hydrophilic molecules                                            |
| **49** Apolipoprotein D (apoD)            | P05090                     | 21.2                      | 5; 21.2%                             |          | Endocytosed; lipid metabolism                                                            |
| **50** Cystatin C                        | P01034                     | 15.8                      | 4; 40.9%                             |          | Cysteine protease inhibitor; amyloidogenic properties                                      |
| **Mitochondrial proteins**               |                            |                |                                       |          |                                                                                             |
| **51** Similar to ATP synthase, mitochondrial F1 complex, α subunit | Q96FB4                     | 59.8                      | 4; 14.5%                             | Mt, Ly   | F1, ATPase subunit                                                                         |

**Subcellular Proteomics of Neuromelanin Granules**

**TABLE I—continued**
from NM granules were further fractionated by 1-D SDS-PAGE into a relatively large number of protein bands, although many of those were poorly resolved. Thus, we cut the entire gel lane into 16 slices and generated peptides from each slice by in-gel digestion with trypsin. These peptides were consequently analyzed in a second dimension by nano-LC coupled to ESI-MS/MS, which gave positive identification of 72 proteins (this experiment was repeated five times with comparable results). The proteins identified by mass spectrometry and the protein characteristics are compiled in Table I. Examples of mass spectral analyses depicting the identification of lysosome integral membrane protein (LIMP) II (Protein 2), subunit A of the vacuolar ATPase (Protein 4), cathepsin D (Protein 8), and calnexin (p90) (Protein 70) are shown in Fig. 6.

We found a variety of transmembrane proteins specific for

| Protein no. and name | Swiss-Prot accession number | Molecular mass | Peptides sequenced; sequence coverage | Location | Comments |
|----------------------|-----------------------------|----------------|--------------------------------------|----------|----------|
| 52 ATP synthase β chain | P06576                     | 56.5 kDa       | 12; 42.9%                           | Mt       | F1 ATPase subunit |
| 53 ATP synthase D chain | O5947                      | 18.4 kDa       | 1; 12.4%                            | Mt       | F1 ATPase subunit |
| 54 ATP synthase γ chain | O75964                     | 11.4 kDa       | 1; 14.6%                            | Mt, Ly   | F1 ATPase subunit |
| 55 60-kDa heat shock protein (HSP60) | P10809                   | 61.1 kDa       | 2; 7.7%                             | Mt       | Mitochondrial matrix; protein folding |
| 56 Phospholipid-hydroperoxide glutathione peroxidase (PHGPx) | P36969                  | 22.1 kDa       | 2; 13.2%                            | Mt, cytosol | Protects against oxidative damage and the toxicity of ingested lipid hydroperoxides |

