Site of Docking and Fusion of Insulin Secretory Granules in Live MIN6 β Cells Analyzed by TAT-conjugated Anti-syntaxin 1 Antibody and Total Internal Reflection Fluorescence Microscopy*

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Insulin is stored in large dense core vesicles in pancreatic β cells and is released by exocytosis when glucose levels rise (1). We (2) and others (3, 4) have demonstrated previously that soluble N-ethylmaleimide-sensitive fusion protein attachment (SNAREs)³ are expressed in pancreatic β cells and play an important role in the insulin exocytotic process (2, 5, 6). However, it is not known how t-SNAREs are structured and distributed in the pancreatic β cell plasma membrane and how they spatially interact with the insulin granule during docking and fusion. A recent study described the distribution and spatial organization of t-SNAREs using the procedure of plasma membrane sheets derived from PC12 cells (7, 8), but technical difficulties have prevented such a study in pancreatic β cells.

Recently, we developed an approach using a green fluorescent protein (GFP)-tagged insulin granule system combined with total internal reflection microscopy (TIRFM) (9). Using this system, we were able to observe the motion of single insulin granules such as in docking and fusion in the exocytotic process during physiological stimulation. TIRF illuminates fluorophores close to the plasma membrane (within −100 nm) (10), allowing us to observe with high resolution not only the single insulin granules approaching, docking, and fusing with the plasma membrane but also the spatial localization of endogenous t-SNAREs such as syntaxin 1 and SNAP-25 in the plasma membrane of live cells. Therefore, if we could label the endogenous t-SNAREs with fluorophore in live cells, we could reveal the dynamic relationship between insulin exocytosis and t-SNAREs. The powerful strategy of a protein transduction domain has been reported previously (11, 12) in studies of the protein transduction domain of HIV-1 TAT protein, which has been shown to cross biological membranes efficiently and to promote delivery of peptides and proteins into cells. Moreover, intracellular delivery of antibodies by chemically conjugating with TAT has been reported (13–15). We have succeeded previously in delivering TAT-fused peptide into live MIN6 cells (16). Here, we applied this technique modified to TAT-conjugated antibody (Ab) against syntaxin 1, resulting in successful labeling of the endogenous syntaxin 1 in living cells.

To determine the insulin exocytotic site in living cells, we took advantage of the techniques for both TAT-conjugated Cy3-labeled Ab and GFP-tagged insulin granules combined with TIRFM to refine the in vivo spatial organization and distribution of the t-SNARE, syntaxin 1, in the intact plasma membrane and topological interaction of syntaxin 1 with the docking and fusion of insulin granules. Our results demonstrate...
that syntaxin 1 is distributed in separate clusters in the intact plasma membrane where insulin granules are preferentially docked and fused, indicating that syntaxin 1 clusters are requisite for effective insulin exocytosis.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The construction of GFP-insulin in expression vector has been described previously (9).

**Antibodies**—Mouse monoclonal Ab was used for the following proteins: syntaxin 1 (obtained from Sigma), SNAP-25 (obtained from WAKO Co. Ltd., Osaka, Japan), and flotillin-1 (obtained from BD Biosciences, San Jose, CA). Rabbit polyclonal sera were used for syntaxin 1 (5). Goat anti-rabbit IgG conjugated to Cy3, and fluorescent goat anti-mouse IgG conjugated to Cy3 and fluorescein were from Jackson Immunoresearch Laboratories (West Grove, PA).

**TAT-conjugated Ab**—Monoclonal anti-syntain 1 Ab was labeled with Cy3 using a Fluoro Link-Ab Cy3 labeling kit (Amersham Biosciences) according to the manufacturer’s instruction. The Cy3-labeled Ab was dialyzed against 0.1 M borate buffer and incubated with a 5-fold molar excess of a cross-linker, sulfo-succinimidyl 6-[3-(2-pyridyldithio)-propionamido] hexanoate (Pierce) for 3 h at room temperature. The conjugated Ab was separated from free cross-linker by gel filtration on a Sephadex G-100 (Pharmacia, Piscataway, NJ) column equilibrated with Hanks’-HEPES (5 mM) buffer.

