Syntaxin 6 is Involved in the Maintenance of Secretory Granules in Parotid Acinar Cells

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Abstract
Secretory granules (SGs) of salivary glands are considered to be generated as an immature granule and to mature by condensing their contents. Syntaxin 6 and vesicle associated membrane protein 4 (VAMP4) are detected in the membrane fractions of immature but not mature SGs, suggesting that syntaxin 6 and VAMP4 are transported from SGs to other organelles during the process of granule maturation. To study the role of syntaxin 6 in the biogenesis of granules, we transfected syntaxin 6 fused with enhanced green fluorescent protein (EGFP) into primary cultured parotid acinar cells. The EGFP-syntaxin 6 signal overlapped with that of an early endosome marker, early endosome antigen 1 (EEA1), which was detected using immunofluorescence. The localization of EGFP-fused VAMP4 was also similar to that of EEA1, suggesting that syntaxin 6 and VAMP4 are transported from immature SGs to early endosomes. Transfection of a syntaxin 6 mutant that lacks a SNARE-binding domain caused a decrease in the number of SGs although the localization of the mutant was unchanged from that of the wild type syntaxin 6. These results suggest that syntaxin 6 has an important role in the maintenance of SGs in parotid acinar cells.

Keywords:
salivary glands, secretory granules, syntaxin 6, VAMP4, early endosomes

Introduction
Secretory granules (SGs) of parotid acinar cells are generated in the trans-Golgi network (TGN) as immature SGs and mature by condensation of their contents. Since the density of mature granules is higher than that of immature granules, mature and immature granules can be separated by density-gradient centrifugation (1). To study the mechanism of the generation and maturation of SGs in parotid acinar cells, we previously examined the presence of membrane proteins in the SG fractions prepared by Percoll-density gradient centrifugation. We found that the distribution of membrane proteins among the fractions is dramatically different (2). Syntaxin 6 and vesicle associated membrane protein 4 (VAMP4) were abundant in immature granule fractions while they were not detected in mature granule fractions. Instead, the concentration of VAMP2 was higher in high-density fractions in matured granules. Syntaxin 6 and VAMP4 may be transported from immature SGs to other organelles during the process of SG maturation (3).

Syntaxin 6 has been reported to be involved in multiple membrane fusion events. Syntaxin 6 mediates homotypic fusion of immature SGs in chromaffin cells, which is required for the maturation of SGs (4). Dominant-negative syntaxin 6 suppressed post-Golgi trafficking of vascular endothelial growth factor receptor and decreased its intracellular pool in endothelial cells (5). Syntaxin 6 regulates exocytosis of tumor necrosis factor-α in macrophages (6) and insulin-responsive recycling of glucose transporter 4 in muscle cells (7). The delivery of some lipids and proteins from the TGN to plasma membranes is controlled by syntaxin 6 (8). Although inhibition of syntaxin 6 function did not interfere with regulated and constitutive secretion in cultured pancreatic β-cells (9), an antibody against syntaxin 6 prevented granule exocytosis in neutrophils (10). Although

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syntaxin 6 has various functions, which may be cell-type specific, a role for syntaxin 6 in exocrine cells has not been reported.

Stable cell lines of salivary acinar cells that generate SGs and retain stimulus-dependent secretion have not been established. SGs of salivary acinar cells are so large that maintaining these granules seems incompatible with immortalization. The lack of stable salivary acinar cell lines is a major obstacle to carrying out molecular studies of salivary acinar cells. We have established a system for primary culture of parotid acinar cells (11). These cells retain SGs and stimulus-dependent secretion of amylase for at least 3 days. Furthermore, we have constructed a VAMP2 and enhanced green fluorescence protein (EGFP) fusion protein, which enabled clarification of the localization of secretory granules in these primary cultured cells (11). In the present study, we constructed VAMP4-EGFP and wild type and mutant syntaxin 6-EGFP fusion proteins to investigate the behavior and function of syntaxin 6 in primary cultured parotid acinar cells.