**Table I—continued**

| Protein no. and name | Swiss-Prot accession number | Molecular mass | Peptides sequenced; sequence coverage | Location | Comments |
|----------------------|-----------------------------|----------------|--------------------------------------|----------|----------|
| 57 Tubulin α 6         | Q9BQE3                      | 49.9 kDa       | 4; 20.8%                             | Major constituent of microtubules |
| 58 Tubulin β 2         | P05217                      | 49.8 kDa       | 5; 19.8%                             | Major constituent of microtubules |
| 59 Microtubule-associated protein 2 | P11137         | 202.8 kDa      | 11; 10.0%                            | May stabilize the microtubules against depolymerization |
| 60 Microtubule-associated protein tau | P10636         | 78.7 kDa       | 2; 6.1%                              | Microtubule assembly |
| 61 Clathrin heavy chain | Q00610                     | 191.6 kDa      | 1; 1.1%                              | Cytoplasmic face of coated pits and vesicles |
| 62 Phosphatidylinositol-binding clathrin assembly protein (CALM, AP180-2) | Q13492          | 70.7 kDa       | 1; 3.4%                              | Assembly of clathrin, clathrin coated-vesicle formation; membrane recycling |
| 63 Phosphatidylinositol 4-phosphate 5-kinase type II α | P48426          | 46.2 kDa       | 2; 8.1%                              | E, Ly    | Associated with membranes of the late endocytic pathway |
| 64 α-SNAP | P54920                      | 3; 14.2%       | Vesicles                             | Participates in various endocytotic and vesicular traffic |
| 65 Syntaxin-binding protein 1 (Hunc18) | P61764          | 67.6 kDa       | 3; 7.9%                              | Vesicles | Participates in various endocytotic and vesicular traffic |
| 66 Dynamin-1, neural isoform | Q05193          | 97.4 kDa       | 3; 3.5%                              | E, vesicles | Participates in various endocytotic and vesicular traffic |
| 67 NipSnap1 protein | Q9BPW8                      | 33.3 kDa       | 3; 16.6%                             | Vesicles? | Putative involvement in vesicular traffic |
| 68 NipSnap2 protein | O75323                      | 33.8 kDa       | 4; 31.8%                             | Vesicles? | Transmembrane domain; putative involvement in vesicular traffic |
| 69 Calreticulin | P27797                      | 46.8 kDa       | 2; 7.2%                              | ER, cytosol, ECM | Chaperone promoting folding, assembly, and quality control in the ER via the calreticulin/ calnexin cycle; associated with the lytic granules |
| 70 Calnexin (p90) | P27824                      | 67.6 kDa       | 4; 9.8%                              | ER       | Type I membrane protein; melanogenic chaperone; not associated to classical lysosomes but to some lysosome-related organelles |
| 71 Dolichyl-diphosphooligosaccharide-protein glycosyltransferase (ribophorin I) | P04843          | 68.6 kDa       | 3; 7.1%                              | ER       | Type I membrane protein; N-oligosaccharyltransferase enzyme (transfer of a high mannose oligosaccharide from a lipid-linked donor to an asparagine residue) |
| 72 Protein-disulfide isomerase A3 (ERp57) | P30101          | 54.3 kDa       | 4; 12.0%                             | ER       | Protein-disulfide isomerase family |

* These proteins were not identified in all five neuromelanin granule preparations.
organelles originating from the endosome-lysosome lineage, such as LIMP II (Protein 2) and LAMP-1 (Protein 3) but also subunits of the vacuolar ATPase (Proteins 4–6) responsible for the acidification of intracellular compartments (Fig. 6 and Table I). LIMP II was the only lysosomal marker membrane protein unequivocally identified by mass spectrometry. Although our analytical approach is compatible with membrane proteins (25, 32), LAMP-1 (Protein 3) and LAMP-3 (LIMP I)
(Protein 1) could not be identified by a sufficient amount of peptides by mass spectrometry. As both proteins are glycosylated, LAMP-1 and -3 might be present in a much lower amount in NM granules than LIMP II. The presence of LAMP-1 was substantiated by Western blot analysis (Fig. 4A, e). We further identified several proteases known to be localized in the lysosome (e.g. cathepsin B (Protein 7) (Fig. 4A, d), cathepsin D (Protein 8), and tripeptidyl-peptidase I (Protein 10)) and enzymes mediating the catabolism of sphingolipids or glycoproteins (e.g. acid ceramidase (Protein 15), palmityl protein thioesterase 1 (Protein 17), and chondroitinase (Protein 22)).

We also identified a few proteins with unknown localization and function, especially of the high mobility group protein family (Proteins 26 and 27) but also some cytosolic proteins. Cytoskeletal components such as microtubule-associated protein 2 (Protein 59), tau protein (Protein 60), α- and β-tubulin (Proteins 57 and 58), necessary for vesicular traffic, were also identified. NM granules exhibit proteins that are involved in vesicular traffic and are associated to endosomal membranes, e.g. clathrin (Protein 61), phosphatidylinositol-binding clathrin assembly protein (Protein 62), and dynamin (Protein 66) (Fig. 4A, f and g) (33–35) but also α-soluble NSF attachment protein (α-SNAP) (Protein 64) and phosphatidylinositol-4-phosphate 5-kinase type II (Protein 63). γ-Glutamyl hydrolase (Protein 11) (peptidase family C26) and lysosomal sialic acid 9-O-acetyler esterase (Protein 43) are likely to be localized in organelles derived from the endosome-lysosome lineage (36–38).

