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その他のタイトル	ラット膵島の一次および逐次開口放出における Munc18bの役割に関する2光子顕微鏡画像による検討
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Abbreviated Words

*Ad*GFP: adenovirus vector including GFP
*Ad*Munc18b: Adenovirus vector including GFP gene and Munc18b
cAMP: cyclic adenosine monophosphate
CFP: cyan fluorescent protein
Diabetes: diabetes mellitus
GFP: green fluorescent protein
GLP-1: glucagon-like peptide-1
GLUT: glucose transporter
GSIS: glucose-stimulated insulin secretion
IBMX: isobutylmethylxanthine
IP: immunoprecipitation
lenti: lentivirus vector
PM: plasma membrane
SG: secretory granules
SG-SG fusion: secretory granule fuse with a secretory granule fusion
shRNA: short hairpin ribonucleic acid
siRNA: small interfering ribonucleic acid
SM (protein): Sec1/Munc18 protein
SNAP25: synaptosomal-associated protein of 25 kDa
SNARE (protein): soluble N- ethylmaleimide-sensitive factor attachment protein receptor protein
Syn: Syntaxin
VAMP: vesicle-associated membrane proteins

ABSTRACT

Sec1/Munc18 proteins facilitate the formation of *trans*-SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complexes that mediate the fusion of secretory granules (SG) with the plasma membrane (PM). The ability of pancreatic β-cells to exocytose insulin is compromised in diabetes. β-cells express three Munc18 isoforms, Munc18a, b, and c. Munc18b was found in cells of many tissues that mainly indicated secretion model is sequential exocytosis, such as platelet cell and pancreatic acinar cell. The role of Munc18b was unknown. We found that Munc18b depletion in rat islets disrupted SNARE complex formation by Syntaxin (Syn)-2 and Syn-3. Two-photon imaging analysis showed that gain-of-function expression of wild-type Munc18b or the dominant-positive K314L/R315L mutant promoted the assembly of cognate SNARE complexes, which potentiated biphasic GSIS. The latter was attributed to a more than three-fold enhancement of both primary exocytosis and sequential SG-SG fusion, including long-chain fusion (6–8 SGs), which is not normally observed (normally 2–3 SG fusion). In contrast, two-photon imaging analysis showed that Munc18b depletion in β-cells reduced primary exocytosis (SG-PM fusion) by 40% and abrogated almost all sequential exocytosis (SG-SG fusion events), accounting for a 41% reduction in glucose-stimulated insulin secretion (GSIS). Thus, Munc18b-mediated

exocytosis might be used to increase the secretory efficiency of SGs in deeper cytosolic layers of β-cells and increase primary exocytosis. Munc18b may open new avenues for the development of therapy for some of diabetes patients characterized by a dysfunction of insulin secretion.

INTRODUCTION

Glucose stimulation of islet β-cells triggers an initial robust first phase of glucose-stimulated insulin secretion (GSIS), followed by a diminished but sustained second phase of GSIS. In type 2 diabetes, the islet insulin secretory capacity cannot meet the increasing insulin demand caused by insulin resistance. β-cells eventually decompensate with loss of first-phase GSIS, and second-phase GSIS becomes defective (1).

Although previous studies have identified the molecular circuitry underlying β-cell stimulus-secretion coupling (reviewed in 2,3), the precise molecular determinants of the complex steps of insulin SG exocytosis at the plasma membrane (PM), termed primary exocytosis, remain unclear. In mast cells, eosinophils (4,5), and pancreatic acinar cells (6), rapid and extensive sequential SG-SG fusion accounts for the cells' high secretory efficiency. Sequential SG-SG fusion also occurs in β-cells, but at a reduced frequency (1.9%–4.5%) and to a reduced extent (only 2–3 SGs) (7,8). Much less is known about the molecular machinery driving SG-SG fusion in β-cells.

The membrane fusion machinery requires two key components: SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins and Sec1/Munc18 (SM) proteins (9). The SNARE paradigm dictates that cognate vesicle

(v)-SNAREs (vesicle-associated membrane proteins [VAMPs]) and target membrane (t)-SNAREs (syntaxins [Syn] and synaptosomal-associated protein of 25 kDa [SNAP-25]) assemble into complexes that mediate different fusion events. The assembly of distinct SNARE complexes is regulated by cognate SM proteins to ensure subcellular compartmental specificity and that fusion only occurs in response to cellular needs and demands (3,10). Of three SM proteins, Munc18a is the best studied (10–12). Its roles include orchestrating SG docking and priming (13,14), chaperoning and inducing the activated conformation of Syn-1A (15–17), and facilitating membrane fusion (18,19). In β-cells, much is known about the roles of Munc18a and Syn-1A in mediating insulin exocytosis (20,21). Munc18c and the cognate molecule Syn-4 mediate GLUT-4 translocation to the PM in adipose tissue and muscle and also mediate some aspects of GSIS (3). Relatively little is known about Munc18b, which preferentially binds Syn-2 and Syn-3 (22).