Materials and Methods

Vector construction
cDNA fragments encoding syntaxin 6 (Accession No. NM_031665.2) and VAMP4 (Accession No. NM_001108856.1) were amplified using the polymerase chain reaction (PCR) and subcloned into the pEGFP-C1 vector (Clontech, Mountain View, CA). The primer sets used for amplification of the genes were: 5’-GGA ATT CCA TGT CCA TGG AGG ACC CCT TC-3’ and 5’-CGG GAT CCT CAC AGC ACT AGG AGG GTC AG-3’ for syntaxin 6, and 5’-GGA ATT CTG TGC CTC CCA AGT TCA AGC G-3’ and 5’-CGG GAT CCT CAA GTA CGG AAT TTC ACA ACT ATA AG-3’ for VAMP4. An N-terminal deletion mutant of syntaxin 6, stx6ΔN (Δ1-130), was amplified by PCR of the same cDNA with the primers 5’-GGA ATT CCA TTG GGA TGC TCA AGC GGA T-3’ and 5’-CGG GAT CCT CAC AGC ACT AGG AGG GTC AG-3’ and was subcloned into the EcoRI-BamHI site of pEGFP-C1. Another deletion mutant of syntaxin 6, stx6ΔC (Δ143-233), was constructed by deletion of the Nari-Nari region of pEGFP-stx6. The EGFP sequence is located at the 3’ end of each gene. pEYFP-Golgi was purchased from Clontech.

Culture of rat parotid primary cells and transfection
Parotid glands were taken from male Sprague-Dawley rats (150-200 g each) under anesthesia. This experiment conformed with the Institutional Guidelines for the use of experimental animals, and was approved by the Experimental Animal Ethical Committee of the Nihon University School of Dentistry at Matsudo. Acinar cells were isolated from the glands and were cultured as previously reported (11). For gene transfection, plasmids and the DMRIE-C transfection reagent (Thermo Fisher Scientific, Waltham, MA) were mixed and kept at room temperature for 30 min in OPTI-MEM I reduced serum medium (Thermo Fisher Scientific). After incubation, the isolated parotid acinar cells were mixed with the reagent and were plated on collagen I-coated glass-based dishes (Iwaki, Tokyo, Japan). After incubation at 37 °C in 5% CO2 for 4 h, Waymouth’s medium (Thermo Fisher Scientific) containing 10% rat serum was added to each dish and the cells were cultured. The efficiency of transfection was checked by counting the number of cells that expressed EGFP under a fluorescent microscope.

Immunofluorescence microscopy
After culture for 48 h, the cells were fixed with 10% formalin in Phosphate-buffered saline (PBS) and permeabilized with 0.2% Triton X-100 in PBS. After blocking with 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) and 0.05% preimmune goat IgG (Sigma-Aldrich), the cells were labeled with mouse monoclonal anti-syntaxin 6 (BD Biosciences, San Jose, CA) or anti-EEA1 (BD Biosciences) antibodies, or with rabbit polyclonal anti-amylase (Sigma-Aldrich) antibodies followed by the appropriate secondary Alexa Fluor-conjugated antibodies. Fluorescence images were acquired with μRadiance MR/AG-2/S confocal microscopy (Bio-Rad, Hercules, CA). The number of SGs that had a diameter greater than 250 nm was counted. When the signals of SGs overlapped with each other, we measured the area of the SGs present and divided it by the estimated area of the cross section of the SGs, assuming that the average diameter of a SG is 0.5 μm.

Results
The localization of syntaxin 6 fused with EGFP in parotid acinar cells in primary culture
To investigate the localization of syntaxin 6 in parotid acinar cells, an EGFP-fused syntaxin 6 (EGFP-stx6) gene was transfected into acinar cells in primary culture. We previously reported that these acinar cells in primary
culture generate new SGs after transfection of exogenous genes (11). Immunofluorescent analysis of the localization of EGFP-stx6 at 2 days after transfection indicated that EGFP-stx6 was present in patches similar to endogenous syntaxin 6 (Figs. 1A and 1B). These patches were not SGs because they showed little overlap with the staining pattern of an anti-amylase antibody. Syntaxin 6 was previously reported to be localized in the Golgi apparatus, especially at the TGN, in some cell types (12–14). To examine whether syntaxin 6 is localized in the Golgi in parotid acinar cells, pEYFP-Golgi, which encodes a fusion protein of enhanced yellow fluorescent protein (EYFP) and the N-terminal region of human β1,4-galactosyltransferase (15), which is considered as a Golgi marker, was transfected. The pattern of fluorescence of EGFP-stx6 partially overlapped with that of EYFP-Golgi (data not shown). Thus, most of the patches of syntaxin 6 may not indicate either the Golgi apparatus or in SGs, but may be other compartments such as intracellular vesicles. Localization of syntaxin 6 at early endosomes (EEs) has also been reported (14). The immunofluorescent staining pattern of EEA1, an early endosome marker, showed a good overlap with EGFP-stx6 (Fig. 1C) indicating that syntaxin 6 is mainly localized in early endosomes. Transfected EGFP-VAMP4 also overlapped with EEA1 staining (Fig. 1D). These results suggested that syntaxin 6 and VAMP4 were translocated from immature SGs to EEs as a complex and that they remained in EEs in a stable manner.

