Synaptotagmin V Is Targeted to Dense-core Vesicles That Undergo Calcium-dependent Exocytosis in PC12 Cells*

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Synaptotagmins (Syts) III, V, VI, and X are classified as a subclass of Syt, based on their sequence similarities and biochemical properties (Ibata, K., Fukuda, M., and Mikoshiba, K. (1998) J. Biol. Chem. 273, 12267–12273; Fukuda, M., Kanno, E., and Mikoshiba, K. (1999) J. Biol. Chem. 274, 31421–31427). Although they have been suggested to be involved in vesicular trafficking, as in the role of the Syt I isoform in synaptic vesicle exocytosis, their exact functions remain to be clarified, and even their precise subcellular localization is still a matter of controversy. In this study, we established rat pheochromocytoma (PC12) cell lines that stably express Syts III, V, VI, and X-GFP (green fluorescence protein) fusion proteins, respectively, to determine their precise subcellular localizations. Surprisingly, Syts III-, V-, VI-, and X-GFP proteins were found to be targeted to specific organelles: Syt III-GFP to the plasma membrane, Syt V-GFP to dense-core vesicles, Syt VI-GFP to endoplasmic reticulum-like structures, and Syt X-GFP to vesicles (other than dense-core vesicles) present in cytoplasm. We showed that Syt V-containing vesicles at the neurites of PC12 cells were processed to exocytosis in a Ca\textsuperscript{2+}-dependent manner. Immunohistochemical analysis further showed that endogenous Syt V was also localized on dense-core vesicles in the mouse brain and specifically expressed in glucagon-positive α-cells in mouse pancreatic islets, but not in β- or δ-cells. Based on these results, we propose that Syt V is a dense-core vesicle-specific Syt isoform that controls a specific type of Ca\textsuperscript{2+}-regulated secretion.

Regulated vesicle trafficking is utilized for diverse cellular processes, including secretion of peptide hormones, neurotransmitter release, outgrowth of neurites in neurons, egg fertilization, and plasma membrane repair, and it is often triggered by Ca\textsuperscript{2+} ions. Synaptic vesicle exocytosis, one of the well-characterized forms of regulated vesicle trafficking, is strictly regulated by a rapid increase in Ca\textsuperscript{2+} ions (10–200 μM) entering through voltage-gated Ca\textsuperscript{2+} channels (1–4). Although the precise mechanism by which synaptic vesicles sense such rapid increases in Ca\textsuperscript{2+} ions remains unclear, synaptotagmin (Syt) I, a Ca\textsuperscript{2+} - and phospholipid-binding protein in synaptic vesicles, has been shown to regulate Ca\textsuperscript{2+}-dependent neurotransmitter release (for review, see Refs. 5–8) and has therefore been proposed to be a major “Ca\textsuperscript{2+} sensor” for neurotransmitter release (for review, see Refs. 9 and 10).

Syt constitutes a large family in both vertebrates and invertebrates, and at least 13 members have been described in the rat, mouse, and human (8, 11–13 and references therein). Syt protein is basically composed of an intravesicular N terminus, a single transmembrane domain, and tandem C2 domains (named the C2A domain and C2B domain) in the cytoplasmic region (5–8). We demonstrated previously that Syt members can be classified into several groups based on their biochemical properties (14–21), gene structure (8, 11, 22), and sequence similarity (12, 17) and suggested that there is functional diversity among Syt family members with regard to vesicular trafficking (14). Consistent with this notion, several Syt isoforms (e.g. Syts III, IV, VI, and VII) are localized at membranes other than classical secretory vesicles and regulate distinct types of Ca\textsuperscript{2+}-dependent exocytosis (e.g. lysosomal exocytosis, plasma membrane repair, and acrosomal reaction) (23–33).