To further examine the lysosomal traits of NM granules, we used GNA, a mannose-binding lectin, to identify proteins targeted to lysosomal compartments. Because of their selective binding to glycan structures lectins are a powerful tool in the analysis of glycoproteins (39, 40). As shown in Fig. 4B, numerous mannosylated proteins were found in NM granules and SN tissue homogenate. However, only a subset of mannosylated proteins was found in NM granules compared with the total tissue homogenate.

Interesting findings, however, are the obvious presence of four ER-derived proteins (Proteins 69–72, most notably calnexin (Figs. 6 and 4A, h), whereas by Western immunoblotting we could not show the presence of the ER marker protein 78-kDa glucose-regulated protein (BiP/grp78) (Fig. 4A, i).

**DISCUSSION**

This study demonstrated the utility of a new approach to analyze purified NM granules that sheds light on the biogenesis and biology of these unique organelles. We developed a new method for isolation of intact and pure NM granules from human SN without using detergents, organic solvents, and proteases as applied in the isolation of NM from crude tissue homogenates (9). Then we applied 1-D SDS-PAGE followed by in-gel digestion and nano-HPLC coupled to ESI-MS/MS to identify novel constituents of NM granules. This approach overcomes the difficulties in separation of basic and hydrophobic proteins as with two-dimensional electrophoresis (32, 41). In addition, we used Western immunoblot analysis to investigate the potential link to lysosomes. Table 1 summarizes the proteins identified by ESI-MS/MS. The function and the subcellular localization of some proteins identified by our approach are not yet entirely known.

By using this approach we identified numerous proteins attributable to the endosome-lysosome lineage as well as four proteins from the ER. A link of NM granules to lysosomes has been assumed previously; however, the morphological appearance and the phylogenetic limitation of the pigment NM are their most striking differences to conventional lysosomes.

In eukaryotic cells, lysosomes serve as the terminal sites for delivery of material targeted for removal (42–44). For this purpose, lysosomes are equipped with a variety of more than 50 soluble acid-dependent hydrolases engaged in final degradation of both exogenous and endogenous macromolecules. Lysosomes are highly dynamic and heterogeneous organelles and thus cannot be identified solely by morphology (45–48). Therefore, lysosomes are described by the presence of highly glycosylated integral membrane proteins known as LAMPS, LIMPs, and lysosomal membrane glycoproteins. In contrast to late endosomal compartments, they lack mannose 6-phosphate receptors. Lysosomes maintain an acidic pH (4.5–5.0) required for the hydrolytic activities of the luminal enzymes. All or most of these features are shared with specialized cell type-specific “lysosome-related organelles” including melanosomes, platelet-dense granules, lytic granules, and major histocompatibility complex class II compartments among others, which mostly perform physiological functions different from biomolecular degradation (49). Various studies on genetic multiorganellar disorders substantiated the biogenetic relationship between lysosome-related organelles and lysosomes.

Recently various models of lysosomal biogenesis have been established (46–48), but all of them include the existence of very specific sorting mechanisms. Membrane proteins such as LAMP-1 and LIMP II exit the trans-Golgi network on a secretory route to the endosomal compartments that is mediated by recognition of their tyrosine-based (LAMP-1) and dileucine-based (LIMP II) sorting motifs (50–53) by adaptor complex AP-3 (51). This adaptor complex AP-3 is also crucial for the biogenesis of lysosome-related melanosomes by targeting tyrosinase gene family proteins according to their dileucine-based sorting motifs (54). However, in our study on NM granules we did not find the melanogenic enzymes tyrosinase and tyrosinase-related proteins 1 and 2, which are located in melanosomes of cutaneous melanocytes and retinal pigment epithelial cells. Although low levels of tyrosinase mRNA have been reported in human SN (55), tyrosinase protein does not appear to be expressed in the human brain (56).2