**Hanks’-HEPES**—HEPES (5 mM) buffer in Hanks’ saline at pH 7.4, containing 10% sucrose, 1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml antipain on ice for 30 min. The suspension was mixed with 600 μl of cold OptiPrep170 (Nycoderm-Pharma, Oslo, Norway). The mix was transferred to two centrifuge tubes and was overlaid with a 250-μl step each of 35, 30, 25, 20, and 15% OptiPrep170 in the Tris buffer. The gradients were spun for 2 h at 50,000 rpm at 4°C. The fractions were collected, precipitated with trichloroacetic acid, and analyzed by immunoblotting as described previously (16) or cholesterol assay with cholesterol oxidase using Cholesterol ClI assay kit (WAKO).

**Insulin Release Assay**—Batch experiments using MIN6 cells were performed as described previously (9), and the amounts of insulin released into the media and cell extracts were analyzed by radioimmunoassay.

**RESULTS AND DISCUSSION**

**t-SNAREs Are Distributed in Separate Clusters in the Plasma Membrane of MIN6 β Cells**—To analyze the localization of t-SNAREs and their association with insulin granules in the plasma membrane of MIN6 β cells, GFP-tagged insulin-transfected cells were fixed, immunostained with anti-syntaxin 1 and anti-SNAP-25 antibodies, and observed by both EPIF (common) microscopy and TIRFM of the same cell. Under EPIF microscopy, the entire cell was visible; the immunofluorescence of syntaxin 1 and SNAP-25 was observed to be mainly subplasmalemmal (Fig. 1, **EPIF**). In contrast, TIRFM, which selectively illuminates subcellular features just beneath the plasma membrane at the cell glass coverslip contact region (within ~80 nm), showed that the immunofluorescence of syntaxin 1 and SNAP-25 was distributed in numerous spots, which suggests that t-SNAREs are concentrated in separate clusters in the plasma membrane of MIN6 cells (Fig. 1, **TIRF**). The SNAP-25 clusters were more dense than those of syntaxin 1 (syntaxin clusters, 179 ± 14/200 μm2; SNAP-25 clusters, 329 ± 18/200 μm2; n = 8 cells). Dual stained immunohistochemical studies for syntaxin 1 and SNAP-25 showed that most of the syntaxin 1 clusters were colocalized with the SNAP-25 clusters (93 ± 3.0%; n = 4 cells) (Fig. 1C). The apparent diameters of the syntaxin 1 and SNAP-25 clusters were 416 ± 11 nm and 429 ± 18 nm, respectively (n = 6 cells each). To obtain the estimation of real diameter of the clusters, the point spread function (PSF) was determined by measuring the diameter of 90-nm fluorescent beads, which was 340 ± 27 nm (35 beads) under our experimental conditions.

$$PSF = (\text{beads measured diameter}^2) - (\text{beads real diameter})^2$$

(Eq. 1)

The real diameter of the spots was calculated by subtraction of the PSF.

$$\text{cluster real size} = \left(\frac{(\text{cluster measured size})^2}{2} - (\text{PSF})^2\right)^{\frac{1}{2}}$$