Syts III, V, VI, and X belong to the same branch in the phylogenetic tree and are classified as a subclass of Syt characterized by having almost no ability to bind inositol 1,3,4,5-tetakisphosphate (17) and an N-terminal Cys cluster responsible for dimer formation via disulfide bonding (12, 19). However, the exact functions and subcellular localization of these Syts are still a matter of controversy. For instance, Südhof and co-workers (8) recently proposed that Syts III and VI function as plasma membrane Ca\textsuperscript{2+} sensors, whereas others have shown that Syt III is localized on insulin-containing vesicles (34–36) and that Syt VI is attached to various membrane fractions (23, 24). Therefore, it is quite important to determine the exact localization of Syts to understand the functional diversity or functional redundancy of these Syt isoforms (III, V, VI, and X).

In this study, we investigated the subcellular distributions of the subclass of Syt (III, V, VI, and X), focusing especially on Syt V, which has never been characterized. Two different methods (stable expression of green fluorescence protein (GFP)-tagged Syt in PC12 cells and subcellular fractionation of brain) demonstrated that Syt V is localized predominantly on dense-core vesicles but that others are not enriched in these vesicles. In addition, Syt V was found to be specifically expressed in pancreatic α-cells. Based on our findings, we propose that Syt V is

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‡ The abbreviations used are: Syt(s), synaptotagmin(s); Ab, antibody; GFP, green fluorescence protein; GST, glutathione S-transferase; NGF, nerve growth factor; PBS, phosphate-buffered saline.
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EXPERIMENTAL PROCEDURES

Construction of the Expression Vectors—The cDNA fragments encoding Syts III, V, VI, and X were prepared as described previously (12, 14). Construction of the expression vector encoding FLAG-Syt-GFP (named pHShooter-FLAG-Syt-GFP) (Invitrogen, Carlsbad, CA) was essentially carried out by PCR as described elsewhere (see Fig. 1A) (12, 37). Briefly, the 5′-end of each Syt cDNA was ligated to the 5′-end of GFP cDNA to encode a fusion protein of Syt-GFP, and the sequences encoding the FLAG tag were inserted into the 5′-end of each Syt cDNA. A glycine linker was inserted between Syt and GFP to minimize interactions affecting the proper folding of the C2B domain (38). The cDNA fragments encoding these fusion proteins were subcloned into the NotI site of the modified pHShooter vector (37) and verified by DNA sequencing as described previously (12).

Screening of Cell Lines Stably Expressing FLAG-Syt-GFP Protein—PC12 cells (2 × 10^6 cells, the day before transfection) were cultured on 10-cm dishes in Dulbecco’s modified Eagle’s medium containing 10% horse serum and 10% fetal bovine serum at 37 °C under 5% CO_2_. The expression vectors encoding the Syt-GFP fusion protein were transfected with LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer’s instructions. The transfected cells expressing neomycin-resistant gene were selected using Geneticin (Invitrogen) at a concentration of 400 μg/ml. Production of Syt-GFP fusion protein in each established cell line was verified by both immunoblot and immunocytochemical analyses.

Immunocytochemistry—The cloned PC12 cells (5 × 10^4 cells/35-mm dish) stably expressing each Syt-GFP fusion protein were cultured with or without 100 ng/ml nerve growth factor (NGF) (Merck KGaA, Darmstadt, Germany) for 3 days. After washing twice with phosphate-buffered saline (PBS), the cells were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 20 min at room temperature, and the fixation was stopped with 0.1 M glycine. The cells were then permeabilized with 0.3% Triton X-100 in PBS for 1 h at room temperature and then incubated with Alexa 568-conjugated anti-mouse IgG (1/5,000 dilution, Molecular Probes, Eugene, OR) for 1 h at room temperature. Immunoreactivity was analyzed with a confocal microscope (Fluoview, OLYMPUS, Tokyo, Japan) and Adobe Photoshop. Studded with the homogeneous buffer, the immunoprecipitates were subjected to 7.5% SDS-PAGE followed by immunoblotting with anti-Syt VAC2AB Ab.