---

2 F. Tribl, M. Gerlach, T. Arzberger, and P. Riederer, unpublished observation.
The current evidence thus suggests that tyrosinase does not appear to have a role in the synthesis of human brain NM. Regardless it cannot be excluded that such an enzyme is covalently bound to the framework of the highly abundant pigment, which renders the protein insoluble and inaccessible to detergents (58).

In the context of membrane protein sorting, we want to point out that surprisingly we did not detect the “classical” lysosomal marker protein lysosomal acid phosphatase. At first, lysosomal acid phosphatase is transiently integrated into the limiting membrane as a type I membrane protein and is further proteolytically processed to gradually release the luminal active site of the enzyme into the lysosomal matrix (59). A distinct pathway guides mannose 6-phosphate-tagged acid-dependent hydrolases to endosomes and lysosomes in clathrin-coated vesicles, which bud from the trans-Golgi network as the mannose 6-phosphate tag is recognized by mannose 6-phosphate receptors. Dynamin has been suggested recently to be associated with late endosomes, tubulovesicles, and clathrin-coated vesicles as well as being involved in the recycling of mannose 6-phosphate receptors after cargo delivery (60). Interestingly by proteomic mapping we identified an unexpectedly small repertoire of mannosylated lysosomal enzymes (Table I), such as glycosyl hydrolases (EC 3.2.1.) (61). Again a similar situation occurred showing a small subset of proteins when tested for mannosylation by GNA recognition (Fig. 4B).

A few proteins specifically facilitating the degradation of glycosphingolipids (62) were identified, such as acid ceramidase, etc. (Table I). Glycosphingolipids are predominantly found in neuronal tissue; it will be the task of further investigations to elucidate whether or not sphingolipids are found in the lipidic bulbs of the NM granules. Mutated forms of tripeptidyl-peptidase I (Protein 10) and dipeptidyl-peptidase II (Protein 14) have been found to be involved in some types of neuronal ceroid lipofuscinosis, characterized by an accumulation of autofluorescent inclusion bodies (63). These forms of neurodegenerative disorders are regarded as lysosomal storage diseases evoked by defective proteolysis.

Our findings of ER proteins associated with NM granules substantiate their multitopological subcellular localization. We assume a specific localization of the ER proteins in NM granules because major ER constituents, e.g. cytochrome P-450 isoforms or NADPH cytochrome c reductase (64), have not been detected, and we could not identify BiP/grp78 by MS and Western immunoblotting (Fig. 4B). This is in line with recent findings that suggest a direct involvement of the ER in the establishment of early lysosomal structures including phagosomes in neutrophiles (65) and stage I melanosomes in melanocytes (14, 66). During the maturation of phagosomes to phagolysosomes the quantity of ER proteins gradually decreases; calnexin especially is lost rapidly (67). In classical lysosomes calnexin is absent but is rather associated with lysosome-related organelles such as melanosomes (68–70). There is increasing evidence that calnexin functions as a melanogenic chaperone (54, 71) currently suggested to mediate the proper folding and sorting of the melanogenic enzymes toward their target compartment (70, 72).
Subcellular Proteomics of Neuromelanin Granules

In summary, our results provided important insight into the cellular machinery used to generate NM granules, revealing an admirable conservation of cellular processes. At this stage of analysis, it has become clear that the majority of the components of NM granules, similar to those of melanosomes, are shared with lysosomes (Fig. 7). The putative precursor compartment may have endosomal traits; however, an additional direct involvement of the ER is suggested to contribute to the constitution of NM granules. Future studies are warranted to clarify the nature of the lipidic bulbs attached to NM granules.

