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Difference in distribution of membrane proteins between low- and high-density secretory granules in parotid acinar cells

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Abstract

Secretory granules (SGs) are considered to be generated as immature granules and to mature by condensation of their contents. In this study, SGs of parotid gland were separated into low-, medium-, and high-density granule fractions by Percoll-density gradient centrifugation, since it was proposed that the density corresponds to the degree of maturation. The observation with electron microscopy showed that granules in the three fractions were very similar. The average diameter of high-density granules was a little but significantly larger than that of low-density granules. Although the three fractions contained amylase, suggesting that they are all SGs, distribution of membrane proteins was markedly different. Syntaxin6 and VAMP4 were localized in the low-density granule fraction, while VAMP2 was concentrated in the high-density granule fraction. Immunoprecipitation with anti-syntaxin6 antibody caused coprecipitation of VAMP2 from the medium-density granule fraction without solubilization, but not from Triton X-100-solubilized fraction, while VAMP4 was coprecipitated from both fractions. Therefore, VAMP2 is present on the same granules, but is separated from syntaxin6 and VAMP4, which are expected to be removed from immature granules. These results suggest that the medium-density granules are intermediates from low- to high-density granules, and that the membrane components of SGs dynamically change by budding and fusion during maturation.

Keywords: Parotid acinar cells; Secretory granules; Maturation; Syntaxin6; Vesicle-associated membrane proteins 2 (VAMP2)

In the *trans*-Golgi network (TGN), proteins are sorted and packaged into carrier vesicles for distinct destinations, such as plasma membrane, lysosome, or endosome. In addition to such organelles, the secretory granules (SGs) in cells that have regulated secretion systems such as neuroendocrine, endocrine, and exocrine cells are also the destinations of proteins. SG is a highly specialized storage organelle, from which secretory proteins are released upon stimulation. They have SNARE proteins which may mediate the fusion of granules to target plasma membrane. SGs are not only carriers of secretory proteins, but also machinery for signal-dependent exocytosis. The study of how granules are constructed is important for understanding the regulation of exocytosis.

Electron microscopy (EM) studies showed granules with low electron density in neuroendocrine, endocrine, and exocrine glands [1–4]. γ-Adaptin and procathepsin B, but not mature cathepsin B, are specifically localized at the granules, and vesicles were reportedly seen budding from them in pancreatic β-cells, pancreatic and parotid acinar cells [5]. The organelles that have a low electron density are believed to be immature granules (IMGs) in the process of maturing.

On the other hand, SGs can be separated by density-gradient centrifugation in biochemical studies. In PC12 cells, syntaxin6 and VAMP4 are specifically localized in

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low-density granule fractions [5,6]. It has been reported that pulse-labeled proteins were first detected in low-density fractions and were later detected in high-density fractions in parotid gland [7]. This suggests that newly synthesized proteins are transported from immature (low-density) to mature (high-density) granules, or a granule that includes the pulse-labeled proteins itself changed from an IMG to a mature granule (MG). Therefore, a granule is considered to be generated first as an IMG and then to mature gradually.

The morphology and final size of granules are diverse, depending on the cell type and the contents of the granules. While the size of the MGs in PC12 cells is about 120 nm, and that of granules of insulin in β-cells is 200 nm, salivary parotid or pancreatic acinar cells have much larger secretory granules about 1 μm in diameter. The biogenesis of SG has been well investigated in neuroendocrine cells. In PC12 cells, IMGs bud from the Golgi apparatus as small granules. The diameter of IMGs in neuroendocrine cells is about 80 nm, which is the same as that of coated vesicles [8], suggesting that coat proteins are involved in the budding of IMGs from TGN. The small IMGs fuse with each other in a process called "homotypic" fusion because granules of similar size and character fuse [9]. After the fusion, clathrin-coated vesicles bud from fused IMGs [4,10]. Homotypic fusion is considered to be necessary for the maturation of SG in neuroendocrine cells. In contrast, it is unlikely that IMGs in exocrine acinar cells are generated as coated vesicles. Large, irregularly shaped vacuoles are observed near the Golgi apparatus in EM images [1,2]. IMGs in exocrine cells, such as parotid and pancreatic acinar cells, are considered to first separate from the Golgi apparatus as organelles that have larger sizes than complete granules and to then mature. However, the mechanisms of generation and maturation of SG in exocrine cells have not been understood as well as in neuroendocrine cells, although many EM studies on the exocrine glands have been reported.

SGs in parotid acinar cells have a higher density than in other organelles and are easy to separate biochemically. The contamination of other organelles is very low [11], which is an advantage for the biochemical study of not only the regulatory mechanism of exocytosis, but also for the formation and maturation of granules. In this paper, we separated granules by density-gradient centrifugation according to the method reported by von Zastrow and Castle [7], and examined the distribution of membrane proteins to study the mechanisms of generation and maturation of SG in parotid acinar cells.

