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Secretory proteins without a transport signal are retained in secretory granules during maturation in rat parotid acinar cells

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ABSTRACT

Objective: The acinar cells of the parotid gland are filled with numerous secretory granules (SGs), which accumulate the digestion enzyme amylase. SGs mature accompanied with membrane remodelling such as fusion and budding of small vesicles. However, little is understood about the mechanism of the condensation of SG contents during maturation. In this study, we examined whether secretory proteins need a specific signal to be retained in SGs.

Design: To induce internalization of the luminal membrane after exocytosis, we injected the β-adrenergic agonist isoproterenol into rats. Acinar cells were then incubated with Lucifer Yellow (LY) dye as a tracer for 3 h for uptake into immature secretory granules (ISGs). To observe whether LY was retained in SGs after maturation, we continued incubating the cultured acinar cells for 2 days.

Results: The localization of LY into ISGs was confirmed by the following four methods: (1) co-localization of the fluorescence of LY and amylase by confocal laser microscopy, (2) detection of the fluorescence from purified ISGs, (3) secretion of the fluorescence together with amylase upon stimulation, and (4) observation of the intracellular localization of LY by electron microscopy. Moreover, we observed co-localization of some of the SGs with the fluorescence of LY after cell culture.

Conclusions: Although the fusion and budding of small vesicles may contribute to the process of granule maturation, LY remained in the SGs even after maturation. These results suggest that secretory proteins that have no transport signal are not excluded from SGs, and they are retained in SGs during granule maturation in exocrine parotid glands.

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1. Introduction

Secretory proteins are synthesized in the endoplasmic reticulum (ER), and then they are sorted to secretory granules (SGs) from the *trans*-Golgi network via the Golgi complex.[1] SGs immediately after formation are termed immature secretory granules (ISGs), and they become SGs to prepare for exocytosis. The process of granule maturation comprises transport to the apical side along microtubules,[2] homotypic fusion,[3] condensation and acidification,[4] processing of contents,[5] and remodelling of the membrane by budding and fusion of small vesicles.[6]

Parotid glands, one of the exocrine glands, release the digestion enzyme amylase into the saliva. The diameter of each SG is around 1 μm, and therefore they can be clearly observed by light microscopy. Thus, using parotid acinar cells is an advantage for the investigation of granule maturation. It has been reported that the density of SGs increases with the degree of maturation by tracer examination.[7] We have separated low-density granules (LDGs) from high-density granules (HDGs) of parotid glands using Percoll density gradient centrifugation. Syntaxin 6, vesicle-associated membrane protein 4 (VAMP4), and γ-adaptin were concentrated in the membrane fraction of LDGs, whereas VAMP2 was concentrated in HDGs,[8] suggesting that remodelling of the membrane occurred by clathrin transport vesicles during granule maturation.

To examine the time-dependent differences of membrane components during maturation, we injected isoproterenol (IPR) into the rat abdominal cavity.[9] Newly formed SGs were observed in acinar cells 5 h after the injection. Compared with before the injection of IPR, the distribution of newly formed SGs was shifted to the low-density fraction when purified by Percoll density gradient centrifugation. The diameter of newly formed SGs was about one-half the size of mature SGs. Syntaxin 6, VAMP4, and γ-adaptin were concentrated in the membranes of newly formed SGs. These characteristics of newly formed SGs were consistent with LDGs. Furthermore, their characteristics changed to be similar to HDGs at 8 h after the injection of IPR. This was consistent with a previous report that ISGs are converted to SGs with a half-time of approximately 45 min.[10] Both newly formed granules and LDGs may be ISGs. These results suggest that the remodelling of membrane components occurs within 3 h after granule formation.

We wondered how secretory proteins are retained in SGs during maturation, although components of the membrane dynamically change during that process. We expressed HaloTag fused to a signal peptide sequence of amylase that was composed of 25 amino acids (SS25H) in primary cultures of parotid acinar cells.[11] Localization of SS25H was observed in SGs of those cells, and secretion of SS25H was enhanced following stimulation with IPR. However, because it was too difficult to separate ISGs from SGs and to distinguish ISGs from SGs by confocal microscopy, we were not able to examine SG maturation in the cultured acinar cells.

In this study, we synchronized the degree of SG maturation by injection of IPR in vivo to resolve those problems. Simultaneously, a tracer, Lucifer Yellow (LY), was incubated to internalize into ISGs via the endocytosis pathway. LY indicates the contents of SGs because it is a fluorescent dye that has no transport signal and has neither permeability nor affinity to a membrane. Finally, we show the fluorescence of LY in SGs after granule maturation, which suggests that a specific signal of secretory proteins is not necessary for them to be retained in SGs during the maturation of rat parotid acinar cells.

2. Materials and methods

2.1. Reagents and antibodies

Anti-α-amylase polyclonal antibody (anti-amylase pAb), foetal bovine serum (FBS), and hyaluronidase were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Alexa Fluor 568 anti-rabbit immunoglobulin G (IgG), LY potassium salt, DiI, Dulbecco's modified Eagle's medium (DMEM)/F-12 medium, Insulin-Transferrin-Selenium-X supplement (ITS-X), and the metal-enhanced diaminobenzidine (DAB) substrate kit were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Percoll and Triton X-100 (TX-100) were from GE Healthcare UK Ltd. (Buckinghamshire, UK). Complete ethylenediaminetetraacetic acid (EDTA)-free was from F. Hoffmann-La Roche, Ltd. (Basel, Switzerland) and collagenase type 2 was from Worthington Biochemical Corp. (Lakewood, NJ, USA).

2.2. Dispersion of parotid glands and primary culture of acinar cells

Animal protocols were approved by the Nihon University Animal Care and Use Committee. Male Sprague-Dawley rats (200–250 g each) were injected with IPR (5.5 mg/kg) into the abdominal cavity. Parotid glands were removed from rats anesthetized with sodium pentobarbital (50 mg/kg) 2 h after the injection of IPR. Parotid glands were treated with collagenase (1.4 mg/ml) and hyaluronidase (0.2 mg/ml) in Krebs-Ringer bicarbonate buffer (KRB buffer; 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 0.96 mM NaH_2PO_4, 25 mM $NaHCO_3$, 5 mM HEPES, pH 7.4, and 11.1 mM glucose) with 0.5% bovine serum albumin (BSA) as described previously.[12] In our experiments, KRB buffer including 0.2 mM LY was used during dispersion. The viability of cells was >90% as determined by trypan-blue exclusion.

For primary culture, acinar cells that have no SGs after exocytosis were cultured in DMEM/F-12 medium (containing 10% FBS, ITS-X supplement, 1 μM hydrocortisone, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10 nM cystatin) including 1 mM LY. They were cultured on glass-base dishes coated with collagen I (Iwaki, Japan) at 37 °C under 95% O_2/5% CO_2.[13] On the second day, the medium was changed to fresh medium without LY. We observed the localization of LY and amylase in the cells using confocal laser microscopy on the third day.

2.3. Purification of ISGs and isolation of granule contents

ISGs were purified using 57% Percoll density gradients as described previously.[14] Briefly, homogenization of parotid glands was performed in buffer H (300 mM sucrose, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1× complete EDTA-free, and 20 mM MOPS-NaOH, pH 7.0). The postnuclear supernatant was centrifuged twice with Percoll at 16,400 × *g* for 30 min. Purified ISGs were collected by centrifugation at 4270 × *g* for 10 min.