Acknowledgments—We thank Siglind Schenk for valuable advice and assistance with the electron microscopy work and Dr. Anne Price Mortimer for critically reading the manuscript.

* This work was supported by the BrainNet Europe II and the BMBF Project “Human Brain Proteome Project” (HUPO) and by BMBF Grant 031U102F, the Deutsche Parkinson Vereinigung, and the Fond der Chemischen Industrie (to G. B.) This research was completed within “The National Parkinson Foundation Center of Excellence Research Laboratories,” Miami, Florida, at the Clinic and Polyclinic for Psychiatry and Psychotherapy of the University of Würzburg (awarded to P. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Recipient of the Ph.D. scholarship DOC from the Austrian Academy of Sciences. To whom correspondence should be addressed.

Tel.: 49-931-201-77300; Fax: 49-931-201-77220; E-mail: florian.tribi@mail.uni-wuerzburg.de.

Present address: Inst. of Neuropathology, Ludwig-Maximilians-Universität, Feodor-Lynen-Strasse 23, 81377 Munich, Germany.

REFERENCES

1. Prota, G. (1992) Melanins and Melanogenesis, Academic Press Inc., San Diego, CA
2. Lillie, R. D. (1957) Metal reduction reactions of the melanins: histochemical studies. J. Histochem. Cytochem. 5, 325–333
3. Hirsch, E., Graybiel, A. M., and Agid, Y. A. (1988) Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson’s disease. Nature 334, 345–348
4. Jellinger, K., Kienzl, E., Rumpelmair, G., Riederer, P., and Stachelberger, H. (1989) Electron microscopic studies of pigment in human and rhesus monkey substantia nigra and locus coeruleus. Anat. Rec. 155, 187–193
5. Duffy, P. E., and Tennyson, V. M. (1965) Phase and electron microscopic studies of pigment in human and rhesus monkey substantia nigra and locus coeruleus. J. Comp. Neurol. 124, 99–107
6. McRitchie, D. A., Halliday, G. M., and Cartwright, H. (1995) Quantitative analysis of pigmented neurons are differentially susceptible to degeneration in Parkinson’s disease. J. Histochem. Cytochem. 43, 990–995
7. Moses, H. L., Ganote, C. E., Beaver, D. L., and Shannon, S. S. (1966) Light and electron microscopic studies of pigment in human and rhesus monkey substantia nigra and locus coeruleus. Anat. Rec. 155, 167–183
8. Borden, K. L., Zecca, L., Costi, P., Mauer, M., Griesinger, C., Ito, S., and Schmitz, H. (2000) Melanins and melanogenesis: identification of novel melanosomal proteins. J. Proteome Res. 2, 69–79
9. Dzierzega-Lecznar, A., Kurkiewicz, S., Stepien, K., Chodurek, E., Wilczok, J., Pinals, T., Arzberger, T., and Streb, M. (2004) GC/MS analysis of thermally degraded neuromelanin from the substantia nigra. J. Am. Soc. Mass Spectrom. 15, 920–926
10. Zecca, L., Costi, P., Mecacci, C., Ito, S., Tereni, M., and Sonnio, S. (2000) Interaction of human substantia nigra neuromelanin with lipids and peptides. J. Neurochem. 74, 1758–1765
11. Fedorow, H., Pickford, R., Hook, J. M., Double, K. L., Halliday, G. M., Gerlach, M., Riederer, P., and Garner, B. (2005) Dolichol is the major lipid component of human substantia nigra neuromelanin. J. Neurochem. 92, 990–995
12. Fedorow, H., Tribi, F., Halliday, G., Gerlach, M., Riederer, P., and Double, K. L. (2005) Neuromelanin in human dopamine neurons: comparison with peripheral melanins and relevance to Parkinson’s disease. Prog. Neurobiol. 75, 109–124