**Mutation of syntaxin 6 decreased the number of SGs in parotid acinar cells**

To investigate the role of syntaxin 6 in parotid acinar cells, genes encoding syntaxin 6 mutants fused with EGFP were transfected into the primary culture of parotid acinar cells. Syntaxin 6 contains two predicted helical domains, H1 and H2, which are considered to form coiled coil interactions with α-SNAP and SNARE proteins, respectively (Fig. 2D) (13). Two kinds of syntaxin 6 deletion mutants were constructed: one that lacks the H1 domain (stx6ΔN) and another that is an H2 domain-deleted mutant (stx6ΔC). Although the transfection efficiency was not high, cells that expressed the exogenous genes were easily recognized by fluorescence detection of EGFP expression. To visualize SGs, cells were fixed and stained with an anti-amylase antibody. On the first day after transfection, there was no apparent difference between cells expressing the syntaxin 6 wild type EGFP-stx6 and cells expressing either of the two syntaxin 6 mutants (data not shown). The transfected cells were round in shape and contained granules similar to those in the cells that did not express EGFP. However, at 2 days after transfection, most of the cells expressing EGFP-stx6ΔC contained fewer SGs than cells that were not transfected with exogenous genes or than cells expressing EGFP-stx6 or EGFP-stx6ΔN (Figs. 2A–C). Quantification of the number of SGs in the cells expressing EGFP-stx6ΔC confirmed that the number of SGs in these cells was significantly decreased compared with that in the cells transfected with other genes (Fig. 2E). Although the cells that were transfected with wild type syntaxin 6 or with the other stx6 deletion mutant, stx6ΔN, also showed a decrease in the number of SGs in comparison to non-transfected cells, the degree of this decrease was the same as that of the cells expressing EGFP alone. Therefore, the reason for the decrease in SGs in cells expressing full-length syntaxin 6 or stx6ΔN was due to expression of the exogenous EGFP gene, but not to expression of the syntaxin 6 gene. These data suggest that syntaxin 6 has a role in maintaining the cellular number of SGs. The cellular distribution of EGFP-stx6ΔC was not different from that of full-length syntaxin 6.

**Discussion**

In this study, both the signals of EGFP-fused syntaxin 6 and VAMP4 overlapped with EEA1 signals, which suggests that most of the syntaxin6 and VAMP4 are localized in EEs. We previously reported that syntaxin 6 and VAMP4 form a complex on the membrane of immature SGs (2). It is likely that syntaxin 6 and VAMP4 were transported from immature SGs to EEs as a complex during maturation of the SG. Although we could not discriminate between immature and mature SGs using immunofluorescence microscopy, immature SGs can be stained by anti-amylase antibody. Therefore, some of the signals of syntaxin 6 and VAMP4 that overlapped with amylase signals may indicate their presence on immature SGs.