(Eq. 2)

resulting in 256 ± 18 nm (syntaxin) and 277 ± 27 nm (SNAP-
Fig. 1. Colocalization of t-SNAREs and insulin granules in the plasma membrane of MIN6 cells observed by EPIF microscopy and TIRFM. After MIN6 cells were transfected with expression vector insulin-GFP (for A and B but not for C), they were fixed and immunostained. A and B, colocalization of syntaxin 1 or SNAP-25 and GFP-tagged granules. Fixed cells were immunostained for syntaxin 1 (A) or SNAP-25 (B) and viewed in the red (syntaxin 1 and SNAP-25) or the green (GFP-tagged insulin granules) channel. The colocalization of syntaxin 1 or SNAP-25 and GFP-tagged insulin granules is demonstrated by the superposition (yellow color) of red and green channel images. C, colocalization of syntaxin 1 and SNAP-25. Fixed cells were immunostained for syntaxin 1 and SNAP-25 simultaneously and viewed in the red (syntaxin 1) or the green (SNAP-25) channel. The overlay of the images (yellow color) shows the colocalization of syntaxin 1 and SNAP-25. Images in EPIF were observed by inverted epifluorescence microscopy and those in TIRF by simply shifting the mirror in the same cell.

25); these measurements are within the range similar to that seen in PC12 cells reported elsewhere by different procedures (7). As shown in Fig. 1, A and B, many GFP-labeled insulin granules appeared to be colocalized with syntaxin 1 and SNAP-25 clusters. However, we cannot conclude that t-SNAREs clusters are definitely colocalized with insulin granules on the basis of these data because it cannot be ruled out that the colocalization of t-SNARE proteins with the insulin granules shown in Fig. 1 may be the result of the amplification used in the immunostaining and protein clumping caused by fixation. To overcome these problems, we tried to label the syntaxin 1 and insulin granules in living cells without the fixation procedure.

Colocalization of TAT-conjugated Ab-labeled Syntaxin 1 and Insulin Granule in the Plasma Membrane—To determine whether syntaxin 1 clusters are colocalized with insulin granules in the plasma membrane of living cell and whether the fusion of insulin granules occurs at the syntaxin 1 clusters, a method for labeling endogenous syntaxin with fluorophore in live cells was needed. We visualized the endogenous syntaxin 1 with Cy3-labeled anti-syntaxin 1 Ab by conjugating with HIV-1 TAT in live cells (Fig. 2). In agreement with the results of the previous experiments using TAT-fused peptide (16) and TAT-conjugated Ab (13–15), TAT-conjugated Cy3-labeled anti-syntaxin 1 Ab was rapidly transduced into the cells, reaching near maximum intracellular concentration in less than 20 min (Fig. 2, A and B). 20 min after treatment with TAT-conjugated Cy3-labeled Ab, Cy3 fluorescence was localized primarily within the subplasmalemmal region and was also observed as dots in the cytosol as well as dispersion in the cytoplasm of the cells under confocal laser scanning microscopy (Fig. 2A). Although the mechanism of the TAT-mediated delivery of proteins into cell has not been fully defined yet (17), it is likely that TAT-fused protein enters cells by uncharacterized pinocytosis/endocytosis and is initially within endosomal compartment (18, 19). Indeed, Fig. 2A displays Cy3 fluorescence strongly in endosomes and the plasma membrane, as well as weakly in the cytoplasm. To ensure that the TAT-conjugated Cy3-labeled anti-syntaxin 1 Ab specifically binds endogenous syntaxin 1 clusters in the plasma membrane, we performed experiments using TAT-conjugated monoclonal anti-syntaxin 1 Ab and polyclonal anti-syntaxin 1 Ab. MIN6 cells treated with TAT-conjugated Cy3-labeled Ab (monoclonal) for 30 min were fixed with parafomaldehyde and immunostained with anti-syntaxin 1 polyclonal Ab (Fig. 2C). There was a significant overlap of syntaxin clusters labeled with TAT-conjugated Cy3-labeled Ab (red) and those stained with polyclonal Ab (green), which suggests that TAT-conjugated Cy3-labeled Ab was actually localized at the endogenous syntaxin. Furthermore, highly magnified viewing revealed that spots for syntaxin clusters labeled with TAT-conjugated Cy3-labeled Ab were precisely coincided with those stained by anti-syntaxin 1 polyclonal Ab (Fig. 2D). Thus, the transduction system with TAT-conjugated anti-syntaxin 1 Ab is highly efficient for labeling the endogenous syntaxin 1 in the plasma membrane in living MIN6 β cells.

