Transduction of MIN6 β Cells with TAT-Syntaxin SNARE Motif Inhibits Insulin Exocytosis in Biphasic Insulin Release in a Distinct Mechanism Analyzed by Evanescent Wave Microscopy*

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To investigate the in vivo interaction of syntaxin-mediated soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) assembly and insulin exocytosis in biphasic release, we examined the dynamics of insulin granule motion such as docking and fusion with the plasma membrane when the syntaxin SNARE motif (H3 domain) was transduced into living MIN6 β cells. TAT-H3, produced by fusion of the protein transduction domain of human immunodeficiency virus-1 TAT to the syntaxin-H3 domain, was rapidly transduced into the subplasmalemmal region in living MIN6 cells. Immunoblotting analysis followed by immunoprecipitation on TAT-H3-treated MIN6 cells showed that TAT-H3 binds SNAP-25 and VAMP-2 in vivo. Transduction of MIN6 cells with TAT-H3 caused a decrease in both the first and second phase of insulin release. We therefore quantitatively analyzed approaching, docking, and fusing of green fluorescent protein-labeled single insulin granules in TAT-H3-transduced MIN6 cells by evanescent wave microscopy. Under high glucose stimulation, TAT-H3 treatment not only reduced the fusion events from previously docked granules for the first 120 s (first phase of release) but also strongly inhibited the docking and fusion from newly recruited insulin granules after this point (second phase of release). During the second phase of release we observed a marked reduction in the accumulation of newly docked insulin granules; subsequently, fusion events were significantly decreased. TAT-H3 treatment by itself, however, did not alter the number of previously docked granules without stimulation. We conclude that introduction of the H3 domain into MIN6 cells inhibits biphasic insulin release by two mechanisms. 1) In the first phase of insulin release, the H3 domain interferes with previously docked granules to be fused, and 2) in the second phase of insulin release reduced fusion events result from a marked decline of newly docked granules. Thus, syntaxin-mediated SNARE assembly modulates insulin exocytosis in biphasic insulin release in a distinct way.

In pancreatic β cells, the hormone insulin is stored in large dense-core vesicles and is released by exocytosis when glucose levels rise (1). Soluble N-ethylmaleimide-sensitive fusion protein (NSF) attachment protein receptors (SNAREs) are believed to be required for vesicular trafficking such as endoplasmic reticulum to Golgi, Golgi to the plasma membrane, and exocytosis in general (2, 3). Syntaxin is a key component of the SNARE complex involved in synaptic vesicle docking and fusion, and it functions as an organizing center for exocytosis (4, 5). Syntaxin 1 consists of four α-helical domains (Habc domain and SNARE motif (H3 domain)) and a short C-terminal transmembrane domain (6, 7). The syntaxin-H3 domain contributes to one of the four α-helical bundles in the SNARE core complex; that is, one helix each from syntaxin-H3 and VAMP and two helices from SNAP-25 (8), suggesting the H3 domain is the most critical domain in the molecular function of syntaxin.

SNAREs are expressed in pancreatic β cells (9, 10). We and others previously demonstrated that the SNARE hypothesis may be applicable to insulin exocytosis in pancreatic β cells (11–13). Although the central role of syntaxin 1 in insulin exocytosis has been extensively studied in β cells (11, 12, 14), the precise interaction between the syntaxin-mediated SNARE complex and the dynamics of docking and fusing insulin granules in living pancreatic β cells remains unclear.

Recently, we developed a new approach using a green fluorescent protein (GFP)-tagged insulin granule system combined with evanescent wave microscopy (15). Using this system, we were able to observe the motion of single insulin granules such as docking and fusion in the exocytotic process during physiological stimulation. The fluorescence imaging excited close to the plasma membrane (within ~100 nm), allowing us to observe with high resolution the single insulin granules approaching, docking, and fusing with the plasma membrane. In the current study, in addition to the above-described system we used the powerful strategy of a protein transduction system; that is, the protein transduction domain (PTD) of the human immunodeficiency virus-1 TAT protein, which has been shown to cross biological membranes efficiently and independently via transporters and specific receptors and to promote delivery of peptides and proteins into cells (16, 17). We produced expression vectors encoding GFP- or syntaxin-H3-domain-fused TAT PTD. We found that TAT PTD fusion proteins rapidly transduced into nearly 100% of MIN6 β cells.