Immunohistochemistry—An ICR adult mouse was perfused with 4% paraformaldehyde, and the brain was excised and postfixed in the same fixative. It was cryosectioned into 10-μm thick sections, dried, and rehydrated in PBS, and fixed in 4% paraformaldehyde. After permeabilization in methanol for 30 min at −20 °C, the sections were incubated in 2% skim milk, 0.1% Triton X-100, and 0.1% donkey serum in PBS for 1 h at room temperature and then with the following primary Abs: 5 μg/ml anti-Syt VAC2AB Ab (described above), 10 μg/ml anti-glucagon goat Ab (Santa Cruz Biotechnology, Inc.), anti-insulin guinea pig Ab (1/1 dilution, Zymed Laboratories, Inc.), or 2 μg/ml anti-somatostatin goat Ab (Santa Cruz Biotechnology, Inc.). Positive immunoreactivity was visualized with the following secondary Abs: Alexa 488-conjugated antirabbit IgG, Alexa 594-conjugated anti-goat IgG, or Alexa 568-conjugated anti-guinea pig IgG (1/1,000 dilution, Molecular Probes).

RESULTS

To investigate the subcellular localization of the subclass of Syts (Syts III, V, VI, and X) (12, 17), we constructed expression vectors encoding each Syt protein with the C-terminal GFP tag (Fig. 1A) to detect its expression by fluorescence because N-terminal GFP tags have sometimes resulted in incorrect localization of certain isoforms. Because PC12 cells are often used to study Ca^2+^-regulated exocytosis (41–46) and are known to express Syts I, III, IV, and IX (25, 41–43, 47, 48), we selected PC12 cells for our initial study. Although the Syt family lacks a signal peptide sequence and should contain a specific mem-

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brane targeting signal itself (49), transient overexpression of some Syt-GFP proteins (especially Syt V) in PC12 cells was found to induce abnormal membranous structures (aggregation of GFP fluorescence) or abnormal cell shapes (data not shown). These effects are unlikely to be attributable to an artifact induced by fusion of GFP because transient overexpression of FLAG-Syt alone produced the same effects (data not shown). To eliminate the artifacts induced by forced overexpression, PC12 cell lines stably expressing each Syt-GFP or GFP alone, as a control, were established (Fig. 1A, left lanes), indicating that the endogenous levels of expression of Syt III, V, VI, or X in normal PC12 cells are very low, if they are expressed at all.

We then attempted to determine the organelles to which the Syt-GFP fusion proteins are targeted by confocal microscopy (Fig. 2). The stable cell lines were also immunostained with anti-Syt I Ab, a marker for dense-core vesicles and synaptic-like microvesicles in PC12 cells (41, 42, 50) to compare the distribution pattern of Syt-GFP with that of Syt I. To our surprise, each Syt-GFP had a distinct distribution, despite the well conserved amino acid sequences in the subclass (12). The Syt V-GFP protein had accumulated at the neurites (Fig. 2A), and barely colocalized with Syt I (Fig. 2B). Syt III-GFP protein was localized preferentially near the plasma membrane (Fig. 2D) –F, respectively. Syt III is localized at the plasma membrane, not at secretory granules (23, 51). Interestingly, the cell bodies of all the Syt III-GFP stable cell lines were much smaller than those of other Syt-GFP stable cell lines (round shape), and they hardly extended their neurites in response to NGF (Fig. 2, A–C). At this stage, we could not determine whether these phenotypes were involved in certain biological events or were just expression artifacts. The Syt V-GFP protein had accumulated at the neurites (Fig. 2D, arrows) and was closely colocalized with Syt I (Fig. 2, E and F). The blots were then incubated with anti-actin Ab as an internal control to confirm that equivalent amounts of total proteins were loaded on each lane (bottom panels). The signals for each FLAG-Syt-GFP fusion protein were detected at the positions expected from their calculated molecular weight, and the amounts of each fusion protein did not vary much among three independent cell lines. Stable cell lines expressing each Syt-GFP fusion protein were targeted by confocal microscopy (Fig. 2A). 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vesicles. V-GFP migrated predominantly in the fractions containing dense-core vesicles (DCV). The positions of synaptic-like microvesicles (SLMV), and anti-synaptophysin Abs (bottom panel) based on the distributions of synaptophysin and secretogranin II, respectively, are indicated by bars. Note that Syt V-GFP migrated predominantly in the fractions containing dense-core vesicles.