13. Zecca, L., Tampellini, D., Gerlach, M., Riederer, P., Fariello, R. G., and Sulzer, D. (2001) Substantia nigra neuromelanin: structure, synthesis, and molecular behaviour. Mol. Pathol. 54, 414–418
14. Basur, V., Yang, F., Kushimoto, T., Higashimoto, Y., Yasumoto, K., Valencilla, J., Muller, J., Veira, W. D., Watabe, H., Shabanowitz, J., Hearing, V., Neupert, D. F., and Ehrhardt, W. (1998) Improved staining of early melanosomes: identification of novel melanosomal proteins. J. Proteome Res. 2, 69–79
15. Dufy, P. E., and Tennyson, V. M. (1965) Phase and electron microscopic observations of Lewy bodies and melanin granules in the substantia nigra and locus coeruleus in Parkinson’s disease. J. Neuropathol. Exp. Neurol. 24, 398–414
16. Johnstone, A. H., Ganote, C. E., Beaver, D. L., and Shannon, S. S. (1966) Light and electron microscopic studies of pigment in human and rhesus monkey substantia nigra and locus coeruleus. Anat. Rec. 155, 167–183
17. Borden, K. L., Zecca, L., Costi, P., Mauer, M., Griesinger, C., Ito, S., and Schmitz, H. (2000) Melanins and melanogenesis: identification of novel melanosomal proteins. J. Proteome Res. 2, 69–79
18. Aebi, F., and Mann, M. (2000) Mass spectrometry-based proteomics. Nature 422, 198–207
19. Birz, M. A., Hochstrasser, D. F., and Appel, R. D. (2003) Mass spectrometry-based proteomics: current status and potential utility in clinical chemistry. Clin. Chem. Lab. Med. 41, 1540–1551
20. Jung, E., Keller, M., Sanchez, J. C., and Hochstrasser, D. F. (2000) Proteomics meets cell biology: the establishment of subcellular proteomes. Electrophoresis 21, 3369–3377
21. Reynolds, E. S. (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17, 208–212
22. Neuhoff, V., Arnold, D. N., Taube, D., and Ehrhardt, W. (1998) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with a cheap background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis 9, 255–262
23. Liao, Y. F., Liu, A., and Moremen, K. W. (1996) Cloning, expression, purification, and characterization of the human broad specificity lysosomal acid α-mannosidase. J. Biol. Chem. 271, 28348–28358
24. Shibuya, N., Golstein, I. J., Van Damme, E. J., and Peumans, W. J. (1998) Binding properties of a mannos-specific lectin from the snowdrop (Galanthus nivalis) bulb. J. Biol. Chem. 263, 728–734
25. Schafer, H., Nau, K., Sickmann, A., Erdmann, R., and Meyer, H. E. (2001) Identification of peroxisomal membrane proteins of Saccharomyces cerevisiae by mass spectrometry. Electrophoresis 22, 2955–2968
26. Marcus, K., Moebius, J., and Meyer, H. E. (2003) Differential analysis of phosphorylated proteins in resting and thrombin-stimulated human platelets. Anal. Bioanal. Chem. 376, 973–993
27. Eng, J. K., McCormack, A. L., and Yates, J. R., III (1994) An approach to correlate tandem mass-spectral data of peptides with amino-acid-sequence in a protein database. J. Am. Soc. Mass Spectrom. 5, 976–989
28. Yates, J. R., III, Eng, J. K., McCormack, A. L., and Schleiert, D. (1995) Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. Anal. Chem. 67, 1426–1436
29. Rogers, S. L., Tint, I. S., Paner, H. C., and Safford, V. J. (1997) Regulated bidirectional motility of melanoliphe pigment granules along microtubules in vitro. Proc. Natl. Acad. Sci. U. S. A. 94, 3720–3725