In the present study, we used TAT PTD fusion protein and GFP-tagged single insulin granule analysis in living pancreatic

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β cells combined with evanescent wave microscopy to refine the in vivo interaction of syntaxin-mediated SNARE assembly and insulin exocytosis. Our results provide the new aspect showing that syntaxin-mediated SNARE assembly modulates docking and fusion of insulin granules in biphasic insulin release.

EXPERIMENTAL PROCEDURES

Plasmid Construction—To generate the construct of GFP-insulin in which GFP is located at the C terminus of preproinsulin, the coding region of human preproinsulin cDNA (a gift from Dr. G. I. Bell, University of Chicago) was amplified by PCR using originally designed primers, cleaved by restriction enzymes, and subcloned into mamma-
ian expression vector pGEX-N1 (Clontech, Palo Alto, CA) as previ-
ously described (15). To produce a construct in which TAT PTD peptide is located at the N terminus of GFP, PCR was performed using an oligonucleotide primer encoding the nucleotide sequence of TAT PTD peptide (YGRKKRRQRRR) (18) and GFP cDNA as a template. The PCR product was digested with appropriate restriction enzymes and subcloned into pProEX™HTa bacterial expression vector (Invitrogen) with an additional His tag to the N terminus. To produce a construct in which the TAT PTD peptide is located at the N terminus of the syntaxin H3 domain (amino acids 202–265), the coding region corre-
sponding to the rat syntaxin-H3 domain was amplified by PCR using an oligonucleotide primer encoding the TAT PTD peptide as described above, and the product was subcloned into pProEX™HTa vector. The resulting products were confirmed by an automated DNA sequencer (Amersham Biosciences).

Isolation of TAT Fusion Proteins—TAT-GFP fusion protein and TAT-H3 fusion protein in the pProEX™HTa vector were expressed in DH5α Escherichia coli strain by induction with isopropyl-β-D-thiogalac-
tospyranoside for 5 h at 37 °C. The recombinant proteins were extracted with 8 m urea in 50 mM Tris and 100 mM KCl, pH 8.0. Urea extracts were incubated with nickel nitriilotriacetic acid-agarose (Qiagen, Valencia, CA) before washing, and stepwise removal of urea was performed to allow renaturation of bound protein. Proteins were eluted from nickel-
agarose by 200 mM imidazole and were desalted on a PD-10 column (Amersham Biosciences) with Hanks’ balanced salt solution (Invitrogen).

Cell Culture, Transfection, and Transduction with TAT Fusion Pro-
teins—MIN6 cells (a gift from Dr. J.-i. Miyazaki, Osaka University, Osaka, Japan) at passage 15–30 were cultured as previously described (12) on fibronectin-coated (KOKEN, Co. Ltd., Tokyo, Japan) glass cham-
ber slides (8 wells; Lab-Tek slides, Nunc, Rochester, NY) for imaging with confocal microscopy and on high refractive index glass (Olympus, Tokyo, Japan) for imaging with evanescent wave microscopy (15). MIN6 cells were transfected with the expression vector encoding the GFP-
tagged human preproinsulin gene using Effectene transfection reagent (Qiagen) according to the manufacturer’s protocol. All experiments were performed between 3 and 5 days after transfection. On the day of experiments using evanescent wave microscopy, MIN6 cells were trans-
duced for 50 min with an appropriate amount of TAT fusion proteins.

To determine the transduction of the TAT fusion protein into the cells, the fluorescence image of the TAT-GFP fusion protein added to the culture media of the cells was observed for 0–50 min using a Zeiss confocal laser-scanning microscope (LSM510, Carl Zeiss, Germany). To analyze the average fluorescence intensity in TAT-GFP treated cells, confocal images were exported as single TIFF files to Metamorph 4.6 software (Universal Imaging, West Chester, PA), and the average of the pixel gray scale values (2 gray scale value/number of pixels) in each whole cell was determined.