FIG. 3. Distribution of Syt V-GFP in sucrose gradient fractions of PC12 stable cells. The PC12 cell line stably expressing Syt V-GFP was fractionated on a 0.6–1.8 m sucrose gradient. The fractions were subjected to SDS-PAGE and analyzed with horseradish peroxidase-conjugated anti-FLAG M2 Ab (top panel), anti-Syt I (second panel), anti-secretogranin II (third panel), and anti-synaptophysin Abs (bottom panel). The positions of synaptic-like microvesicles (SLMV) and dense-core vesicles (DCV) are indicated by bars. Note that Syt V-GFP migrated predominantly in the fractions containing dense-core vesicles.

Ca2+-dependent Exocytosis—Because PC12 cells contain various types of secretory vesicles (52), we next investigated whether Syt V-GFP is localized on Ca2+-regulated secretory vesicles. A positive result would be important evidence in support of Syt V involvement in Ca2+-regulated exocytosis in which Syts I and IX play a role (41–45). To do so, Ca2+-dependent uptake of Ab against the Syt N terminus (anti-FLAG Ab) was performed as described previously (41, 53–55), and we investigated whether Syt V-GFP-containing vesicles undergo exocytosis in response to Ca2+ stimulation. If FLAG-Syt V-GFP-containing vesicles undergo exocytosis in response to Ca2+ stimulation, the N terminus of FLAG-Syt V-GFP would be accessible on the outside surface of the cell membrane and should be recognized by anti-FLAG M2 Ab in the culture medium. The complex of FLAG-Syt V-GFP and anti-FLAG M2 Ab would then be incorporated into the cell by endocytosis. As expected, anti-FLAG M2 Ab was incorporated efficiently into the neurites after high KCl stimulation, and its signals were almost completely colocalized with GFP fluorescence (FLAG-Syt V-GFP) (Fig. 4, A–C). The uptake of anti-FLAG M2 Ab was Ca2+-dependent because neither in high KCl buffer containing 5 mM EGTA (Fig. 4, D–F) nor in low KCl buffer (Fig. 4, G–I), could we observe any anti-FLAG M2 signals in the neurites, and the weak dotted signals were only present at the edge of the neurites (Fig. 4, E and H). Although this might have resulted from nonspecific binding of Ab to the plasma membrane or the presence of a small amount of FLAG-Syt V-GFP at the plasma membrane, the former possibility is unlikely because no uptake of anti-FLAG M2 Ab was observed in control GFP stable cells even in the presence of high KCl buffer (data not shown). We therefore concluded that FLAG-Syt V-GFP is localized on dense-core vesicles that undergo Ca2+-dependent exocytosis.

Expression Patterns of Syt V in Vivo—Finally, we performed immunoprecipitation and subcellular fractionation studies to investigate whether endogenous Syt V is indeed targeted to dense-core vesicles in brain or endocrine tissues (e.g. pancreas). A single immunoreactive band with an apparent molecular weight of 65,000 was detected by anti-Syt V Ab in total homogenates of both pancreas and brain (lanes 1 and 2 in Fig.
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5A). The immunoreactive bands observed in brain and pancreas were specifically concentrated by immunoprecipitation with anti-Syt C2AB Ab, but not with control rabbit IgG (lanes 3 and 4, and data not shown). In addition, the band was negative with anti-Syt I, III, VI, or X Ab, indicating that the signals observed in our experiments represented only Syt V (data not shown).

This specific antibody was used to investigate the localization of Syt V in dense-core vesicles by subcellular fractionation of the homogenate from whole brain. As shown in Fig. 5, B and C, Syt I peaked at around fractions 2 and 7. The lighter peak of Syt I (fraction 2) corresponds to synaptic vesicle-enriched fractions because this peak overlapped that of synaptophysin (data not shown). The denser peak of Syt I (fraction 7) corresponds to dense-core vesicles. Consistent with the results of recombinant Syt V-GFP in PC12 cells, brain Syt V had accumulated in fraction 7, whereas there was only a small amount of Syt V in fraction 2. These results suggested that Syt V is expressed in endocrine and neuroendocrine tissues and is targeted to dense-core vesicles.