Here, my collaborators, Dr. Gaisano and his colleagues, examined the expression and localization of the Munc18 proteins in pancreatic β-cells and INS-1 cells using western blot and confocal microscopy with fluorescent antibodies. Rat islets and INS-1 cells expressed all three SM proteins (Munc18a, Munc18b, Munc18c), the four cognate syntaxins (Syn-1A, -2, -3, -4), SNAP-25, VAMP-2, and VAMP-8 (Appendix

Fig. 1). Syn-1A and Munc18a, which drive primary exocytosis (21), were abundant in the PM. Munc18c is thought to localize to the PM (23). On the other hand, Munc18b, Syn-2, and Syn-3 localized on insulin SGs, though not exclusively (Appendix Fig. 2).

With regard to insulin secretion, they examined the effects of deleting Munc18b and overexpressing Munc18b wild-type and mutant proteins, which either facilitated (Munc18b-K314L/R315L [Munc18b-KR]) or suppressed (Munc18b-E59K) SNARE assembly in INS-1 cells, rat insulin-secreting cell line. In radioimmunoassays (RIA), depletion of Munc18b (86%, on average) with two siRNAs (siMunc18b nos. 1 and 2) in INS-1 cells decreased 10 mM GSIS by ~40% (data not shown). Next they used lenti-shRNAs (co-expressing eCFP) to reduce Munc18b expression in rat islets (24 h, 32%; 48 h, 75%; and 72 h, 97%; all $P < 0.05$; Appendix Fig. 3A). GSIS with 16.7 mM glucose was assessed using the islet perifusion assay. Munc18b depletion resulted in a 44% reduction in GSIS (area under the curve [AUC] analysis: Munc18b shRNA, 25.79 ± 2.49 vs. control, 45.54 ± 2.37; $P < 0.001$). The addition of cAMP-acting GLP-1 (10 nM with 150 μM IBMX) potentiated GSIS approximately three-fold (Appendix Fig. 3B). Munc18b depletion reduced the GLP-induced potentiation by ~52% (Munc18b shRNA, 68.63 ± 19.3 vs. control, 132.14 ± 17.15; $P < 0.05$). IBMX, a phosphodiesterase inhibitor that increases intracellular cAMP levels, was added with

GLP-1 to improve consistency. The results from shRNA in rat islets accorded with siRNA results in INS-1 cells.

My collaborators, Dr. Kauppi and her colleagues, previously reported that Munc18b-WT preferentially binds Syn-2 and Syn-3 and that Munc18b mutants generated by them exhibit weaker binding (Munc18b-KR) or no binding (Munc18b-E59K) to the syntaxins (22), mentioned the details of these mutatnts later in this section. They subcloned these proteins, along with enhanced GFP to identify transfected cells (for two-photon studies), into adenoviral vectors to transduce islets and INS-1 cells. *Ad*Munc18b-WT or mutants expression were ~4–5 times higher than that of endogenous Munc18b expression (Appendix Fig. 4A). The recombinant proteins were expressed in 60%–70% of islet cells (based on visualization of GFP with confocal microscopy). Syn-2 and Syn-3 localization to insulin SGs was not affected by overexpression of any of the three Munc18b proteins (Appendix Fig. 4) (analysis with Pearson correlation (24); data not shown here). Moreover the protein and mRNA expression of endogenous SNAREs and Munc18s was unaffected in transfected islets. Then they examined the effects of the *Ad*Munc18 and *Ad*Munc18b mutants on biphasic 16.7 mM GSIS (Appendix Fig. 5A) and 16.7 mM GSIS with GLP-1 and IBMX (Appendix Fig. 5B). The AUC of insulin secretion on potentiated GSIS was