Endogenous Insulin Release Assay—Control or TAT fusion protein-
treated MIN6 cells (plated on 48-multwell plates Nunc) were preincu-
ated for 15 min in 2.2 mM glucose-KRB and then challenged with 2.2 mM glucose, 22 mM glucose, or 50 mM KCl. At the end of the stimulation period, the cells were treated with acid-alcohol, and aliquots of media and cell extracts were analyzed by enzyme-linked immunosorbent assay as described previously (12). Released insulin is expressed as a percentage of total cellular content.

RESULTS

Rapid Transduction of TAT Fusion Protein into MIN6 β Cells—To analyze the ability of TAT fusion proteins to trans-
duce into MIN6 β cells, TAT-GFP fusion protein was added to the culture media of cells and analyzed by fluorescence using laser-scanning confocal microscopy. As shown in Fig. 1, TAT-
GFP fusion protein was rapidly transduced into the cytosol of nearly 100% of cells, reaching near maximum intracellular concentration in less than 30 min. Thus, the transduction sys-
tem with TAT fusion protein is highly efficient at rapidly trans-
fering the target protein into pancreatic β cells, in agreement with previous reports using rat islets and βTC-3 cells (21) and PC12 cells (22).

Binding of TAT-H3 to SNAREs in Living MIN6 Cells—We next studied the uptake of syntaxin-H3 domain fused to a TAT protein (TAT-H3) in MIN6 β cells (Fig. 2). Cells were treated with TAT-H3 for 50 min, fixed with paraformaldehyde, and
inhibits docking and fusion of insulin granules.

**Fig. 1. Transduction of TAT-GFP fusion protein into intact MIN6 β cells.** The fluorescence image of the TAT-GFP fusion protein (70 µg/ml) added to the culture media of the cells was analyzed using confocal microscopy. A, confocal images of MIN6 cells treated with TAT-GFP for 0–50 min. B, time course of changes in the fluorescence intensity in TAT-GFP-treated cells. Time 0 indicates the addition of TAT-GFP into the culture medium. The average of fluorescence intensity was obtained by measuring the average of the pixel gray scale values in each whole cell using Metamorph software. The background fluorescence intensity at time 0 (gray value, 7.5 ± 1.2) was subtracted from each fluorescence intensity. Data are the mean ± S.E. of 80 cells.

**Fig. 2. Transduction of TAT-H3 into MIN6 β cells.** Control cells and TAT-H3-treated (70 µg/ml for 50 min) cells were fixed with paraformaldehyde and immunostained with anti-syntaxin 1 polyclonal antibody and fluorescein-conjugated anti-rabbit IgG. A, the immunofluorescence staining of control MIN6 cells detected by confocal laser-scanning microscopy. B, immunostaining of TAT-H3-treated MIN6 cells. C, immunoblotting analysis. After immunoprecipitation on TAT-H3-treated MIN6 cells was performed with anti-SNAP 25 and/or anti-VAMP-2 antibodies (Ab), immunoprecipitates were subjected to immunoblot analysis with anti-polyhistidine antibody. A representative blot is shown (n = 3).