The two helical domains of syntaxin 6 are considered to interact with α-SNAP and SNARE proteins, respectively (13). Various SNARE proteins have been reported to interact with syntaxin 6. α-SNAP recruits the N-ethylmaleimide-sensitive factor and consequently mediates dissociation of the SNARE complex. Both helical domains of syntaxin 6 may be essential for intracellular membrane transport. In addition, interactions of syntaxin 6 with other proteins have been reported. EEA1, which is an early
Fig. 1. Immunofluorescent analysis of the cellular localization of endogenous and exogenous syntaxin 6. A: Parotid acinar cells in primary culture were labeled with an anti-syntaxin 6 antibody followed by Alexa Fluor 568-anti-mouse IgG (green), and with an anti-amylase antibody followed by Alexa Fluor 488-anti-rabbit IgG (red). B: Parotid acinar cells were transfected with pEGFP-stx6 (green) and stained with an anti-amylase antibody followed by Alexa Fluor 568-anti-rabbit IgG (red). C: Acinar cells were transfected with pEGFP-stx6 (green) and stained with an anti-EEA1 antibody followed by Alexa Fluor 568-anti-mouse IgG (red). D: Acinar cells were transfected with pEGFP-VAMP4 (green) and stained with an anti-EEA1 antibody followed by Alexa Fluor 568-anti-mouse IgG (red). Bar, 5 μm.
Fig. 2. The effect of transfection of EGFP-fused syntaxin 6 mutants on parotid acinar cells in primary culture. A-C: Immunofluorescence microscopy of parotid acinar cells in primary culture. Acinar cells were transfected with pEGFP-stx6 (A), pEGFP-stx6ΔC (B), or pEGFP-stx6ΔN (C). At 2 days after transfection, the cells were stained with an anti-amylase antibody followed by Alexa Fluor 568-anti-mouse IgG and fluorescent signals of amylase (red) and EGFP (green) were observed. Bar, 10 μm. D: The structure of syntaxin 6 mutants. The H1 domain is an α-SNAP-binding domain and the H2 domain is a SNARE-binding domain. TM, transmembrane domain. E: The number of granules per cell was counted and is shown in a distribution graph. The number of granules in more than 30 cells was counted for each transfection. The number of SGs in the cells transfected with pEGFP-stx6ΔC was significantly fewer than in that in the pEGFP-stx6-transfected cells (p < 0.01, ANOVA and Dunnett’s test).
endosomal marker and is important for endosomal trafficking, directly interacts with syntaxin 6 (14). The N-terminal side of syntaxin 6 binds to the Golgi-associated retrograde protein (16) and is essential for localization of the Golgi apparatus (17). In the present study, deletion of the H2 domain from syntaxin 6 decreased the number of SGs while deletion of the H1 domain seemed to have no effect on SG number. This result does not mean that the H1 domain is not essential for SG biogenesis. It is possible that, following deletion of the H2 domain, the remaining H1 domain might interact with a certain target protein and thereby suppress its function.

There are two possible explanations for the decrease in SGs in stx6ΔC-expressing cells. One is that exocytosis of amylase was enhanced by expression of stx6ΔC, and the generation of SGs lagged behind. Tlg1, a yeast homolog of syntaxin 6, was reported to suppress cis-Golgi fusion by forming a non-fusogenic SNARE complex (18). Syntaxin 6 can bind to both VAMP2 and VAMP4, and VAMP2 probably forms another fusogenic SNARE complex, which is essential for cAMP-dependent amylase secretion in parotid acinar cells (19). Interaction of syntaxin 6 with VAMP2 probably inhibits exocytosis, and interaction of syntaxin 6 with VAMP4 hinders the binding between syntaxin 6 and VAMP2 and consequently leaves VAMP2 free to form the fusogenic SNARE complex. There is a possibility that deletion of the H2 domain suppressed the interaction of syntaxin 6 and VAMP2 and enhanced the exocytosis of immature SGs. Another possibility is that the generation of new SGs was suppressed. Syntaxin 6 and VAMP4 are considered to be involved in TGN/endosome recycling (9, 20). The H2 domain was reported to be required for syntaxin 6 localization in the TGN (17). Although there was no apparent difference between the localization of EGFP-stx6ΔC and the wild type syntaxin 6, the deletion in stx6ΔC may interfere with its return to the Golgi. Syntaxin 6 probably transports some essential factors for generation of new SGs from EEs to the TGN, which may be inhibited by the stx6ΔC mutant.

Although syntaxin 6 binds to VAMP4 on immature granule membranes, its binding partner may be different in other organelles. Identification of the target protein that interacts with syntaxin 6 in the TGN or EE will provide information regarding the mechanism by which SGs are maintained in parotid acinar cells.

Conflicts of interest
The authors have no potential conflicts of interest.

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