To determine further whether Syt V is expressed uniformly (i.e. all dense-core vesicles) or in a cell type-specific manner (i.e. specific population of dense-core vesicles), immunohistochimical analysis was performed with specific anti-Syt VΔC2AB Ab. In mouse brain, positive Syt V immunoreactivity was detected mainly in cells around the interpeduncular nucleus and essentially not at all in other brain regions, suggesting cell type-specific expression of Syt V (data not shown). Although this nucleus has been reported to be immunoreactive to Abs against opioid receptors and suggested to be involved in enkephalin metabolism (56, 57), it is difficult to assign a function of Syt V in the interpeduncular nucleus at present because its neuroendocrine and peptide secretion properties have not been well characterized. We then focused on the expression patterns of Syt V in pancreatic islet cells because islet cells contain three functionally distinct cells (α, β, and δ) that store different peptide hormones in dense-core vesicles (i.e. glucagon, insulin, and somatostatin are specifically produced in pancreatic α-, β-, and δ-cells, respectively). Interestingly, Syt V was expressed specifically in glucagon-containing α-cells in mouse pancreas (Fig. 6, A–C), but not in either insulin- (Fig. 6D) or somatostatin-containing cells (Fig. 6E). We therefore concluded that Syt V is expressed specifically in pancreatic α-cells and may be involved in glucagon secretion.

**DISCUSSION**

We previously identified a subclass of Syt (Syt III, V, VI, and X) which shows relatively high sequence similarity (12) and similar biochemical properties (12, 17, 19). One of the characteristic features of this class is the N-terminal cysteine residues that are responsible for dimer formation via disulfide bonding (12), suggesting a similar or redundant function of this class of Syt in vesicle trafficking. Surprisingly, however, the results of the present study showed that Syts III, V, VI, and X exhibit a distinct subcellular localization when stably expressed in PC12 cells (Figs. 2 and 3).

Syt III was first proposed as a candidate for the Ca2+ sensor for dense-core vesicle exocytosis in insulin-secreting cells (34–36) because Ab against the recombinant GST-Syt III (34, 36) or C-terminal peptide of Syt III (35) recognized Syt molecules on insulin-containing vesicles. However, these studies did not provide sufficient evidence of the specificity of their antibody, and it is possible that their antibody cross-reacts with other Syt isoforms. By contrast, Gut and co-workers (51) recently showed that Syt III is most unlikely to be expressed on insulin-containing vesicles and that Syt III is targeted to the plasma membrane of primary islet cells when exogenous Syt III is transiently expressed. Our present study suggests that Syt III-GFP is localized near the plasma membrane of PC12 cells, consistent with the latter finding. In addition, Butz et al. (23) reported that Syt III is not concentrated in synaptic vesicles in the nervous system but enriched in synaptic plasma membranes. Therefore, Syt III is most likely present at the plasma membrane and may function as a plasma membrane Ca2+ sensor (58) or be involved in neurite formation because PC12 cells...
stably or transiently expressing Syt III-GFP showed abnormal neurite formation (Fig. 2A).²

Almost all of the Syt VI proteins in brain are expressed as a transmembrane-deficient type (Syt VI-TM) (24, 59), and they are attached to various membrane fractions as well as soluble fractions (23, 24). Recently, full-length Syt VI having a transmembrane domain was demonstrated to be localized on the outer acrosomal membrane and to be involved in the Ca²⁺-triggered acrosomal exocytosis of human spermatozoa (29). By contrast, our present study showed that Syt VI-GFP is distributed mainly in endoplasmic reticulum-like structures in PC12 cells (24). These discrepancies may be the result of the difference in cell types; the subcellular localization of Syt VI may be cell type-specific.

The syt X gene was originally isolated from the brain as a seizure-induced gene, suggesting that Syt X protein is involved in neuronal plasticity (60). However, the precise subcellular localization of Syt X has never been elucidated. Syt X-GFP showed a vesicular-like distribution (other than dense-core vesicles) in this study (Fig. 2J), but we could not determine the types of vesicle that contained the Syt X-GFP. Further analysis is required for a full understanding of the subcellular localization and functions of Syt X.