**Inhibition by TAT-H3 of the Docking and Fusion of Insulin Granules in Glucose-induced Biphasic Insulin Release—**To analyze how the H3 domain incorporated into MIN6 cells affects the dynamics of single insulin granule motion, we monitored the real-time docking and fusion process of single insulin granules labeled with GFP-tagged insulin near the plasma membrane in TAT-H3-treated MIN6 cells using evanescent wave microscopy. During 12 min of glucose stimulation, we counted the fusion events from sequential images taken at 100-ms intervals on 200 µm² of MIN6 cell plasma membrane attached to cover glass. As shown in Fig. 4A, TAT-H3 treatment reduced not only the fusion events during the first 120 s (first phase of release) but also strongly inhibited the continuous fusion events throughout 10 min (second phase of release), which was consistent with the data of endogenous insulin release measured by enzyme-linked immunosorbent assay (Fig. 3B). In agreement with our previous report (15), the first phase of glucose-induced fusion events occurred only on previously docked granules (red column in Fig. 4B). TAT-H3 treatment reduced the number of fusion events to ~50% that of control levels by TAT-H3 treatment. In contrast, fusion events in the second phase of insulin release (after 2 min) mostly occurred from newly recruited docked granules and were nearly abolished by TAT-H3 treatment (green column in Fig. 4B).
point is expressed as the percentage of total cellular content. The basal
ured by enzyme-linked immunosorbent assay. Insulin release at each
treated with acid-alcohol at the end of incubation. The amounts of
GFP-tagged insulin were treated with and/or without 70
ules in TAT-H3-treated MIN6 cells by high glucose (22 mM) stim-
ulation with evanescent wave microscopy.
Fig. 5. Time-dependent change of the number of insulin gran-
docked to the plasma membrane during high glucose (22
mM) stimulation. The number of previously docked granules (red line)
and that of newly recruited granules (green line) during 22 mM glucose
stimulation were determined by counting granules on each sequential
image (200 μm², n = 3 cells each) in control cells (A) and TAT-H3-
treated cells (B). The black line shows the total number of docked
granules, corresponding to the sum of red and green lines. Time 0
indicates the addition of high glucose (22 mM). The number of previ-
sously docked granules at time 0 was taken as 100% (45, 57, and 68
granules in each of the 3 cells in A; 51, 60, and 63 granules in each of the
3 cells in B). The typical images before and after high glucose stimula-
tion were superimposed. Data are the mean ± S.E. of eight determinations from individual wells.

Reduction by TAT-H3 of the Accumulation of Newly Docked
Granules on the Plasma Membrane and Subsequent Decrease of
the Fusion Events in the Second Phase of Release—To clarify
the mechanism behind the marked decrease in fusion events
observed in the second phase of insulin release, we examined the
change in total number of insulin granules docked to the
plasma membrane during continuous glucose stimulation by
tracking the motion of each docked granule on sequential im-
ages. In control cells, the number of previously docked granules
slowly decreased due to fusion and retreat (granules paused
and then moved back inside the cell without fusing; red line
in Fig. 5A), whereas the number of newly recruited docked gran-
ules rapidly and progressively increased after glucose stimula-
tion (green line in Fig. 5A). As a result, the total number of
docked granules (previously docked granules plus newly re-
ruited docked granules) increased to ~140% of the initial
number of docked granules during stimulation (black line
in Fig. 5A) in agreement with our previous report (15). The acti-
vation of recruitment of new granules to docking sites may help
to restock the docked granule pool (those ready to be released)
during the second phase of insulin release. In contrast, in
TAT-H3-treated cells, the progressive increase in the number
of newly recruited docked granules observed in control cells
was completely abolished by TAT-H3 treatment (green line
in Fig. 5B), although the decrease in the number of previously
docked granules was affected little by TAT-H3 treatment (red
line in Fig. 5B). Eventually, TAT-H3 treatment reduced the
total number of docked granules to 60% that of the initial level
during glucose stimulation because of the reduced accumula-
tion of newly recruited docked granules (black line in Fig. 5B).
Thus, our image analysis with evanescent wave microscopy
showed that a marked reduction in accumulation of newly
recruited granules docked to the plasma membrane caused a
significant decline of fusion events in the second phase of
release.

Reduction by TAT-H3 of High KCl-induced Fusion Events
from Previously Docked Granules—As mentioned above, in
the second phase of insulin release fusion events in TAT-H3-
treated MIN6 cells were strongly inhibited mostly due to the
marked reduction of newly docked granules transferred from
the intracellular (reserve) pool. As shown in Fig. 4, however,
TAT-H3 treatment also decreased the fusion events even in the
first phase of insulin release. To focus on the mechanism of
TAT-H3-induced decline of fusion events in the first phase, we
performed experiments using 50 mM KCl as a secretagogue,
which evokes rapid and transient insulin release, representing
the first phase of insulin release (13, 15). TAT-H3 treatment
decreased the number of high KCl-induced fusion events to
~50% that of the control level (Fig. 6A), and this reduction of
fusion events mostly occurred from previously docked granules
(Fig. 6B). To determine the mechanism for these declined fusi-
on events, we performed several experiments as follows.