30. Testorf, M. F., Roback, K., Lundstrom, I., and Svensson, S. P., Jr. (2001) Volume changes of individual melanosomes measured by scanning force microscopy. Pigm. Cell Res. 14, 445–449
31. Hori, K. A. (1986) Electron microscopic studies on pigment granules in the substantia nigra and locus coeruleus of the Japanese monkey (Macaca fuscata yakui). Z. Zellforsch. Mikrosk. Anat. 188, 187–203
32. Santoni, V., Molloy, M., and Rabilloud, T. (2000) Membrane proteins and proteomics: an arm impossible? Electrophoresis 21, 1054–1070
33. Hinrichsen, L., Harborth, J., Andres, L., Weber, K., and Ungewickell, E. J. (2003) Effect of clathrin heavy chain- and α-adaptin-specific small inhibitory RNAs on endocytic accessory proteins and receptor trafficking in
HeLa cells. J. Biol. Chem. 276, 45160–45170
34. Iversen, T. G., Skretting, G., van Deurs, B., and Sandvig, K. (2003) Clathrin-coated pits with long, dynamin-wrapped necks upon expression of a clathrin antisense RNA. Proc. Natl. Acad. Sci. U. S. A. 100, 5175–5180
35. Stoovorgel, W., Oorschot, V., and Geuze, H. J. (1996) A novel class of clathrin-coated vesicles budding from endosomes. J. Cell Biol. 132, 21–33
36. Butor, C., Higa, H. H., and Varli, A. (1993) Structural, immunological, and biosynthetic studies of a sialic acid-specific O-acetylcetylase from rat liver. J. Biol. Chem. 268, 10207–10213
37. Galivan, J., Ryan, T. J., Chave, K., Rhee, M., Yao, R., and Yin, D. (2000) Glutamyl hydrolase: pharmacological role and enzymatic characterization. Pharmacol. Ther. 85, 207–215
38. Takematsu, H., Diaz, S., Soddart, A., Zhang, Y., and Varli, A. (1999) Lysosomal and cytosolic sialic acid β-O-acetylcetylase activities can be encoded by one gene via differential usage of a signal peptide-encoding exon at the N terminus. J. Biol. Chem. 274, 25623–25631
39. Hagglund, P., Bunkenborg, J., Elortza, F., Jensen, O. N., and Roepstorff, P. (2004) A new strategy for identification of N-glycosylated proteins and unambiguous assignment of their glycosylation sites using HILIC enrichment and partial deglycosylation. J. Proteome Res. 3, 556–566
40. Medzihradszky, K. F. (2002) Characterization of site-specific glycosylation. Methods Mol. Biol. 194, 101–125
41. Wu, C. C., and Yates, J. R., III (2003) The application of mass spectrometry to membrane proteins. Nat. Biotechnol. 21, 262–267
42. Chataway, T. K., Whittle, A. M., Lewis, M. D., Bindloss, C. A., Moritz, R. L., Wu, C. C., and Yates, J. R., III (2003) The application of mass spectrometry to membrane proteins. J. Cell Biol. 160, 222–229
43. de Duve, C. (1983) Lysosomes revisited.
44. Kornfeld, S., and Mellman, I. (1989) The biogenesis of lysosomes.
45. Bagshaw, R. D., Mahuran, D. J., and Callahan, J. W. (2005) A proteomic analysis of lysosomal integral membrane proteins reveals the diverse composition of the organelle. Mol. Cell. Proteomics 4, 133–143
46. Hunziker, W., and Geuze, H. J. (1996) Intraacellular trafficking of lysosomal membrane proteins. Bioessays 18, 379–389
47. Luzio, J. P., Poupon, V., Lindsay, M. R., Mullock, B. M., Piper, R. C., and Pryor, P. R. (2003) Membrane dynamics and the biogenesis of lysosomes. Mol. Membr. Biol. 20, 141–154
48. Mullins, C., and Bonifacino, J. S. (2001) The molecular machinery for lysosome biogenesis. Bioessays 23, 333–343