The most important and surprising finding was that Syt V is targeted to dense-core vesicles in both brain and neuroendocrine cell and essentially not to synaptic-like microvesicles (Figs. 2–5) because other members of this subclass (Syts III-, VI-, and X-GFP in PC12 cells) did not accumulate in dense-core vesicles. Based on this finding, together with the fact that the C2 domains of Syt V bind phospholipids or syntaxin in a Ca²⁺-dependent manner (58, 61), we propose that Syt V is a dense-core vesicle-specific isoform that regulates Ca²⁺-dependent exocytosis in brain and endocrine tissues (e.g. glucagon release in pancreatic α-cells). The possible involvement of Syt V in dense-core vesicle exocytosis in PC12 cells is being investigated in our laboratory by using recombinant fragments of C2 domains (so-called dominant negative approach) (42, 43, 45, 46, 58) or a functional block antibody specific to Syt V (41). In our preliminary experiments, however, the former strategy seemed unreliable for the following two reasons. First, the effect of the Syt V fragments on Ca²⁺-dependent neuropeptide Y release by Syt V-GFP-stable cells and control cells in which Syt V is not endogenously expressed was indistinguishable. Second, the inhibitory potential of the fragments of the Syt family did not reflect the endogenous expression levels of each Syt isoform (41, 46, 58). For instance, although Syts III and VII are not expressed well in PC12 cells (37, 41), the effect of recombinant Syts III and VII was stronger than that of Syt I, the most abundant Syt isoform in normal PC12 cells (41), suggesting that the blocking site is non-Syt proteins (e.g. soluble NSF attachment protein receptor (SNARE) proteins) rather than endogenous Syt proteins (62). The second approach was also very difficult because we could not obtain highly specific Ab to the C2A domain of Syt V.² We generated four different anti-Syt V-C2A Abs, but all of them cross-reacted with Syts I and IX, both of which regulate dense-core vesicle exocytosis in PC12 cells (41), even after preincubation with an excess amount of GST-Syt I-C2A or -Syt IX-C2A. Thus, additional specific tool(s) are required to elucidate whether Syt V is involved in dense-core vesicle exocytosis.

Another important finding was that the distribution pattern of Syt-GFP fusion proteins (especially Syts V and VII) expressed transiently was absolutely different from the pattern observed in the stable cell lines. For instance, transiently expressed Syt V-GFP fusion protein was often aggregated in cell bodies, whereas the stably expressed Syt V-GFP was localized predominantly on dense-core vesicles in PC12 cells. Similarly, transiently expressed Syt VII-GFP was localized on the plasma membrane (46), whereas the stably expressed Syt VII-GFP was enriched at the tips of the neurites as well as trans-Golgilike network-like structures (37). These discrepancies may be explained as follows. The amount of the exogenous Syt-GFP fusion proteins expressed transiently is often too much, and such an excess protein overflows the normal sorting machinery. We therefore emphasize the advantage of using stable cell lines to evaluate the involvement of Syt in physiological events in cells. Of course, we cannot exclude the possibility that the sorting pathway of Syt-GFP fusion protein is different from that of endogenous Syt protein.

In conclusion, we have demonstrated distinct subcellular localizations of Syts III-, VI-, and X-GFP in stable PC12 cells even though they belong to the same subclass of Syt. The results also suggest that Syt V, which is distributed predominantly in dense-core vesicles, is involved in Ca²⁺-mediated vesicle trafficking.

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FIG. 6. Expression of Syt V in pancreatic α-cells. Mouse pancreas was cryosectioned into 10-μm thick sections and subjected to immunohistochemistry with anti-Syt V/C2A Ab (green in A, D, and E), anti-glucagon Ab (a marker for α-cells; red in D), and anti-somatostatin Ab (a marker for δ-cells; red in E). C is the superposition of A and B. Note that Syt V is expressed in the glucagon-containing cells (A–C), but not in either insulin- or somatostatin-containing cells (D and E, respectively). These results indicate that Syt V is expressed in pancreatic α-cells but not in β-cells or δ-cells. Scale bar = 20 μm.