approximately four- to five-fold higher than that on 16.7mM GSIS without accelerants. *Ad*Munc18b-WT potentiated first-phase GSIS (12–22 min) by 63% (20.1 ± 2.2 vs. *Ad*GFP 12.3 ± 1.6; $P = 0.03$) and second-phase GSIS (22–40 min) by 43% (36.0 ± 1.6 vs. *Ad*GFP 25.2 ± 1.8; $P = 0.02$). *Ad*Munc18b-KR potentiated first-phase GSIS by 95% (24.0 ± 3.1; $P = 0.003$) and second-phase by 83% (46.2 ± 4.2; $P = 0.003$). The effect of *Ad*Munc18b-KR was stronger than that of *Ad*Munc18b-WT. *Ad*Munc18b-E59K was expected to inhibit GSIS, but did not cause a significant reduction (10.1 ± 2.4 and 21.5 ± 3.6 for first and second phases, respectively). The effects on first-phase and second-phase potentiated GSIS (Appendix Fig. 5B) were remarkably similar to those observed with 16.7 mM GSIS. GSIS in cells expressing *Ad*Munc18b-KR (87.0 ± 1.4 and 257.4 ± 5.5) was higher than in those expressing *Ad*Munc18b-WT (56.6 ± 3.4 and 198.1 ± 20.9), which in turn was higher than in cells expressing *Ad*GFP (46.4 ± 3.4 and 136.1 ± 4.3). Potentiated GSIS in cells expressing *Ad*Munc18b-E59K (40.7 ± 3.9 and 162 ± 12.3) and GFP was not significantly different. The effects of the Munc18b proteins on GSIS in INS-1 cells (Appendix Fig. 5B) were similar to those in islets. Basal insulin release was not affected by the *Ad*Munc18b mutants. *Ad*Munc18b-KR expression potentiated GSIS (10 mM; determined as stimulated minus basal values) by 98% (2.14% ± 0.09% vs. *Ad*GFP 1.08% ± 0.80%; $P < 0.05$). *Ad*Munc18b-WT

expression potentiated GSIS by 48% (1.60% ± 0.11%; $P < 0.05$), thus to a lesser extent than *Ad*Munc18b-KR ($P = 0.007$). *Ad*Munc18b-E59K had no significant effect (0.86% ± 0.05%). Potentiated GSIS (10 nM GLP-1 plus 150 μM IBMX) (Appendix Fig. 4B) was approximately four times higher than 10 mM GSIS and had a trend similar, wherein *Ad*Munc18b-KR (7.71% ± 0.59%) and *Ad*Munc18b-WT (5.99% ± 0.19%) increased secretion by 68% and 30%, respectively, over that induced by *Ad*GFP (4.60% ± 0.16%; $P < 0.05$), with *Ad*Munc18b-KR inducing higher secretion than *Ad*Munc18b-WT ($P = 0.012$). *Ad*Munc18b-E59K (4.98% ± 0.22%) did not have a significant effect when compared with *Ad*GFP. Thus, the effects of Munc18b knockdown and the Munc18b mutants on GSIS were similar in INS-1 and β-cells.

Then they examined protein pull down assay to elucidate the relation between Munc18b and Syn, and the formation of distinct SM-activated SNARE complexes. The INS-1 cell line was used as a surrogate for β-cells to provide the amount of protein required for protein binding studies. The coimmunoprecipitation experiments were performed with antibodies to Syn-1A, Syn-2, and Syn-3 using lysates from INS-1 cells transfected with scrambled siRNA (control) or Munc18b-siRNA and cultured under non-stimulatory (0.8 mmol/L glucose) or maximal stimulatory conditions (preincubation with GLP-1 and IBMX followed by incubation with 16.7 mM glucose) (Appendix Fig.

6, *left panels*, coimmunoprecipitation; *right panels*, total lysate input controls). In control scrambled siRNA-treated cells under non-stimulatory conditions, Syn-2 (Appendix Fig. 6B) and Syn-3 (Appendix Fig. 6C) bound Munc18b, but little binding to VAMPs or SNAP25 was observed. Only upon stimulation, which presumably activates Munc18b, did we observe formation of full and distinct SNARE complexes, with Syn-3 preferentially binding VAMP8 and Syn-2 binding both VAMPs (VAMP2 > VAMP8). These results are consistent with our previous findings that VAMP8 forms complexes with Syn-2 and Syn-3 (but not Syn-1), which mediates the primary exocytosis of newcomer insulin SGs (25) and SG-SG fusion. The latter was also observed in pancreatic acinar cells (26). Remarkably, Munc18b depletion with siRNA nearly prevented Syn-2 (Appendix Fig. 6B) and Syn-3 (Appendix Fig. 6C) from forming complexes with VAMPs and SNAP25. The disruption of SNARE complexes coimmunoprecipitated with the Syn-3 antibody matched the ~80% reduction in Munc18b levels analysed by quantitative densitometry from Appendix fig. 6; the disruption of SNARE complexes coimmunoprecipitated with the Syn-2 antibody was slightly lower (~70%) (Appendix Fig. 6B). This is remarkable considering the total cellular levels of the SNARE proteins in the cells, as shown in the inputs. The control cells were unaffected, and the proteins were expected to be available to participate in