It is possible that this reduction of fusion events resulted
from the decreased number of previously docked granules
caused by TAT-H3 treatment during the preincubation period.
We therefore examined whether TAT-H3 treatment by itself
could change the status of previously docked granules during the preincubation period with TAT-H3 under 2.2 mM glucose. As shown in Fig. 7, the number of insulin granules docked to the plasma membrane in control cells (~50 granules/200 μm²) did not differ from that observed in TAT-H3-treated MIN6 cells during the 60-min incubation period, indicating that TAT-H3 could not alter the number of previously docked granules without any stimulation.

Another possibility that should be ruled out is that TAT-H3-reduced fusion events from previously docked granules are associated with the change of intracellular Ca²⁺ concentration [Ca²⁺]. In neurons, the syntaxin-H3 domain interacts not only with the synaptic proteins but also with N-type voltage-dependent Ca²⁺ channels (26, 27). In pancreatic β cells, syntaxin 1 may interact with L_{βγ} and L_{γδ} type voltage-dependent Ca²⁺ channels, although this theory is still controversial (28, 29). In the present study, to rule out the possibility that TAT-H3 treatment changes [Ca²⁺], thereby inhibiting insulin release, we used the Ca²⁺-ionophore ionomycin as a secretagogue in TAT-H3-transduced MIN6 cells, which has been shown to increase cytosolic calcium in insulin-secreting cells (30). After stimulation with ionomycin (5 μM), fusion events were immediately seen from previously docked granules for 1 min in control cells (Fig. 8). In contrast, TAT-H3 treatment reduced the ionomycin-induced fusion events to ~50% that of control cells, a decline similar to that observed after KCl stimulation (Fig. 8). Thus, the inhibitory effect of TAT-H3 on fusion events was not altered by using a Ca²⁺-ionophore as a secretagogue, indicating that TAT-H3 directly acts on the exocytic process.

Finally, we addressed the question of whether TAT-H3 directly affected the kinetics of individual fusion events. During stimulation with high KCl, the fluorescence intensity of granules transiently increased, spread, and then immediately disappeared in both control cells and TAT-H3-treated cells (Fig. 9A). The time course of changes in the fluorescence intensity during fusion did not appear to be affected by TAT-H3 treatment (Fig. 9B). No difference was observed in the mean values for the rise time, half-widths, and fall time of fluorescence intensity between TAT-H3-treated and control cells (Fig. 9C), indicating that TAT-H3 alone does not modify the kinetics of fusion. Considering these results, TAT-H3 seems to prevent the insulin granule from docking to the final fusion stage, that is, the priming stage. Thus, we conclude that TAT-H3 reduces the fusion events in the first phase of insulin release by interrupting the priming stage.

**DISCUSSION**

The importance of SNARE assembly in the secretory system has been suggested using permeabilized cells and electrophysiological analysis with a microinjection system (24, 25, 31, 32). However, the physiological interaction of SNAREs in vivo with vesicular movement in the exocytotic process is yet to be determined because these systems are not suitable to resolve single granules approaching, docking, and fusing with plasma membrane during continuous secretagogue stimulation. Indeed, earlier experiments using these systems could reveal that the recombinant H3 domain inhibited neurotransmitter release by forming nonfunctional SNARE complexes (24, 25); however, these findings were limited to the analysis concerning rapid exocytosis from previously docked synaptic vesicles. To overcome these problems, we have established a GFP-labeled insulin granule system combined with evanescent wave microscopy, which is a powerful system that analyzes the single granule motion in biphasic insulin release as described previously (15). In the present study, we combined this image analysis system with the TAT PTD fusion protein system to quickly deliver the protein into living cells, which allowed us to clarify the SNARE function in docking and fusion of insulin granules that originated from both previously docked granules and newly re-
To identify the mechanism by which introduction of the H3 domain into MIN6 cells inhibits insulin release (slight decrease in the first phase and marked decrease in the second phase), we monitored the behavior of single insulin granule movement using evanescent wave microscopy. The most marked effect of TAT-H3 treatment on docking and fusion of insulin granules was observed during the second phase of insulin release. Notably, our image analysis system allowed us to visualize that the supply of newly recruited granules to the plasma membrane was strongly inhibited by TAT-H3 treatment, which caused an eventual decrease in the number of granules docked to the plasma membrane during continuous glucose stimulation. As a result, fusion events in the second phase of insulin release were markedly diminished. Thus, our image analysis showed that TAT-H3 treatment prevents docking of newly recruited insulin granules to the plasma membrane, which subsequently reduced the fusion events in the second phase of insulin release.