Materials and methods

Materials and antibodies. Antibodies to aquaporin5 (AQP5) and *N*-ethylmaleimide-sensitive factor (NSF) were from Chemicon (Temecula, CA) and Stressgen Biotechnologies, respectively. Antibodies against syntaxin6, α-soluble NSF attachment protein (α-SNAP), γ-adaptin, Munc18, GS28, BiP/GRP78, and EEA1 were purchased from BD Biosciences. Anti-Rab3D antibody was purchased from Alexis Biochemicals; antibodies against SNAP-23 and VAMP4 were from Affinity Bioreagents (Deerfield, IL); anti-α-amylase antibody from Sigma. Anti-VAMP2 antibody (anti-SER4253) was prepared as reported previously [12]. Anti-pantophysin antibody was a kind gift from Dr. B. Cheatham (Harvard Medical School), and anti-Rab3A antibody was kindly given by Dr. M. Takahashi (Kitasato University School of Medicine, Japan). Botulinum neurotoxin B (BNT-B) was kindly given by Dr. S. Kozaki (Osaka Prefecture University, Japan).

Separation of SG fractions and preparation of precipitates of SG fractions by centrifugation. The SGs of the rat parotid glands were isolated as described [7], but with a modification. The parotid glands were taken from male Sprague–Dawley rats (200–250 g) anesthetized by sodium pentobarbital (Dainippon Pharmaceutical Co. Ltd, Osaka, Japan). When rats were injected with 0.4 mg isoproterenol (IPR), parotid glands were taken at 8 h after the injection. Homogenization was performed with buffer A (300 mM sucrose, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1× Complete EDTA-free, and 20 mM MOPS·NaOH, pH 7.0). A part of the postnuclear supernatant was collected, homogenized with low osmotic buffer, added NaCl to 150 mM, and centrifuged at 100,000*g* for 60 min. The pellet was collected as P100 fraction.

The rest of the postnuclear supernatant was centrifuged in 40% Percoll at 16,400*g* for 30 min. The heaviest fraction was collected and recentrifuged in 60% Percoll at 45,500*g* for 30 min. Granule fractions in Percoll-sucrose medium were collected in following three densities: low (1.115–1.125 g/ml), medium (1.125–1.14 g/ml), and high (1.14–1.16 g/ml). Purified granules (Go) were suspended in buffer B (1 mM $MgCl_2$, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1× Complete EDTA-free, and 20 mM Hepes·NaOH, pH 7.4), homogenized, added NaCl to 150 mM, and centrifuged at 100,000*g* for 60 min. The precipitate was used as the granule precipitate fraction (Gp).

Lysosome-rich fraction was prepared as described by Maguire and Luzio [13]. The fraction was homogenized with buffer B, added NaCl to 150 mM, and centrifuged at 100,000*g* for 60 min. The precipitate was suspended in buffer B containing 150 mM NaCl.

Electron microscopy. A pellet of the freshly isolated granules was fixed in modified Karnovsky's fixative solution (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) for 1 h at room temperature. Samples were postfixed in 1% osmium tetraoxide for 60 min, dehydrated with a graded series of ethanol and propylene oxide, and embedded in Epon812. Ultrathin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and then examined with a transmission electron microscope (JEOL JEM-1010).

Particle size analysis by photon correlation spectroscopy. The sizes of SGs were measured by the photon correlation spectroscopy instrument N5 (Beckman Coulter, USA). SGs were suspended in 1 ml of buffer A and transferred to a plastic cuvette at room temperature. The cuvette was set in N5 and a 25 mV Helium–Neon laser beam was directed at it, and scattering light at an angle of 90° was analyzed.

Immunofluorescence microscopy. Granules were attached to coverslips by Cell-TAK. After fixation with 10% formalin/PBS, granules were permeabilized with 0.2% Triton X-100/PBS, blocked with 1% BSA-0.05% goat IgG, and labeled with anti-amylase antibody (1:50) followed by Alexa Fluor® 488-anti-rabbit IgG. Fluorescence images were acquired by μRadiance MR/AG-2/S confocal microscopy (Bio-Rad).

Immunoprecipitation from solubilized Gp fractions. Gp fractions were suspended in solubilizing buffer C (150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 20 mM Hepes·NaOH, pH 7.4) containing 4% Triton X-100 and incubated for 45 min. Unsolubilized materials were removed by centrifugation (20,000*g* for 15 min). The supernatants were dialyzed against buffer C containing 1% Triton X-100 overnight. After centrifugation at 20,000*g* for 15 min, the supernatants were obtained as solubilized Gp fractions. Solubilized Gp fractions (20 μg) were suspended in reaction buffer (150 mM NaCl, 20 mM NaF, 10 mM $MgCl_2$, 2 mM ATP, 1 mM PMSF, 1 mM DTT, 1 mM EGTA, 1% Triton X-100, and 20 mM Hepes·NaOH, pH 7.4). Samples were added to protein A-Sepharose 4FF conjugated with normal mouse or rabbit IgG and were incubated at 4 °C for 60 min. After centrifugation, the supernatants were recovered and incubated with anti-syntaxin6 antibody conjugated with