We then examined whether spots for syntaxin clusters corresponded with the docking sites of insulin granules in living β cells without the fixation procedure to avoid experiment-induced artifacts. For this purpose, MIN6 cells were first transfected with GFP-tagged insulin expression vector and then treated with TAT-conjugated Cy3-labeled anti-syntaxin Ab to visualize the insulin granules and syntaxin 1 clusters in living cells using TIRFM. To analyze the colocalization of the insulin granules with syntaxin 1 clusters, we first selected the area where the docking sites of insulin granules are similar to sites of syntaxin 1 clusters. Then, a dual image of GFP and Cy3 fluorescences was acquired by TIRFM. As shown in Fig. 3A, spots for insulin granules (green) were associated with areas for syntaxin 1 clusters, although those spots appear enlarged because of the fluorescence of GFP and Cy3, and the diffraction
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limited resolution of the objective. Indeed, the diameter of insulin granules observed in TIRF image was 426 ± 13 nm (n = 5 cells), although the real size of insulin granules was calculated as 272 ± 20 nm, using a formula based on PSF, as outlined above, which agreed well with the value (289 nm) measured from electronmicroscopy using mouse pancreas (20). Similarly, the real diameter of syntaxin 1 clusters in living cells was calculated into 261 ± 30 nm, on the basis of the apparent size of TAT-conjugated Cy3-labeled syntaxin 1 clusters (419 ± 19 nm). To correct the apparent sizes to real sizes based on these values, circles (400-nm diameter) drawn around fluorescent spots for GFP-labeled insulin granules and Cy3-labeled syntaxin 1 clusters observed in TIRF images (Fig. 3A) were reduced to 68% (272-nm diameter) and 65% (261-nm diameter), respectively, to correct the real diameters (a′ and b′), and these were transferred to the identical pixel localization (c′). Circles of insulin granules (green) and syntaxin 1 clusters (red) were precisely overlapped.

Syntahin 1 Clusters Are Sites for Fusion of Insulin Granule—Next, to investigate whether fusion from insulin secretory granules occurs at sites of syntaxin 1 clusters, we monitored the motion of GFP-tagged insulin secretory granules and their interaction with Cy3-labeled syntaxin 1 in live MIN6 cells. Under the basal conditions, quantitation by TIRFM imaging revealed that 73.5 ± 5.1% of GFP-tagged insulin granules corresponded to the syntaxin 1 clusters, whereas about 27% of granules were not on syntaxin 1 clusters (Fig. 4A; n = 5 cells). Stimulation with high KCl (50 mEq/L) clearly revealed that the fusion events of insulin granules occurred exclusively at the syntaxin 1 clusters (Fig. 4A; granules to be fused are indicated by arrows; see also Movie 1). Fig. 4B shows sequential images (1 × 1 μm, 300-ms intervals) of the single granule (green) and the syntaxin cluster (red) simultaneously observed during KCl stimulation. Notably, GFP-fluorescent spots, which were present at the syntaxin 1 clusters, suddenly brightened and finally spread as GFP-tagged insulin diffused laterally through fusion on the plasma membrane, whereas no changes were observed in the syntaxin 1 clusters during and after granule exocytosis (see also Movie 1). Because the number of fusion events in the cells transduced with TAT-conjugated Ab was similar to that in control cells, labeling endogenous syntaxin with TAT-conjugated Cy3-labeled Ab does not affect the insulin exocytosis (data not shown). Thus, our results demonstrate that the fusion of insulin granules occurs exclusively at the site of syntaxin 1 clusters.