49. Dell’Angelica, E. C., Mullins, C., Caplan, S., and Bonifacino, J. S. (2000) Lysosome-related organelles. FASEB J. 14, 1265–1278
50. Bonifacino, J. S., and Traub, L. M. (2003) Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu. Rev. Biochem. 72, 395–447
51. Dell’Angelica, E. C., Shotelersuk, V., Aguilar, R. C., Gahl, W. A., and Bonifacino, J. S. (1999) Altered trafficking of lysosomal proteins in Hermany-Pudlak syndrome due to mutations in the β 3 A subunit of the AP-3 adaptor. Mol. Cell 3, 11–21
52. Honing, S., Sandoval, I. V., and von Figura, K. (1998) A δ-tubulin-based motif in the cytoplasmic tail of LIMP-II and tyrosinase mediates selective binding of AP-3. EMBO J. 17, 1304–1314
53. Obermuller, S., Kiecke, C., von Figura, K., and Honing, S. (2002) The tyrosine motifs of Lamp 1 and LAP determine their direct and indirect targeting to lysosomes. J. Cell Sci. 115, 185–194
54. Jimbow, K., Park, J. S., Kato, F., Hirosaki, K., Toyofuku, K., Hara, S., and Itoyama, Y. (2003) Overexpression of high mobility group box-1 in SH-SYSY neuroblastoma cells that express tyrosinase. J. Neurochem. 87, 470–475
55. Muller, S., Scaffidi, P., Degryse, B., Bonaldi, T., Ronfani, L., Agresti, A., Beltrame, M., and Bianchi, M. E. (2001) New EMBO members’ review: the double life of HMG1B1 chromatin protein: architectural factor and extracellular signal. EMBO J. 20, 4337–4340
56. Jimbow, K., Hara, H., Vinayagamoorthy, T., Luo, D., Dakour, J., Yamada, K., Dixon, W., and Munoz, V. (1993) Isolation of a cDNA coding for a Ca 2+ -binding phosphoprotein (p90), calnexin, on melanomas in normal and malignant human melanocytes. Exp. Cell Res. 209, 288–300
57. Salopek, T. G., and Jimbow, K. (1996) Induction of melanogenesis during the various melanoma growth phases and the role of tyrosinase, lysosome-associated membrane proteins, and p90 calnexin in the melanogenesis cascade. J. Invest. Dermatol. Symp. Proc. 1, 195–202
58. Toyokofu, K., Wada, I., Hiroaki, K., Park, J. S., Hori, Y., and Jimbow, K. (1999) Promotion of tyrosinase folding in COS 7 cells by calnexin. J. Biochem. (Tokyo) 125, 82–89
59. Jimbow, K., Hara, H., Vinayagamoorthy, T., Luo, D., Dakour, J., Yamada, K., Dixon, W., and Chen, H. (1994) Molecular control of melanogenesis in malignant melanoma: functional assessment of tyrosinase and lamp gene families by UV exposure and gene co-transfection, and cloning of a cDNA encoding calnexin, a possible melanogenesis “chaperone.” J. Dermatol. 21, 894–906
60. Hasegawa, T., Matsuzaki, M., Takeda, A., Kikuchi, A., Furukawa, K., Shibahara, S., and Itoyama, Y. (2003) Increased dopamine and its metabolites in SH-SYSY neuroblastoma cells that express tyrosinase. J. Neurochem. 87, 470–475
61. Nagatsu, I., Ito, S., King, R. A., Nishimura, H., and Nagatsu, T. (1998) Does tyrosinase exist in neuromelanin-pigmented neurons in the human substantia nigra? Brain Res. Mol. Brain Res. 51, 198–200
62. Ogimoto, T., Komatsu, A. G., and Ohtani, Y. (2002) Structure of the retinal photocycle. J. Biochem. 132, 1720–1728
63. Zhou, Y., Gu, G., Goodlett, D. R., Zhang, T., Pan, C., Monteine, T. J., Monteine, K. S., Abelesbord, R. H., and Zhang, J. (2004) Analysis of α-synuclein-associated proteins by quantitative proteomics. J. Biol. Chem. 279, 39155–39164