How does TAT-H3 inhibit the docking of newly recruited granules to the plasma membrane in the second phase of insulin release? Previous reports using in vitro binding studies demonstrated that disruption of the interaction between syntaxin and SNAP-25 by the recombinant H3 fragment caused the inhibition of the neurotransmitter release (24, 25). In the present study, we also found that TAT-H3 produced the assembled formation of a binary SNARE complex of H3 and SNAP-25. These results indicate that the H3 domain interferes with syntaxin binding to SNAP-25. Because the formation of a binary complex of syntaxin and SNAP-25 is essential before ternary SNARE assembly by the binding of VAMP-2 (34, 35), it is conceivable that the introduction of the H3 domain into cells inhibits the binding of endogenous syntaxin to SNAP-25, which prevents a subsequent binding of VAMP-2. As a result, the newly recruited insulin granules cannot dock to the plasma membrane. Of course, the possibility that the H3 domain interrupts the association of syntaxin and munc18-1 cannot be ruled out. The relationship between syntaxin 1 and munc18-1 might be important; munc18-1, a cytosolic partner of syntaxin 1, is known to function upstream of the SNARE complex and to act as a regulator of large dense-core vesicles docking (36). Possibly, the formation of the munc18-1 and syntaxin 1 complex in its closed configuration, which facilitates the docking of large dense-core vesicles (36), is interrupted by the introduction of a large molar excess of the H3 domain into cells despite the fact that the H3 domain directly cannot bind munc18-1 (37).

The transduction of MIN6 cells with TAT-H3 also inhibited fusion events in the first phase of insulin release from previously docked granules, although it did not have a marked effect during the second phase of insulin release. Because TAT-H3 treatment had no effect on 1) the number of granules previously docked to the plasma membrane without stimulation, 2) intracellular calcium concentrations, and 3) the fusion kinetics of individual insulin granule, TAT-H3-induced reduction of the fusion events from previously docked granules may result from an interference of the priming stage in insulin exocytosis. The recent model of the role of the SNARE complex in exocytosis suggests that SNARE complexes exist in a dynamic equilibrium between a loose, neurotoxin-sensitive state and a tight, neurotoxin-insensitive state (31, 38, 39). Chen et al. (31) suggest that SNAREs partially and reversibly assemble during MgATP-dependent priming in permeabilized PC12 cells. Therefore, when endogenous SNARE assembly forms a loose complex during the priming stage in insulin exocytosis, TAT-H3 may break into these flexible SNARE complexes, thereby replacing the endogenous syntaxin1 with exogenous...
evanescent wave microscopy, we were able to reveal be docked to the plasma membrane during the second phase of release and 2) strong inhibition of newly recruited granules to docked granules to be fused during the first phase of insulin MIN6 cells reduced the number of fusion events of insulin granules, such as docking and fusion in biphasic insulin release because such a nonfunctional SNARE complex cannot be disassembled in the α-SNAP/NSF-mediated process (37, 40). Thus, we propose that TAT-H3 inhibits the fusion from previously docked granules, possibly mediated by interrupting the priming stage.

In conclusion, introduction of the syntaxin-H3 domain into MIN6 cells reduced the number of fusion events of insulin granules by two mechanisms, 1) interfering with previously docked granules to be fused during the first phase of insulin release and 2) strong inhibition of newly recruited granules to be docked to the plasma membrane during the second phase of insulin release. Thus, by combining a TAT fusion protein transduction system with real-time image analysis system using evanescent wave microscopy, we were able to reveal in vitro interaction of SNARE assembly and the exocytotic process of insulin granules, such as docking and fusion in biphasic insulin release.

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