Syntaxin 1 Clusters Are Required for Effective Insulin Exocytosis—We then examined whether t-SNARE clusters are required for insulin exocytosis. For this purpose, we used a methyl-β-cyclodextrin that is known to extract the cholesterol from the plasma membrane selectively and rapidly, in preference to
other membrane lipids (21, 22). Because the formation of syntaxin 1 clusters has been reported to be dependent on the cholesterol in the plasma membrane in other cell types (7), we expected that cholesterol depletion in the plasma membrane of MIN6 cells would disintegrate the syntaxin clusters. Indeed, the treatment of MIN6 cells with methyl-β-CD (10 mM for 30 min) caused the disruption of syntaxin 1 clusters (Fig. 5A). The clusters were disintegrated, and syntaxin was dispersed on the membrane in a methyl-β-CD concentration-dependent manner (data not shown). Along with the disintegration of syntaxin 1 clusters, the number of docked insulin granules was decreased markedly (Fig. 5A) (66 ± 10 granules/200 μm² versus 41 ± 8 granules/200 μm²; n = 6 cells). We then stimulated these cells with 50 mM KCl. As shown in Fig. 5B, the...
methyl-β-cyclodextrin treatment markedly reduced the number of fusion events, consistent with the insulin release data measured by radioimmunoassay (Fig. 5C). On the other hand, no difference was observed in the kinetics of individual fusion events (Fig. 5D). Lang et al. (7) reported that syntaxin 1 clusters are prerequisite for granule docking, but not for fusion itself. Taken together, we concluded that the reduced number of fusion events occurring with the methyl-β-cyclodextrin treatment resulted from the decreased number of docked insulin granules caused by disruption of syntaxin 1 clusters.

Several molecules such as SNAREs and Rabs have been proposed as the candidate responsible for granule docking (23, 24). In pancreatic β cells, SNARE proteins are known to regulate insulin exocytosis (2, 25, 26); however, no specific molecules have been determined to govern insulin granule docking. The present data at least indicate that the presence of syntaxin 1 clusters is a prerequisite for insulin granule docking, followed by fusion.

Syntaxin Clusters Are Distinct from Fattoin-Enriched Lipid Membrane Raft—Recently, lipid microdomains rich in sphingolipids and cholesterol, also known as lipid rafts, have been proposed as the region within the plasma membrane which is important for signal transduction, including the exocytotic and endocytic pathways (27, 28). Indeed, it has been reported that syntaxin 1A/SNAP-25 heterodimers are present in rafts and are implicated in the regulated exocytosis of dopamine from PC12 cells (29). We therefore investigated the relationship between syntaxin 1 clusters and lipid rafts in MIN6 cells.

Cellular fractions of MIN6 cells obtained by discontinuous OptiprepTM-sucrose gradient were analyzed by immunoblotting and cholesterol assay. Although caveolae are a specific form of lipid rafts and implicated in important cellular functions in various cell types (30, 31), caveolin expression was barely detectable in MIN6 cells (data not shown), in agreement with the results of studies of PC12 cells (29, 32, 33). Therefore, we used an Ab against fattoin-1(reggie-2), a protein that is known to be enriched in lipid rafts (34, 35), as a marker of lipid rafts. As shown in Fig. 6A, fattoin-1 was present in the two fractions from the top of the gradient, which confirms that these fractions are rich in lipid rafts. On the contrary, syntaxin 1 was present in the two fractions from the bottom of the gradient with relatively high concentrations of cholesterol. In addition, immunostaining of the cells for syntaxin 1 and fattoin-1 simultaneously revealed that syntaxin 1 clusters do not colocalize with fattoin-1 lipid rafts (Fig. 6B). Our results suggest that syntaxin 1 clusters are cholesterol-dependent, whereas they are distinct from the lipid raft, so there was no clear evidence showing the direct interaction between lipid rafts and insulin exocytosis in MIN6 cells.

In conclusion, insulin secretory granules are docked preferentially on the syntaxin 1 clusters and fused exclusively at the site of syntaxin 1 clusters in MIN6 β cells, which are distinct from fattoin-1 lipid rafts.

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