SNARE complex formation. Munc18b depletion did not affect the amount of coimmunoprecipitated Munc18a, indicating that formation of SNARE complexes by Syn-2 and Syn-3 is primarily mediated by Munc18b. In contrast, Syn-1A coimmunoprecipitated endogenous Munc18a and formed complexes with SNAP25 and VAMP2 (Appendix Fig. 6A) in spite of Munc18b depletion, indicating that Munc18a but not Munc18b is the primary SM protein to activate Syn-1A to form SNARE complexes. Furthermore, the association of Munc18b with native β-cell syntaxin within assembled SNARE complexes was unaffected by Munc18b-WT or Munc18b-KR expression under basal and stimulatory conditions but was compromised by Munc18b-E59K expression (Appendix Fig. 7). These results differ from those of previous IP study (22), in which Munc18b-KR exhibited reduced association and Munc18b-E59K displayed almost no binding to uncomplexed recombinant syntaxin. This emphasizes the importance of analyzing mutants in native cells, where other exocytotic protein cooperate with SNARE and SM proteins on insulin exocytosis, because we also showed that the number of SM-activated SNARE complexes increased under stimulated conditions. From *Ad*Munc18b-KR-expressing and *Ad*Munc18b-WT -expressing cells, more SM-activated SNARE complexes were precipitated than from control cells. Munc18b-KR associated with Syn-1A, Syn-2, and Syn-3 SNARE

complexes to a greater extent than did Munc18b-WT. Syn-3, which putatively mediates SG-SG fusion (26), formed a complex with Munc18b to immunoprecipitate VAMP8 exclusively, along with SNAP25 (Appendix Fig. 7C). A caveat of these coimmunoprecipitation studies is that syntaxin antibodies might pull down SM proteins independently from SNARE complexes. Because Munc18b also binds Syn-1A (27), Munc18b formed SNARE complexes with Syn-1A, SNAP25, and, preferentially, VAMP2 (Appendix Fig. 7A). These actions of Munc18b mutants on Syn-1A mimicked that of Munc18a. Thus, Munc18b likely has functions similar to those of Munc18a in mediating primary exocytosis. The induction of SM-SNARE complex formation by Munc18b proteins followed the order KR > WT > GFP > E59K (detailed data analyzed from Appendix Fig. 7 were not shown here, but in Supplementary Fig. 4 of our published article in *Diabetes* 2013 (24)), corresponding to the abilities of the proteins to potentiate biphasic GSIS (Appendix Fig. 5A). The role of Syn-2 in SNARE complex formation and in the broader field of exocytosis is less clear. The fact that Munc18b proteins (KR > WT) induce Syn-2 to form SNARE complexes with VAMP2 and VAMP8 (Appendix Fig. 7B) and that such complexes are reduced by Munc18b depletion (Appendix Fig. 6B) suggests that Syn-2 might have functions redundant with those of Syn-1A and Syn-3.

Munc18b-E59K bound the syntaxins under basal conditions, but upon stimulation, it did not induce the syntaxins to bind SNAP25 or VAMPs. In addition, the levels of SM-activated SNARE complexes in cells expressing Munc18b-E59K were similar to or lower than those in cells expressing *Ad*GFP. Munc18b-E59K/Syn complexes were essentially functionally inert; they did not markedly compete with endogenous Munc18b in any function essential for secretion. It was concerned that this explains why GSIS in both Munc18b-E59K-expressing cells and GFP-expressing control cells did not indicate a significant change of reduction (Appendix Fig. 5) and why E59K does not work as dominant-negative.

The aforesaid results from perifusion assay and protein assay (24) lead to three points: the post-stimulus assembly of SNAREs in response to glucose stimulation, which is consistent with the formation of SNARE complexes (28,29) in insulin exocytosis, the potentiation of assembly by Munc18b, which is similar to the result of liposome fusion assay of Munc18a associated with SNARE proteins (19), and the role as a positive regulator of Munc18b, as mutated Munc18b that cannot interact with syn-11 cause Familial hemophagocytic lymphohistiocytosis type 5 (FHL5), showing impaired degranulation in NK (Natural killer) cells and CTL cells (cytotoxic T lymphocyte), and severely impaired platelet α (CD 62P)– and δ (CD 63)–granule

secretion in response to thrombin stimulation from FHL-5 patients (30,31).

Munc18b is abundant in the cells that characterized in a sequential secretion with long duration of secretion time scale in many hours, which has Munc18b, Syn-2, Syn-3, SNAP23, VAMPs, such as epithelial cells (e.g. airway and pancreas acini) and platelet. I wonder why Munc18b exhibit in insulin-secreting cells, i.e. INS-1 cell line and rat islets, where exocytosis model is mainly primary exocytosis. I made an assumption that Munc18b worked on only sequential exocytosis on limited frequency when Munc18a worked on only primary exocytosis, and two isoforms shared the respectively different functions. Overexpression of Munc18b may be capable of treating diabetes patients caused by dysfunction of insulin secretion. These assumptions led the idea that the augmentation of insulin exocytosis shown perifusion assay in overexpressed Munc18b islets consisted of the augmentation of sequential exocytosis, and imaging of sequential exocytosis, it might be two-SG sequential exocytosis, was more available in two photon microscopy. Now I used two mutants of Munc18b which enhanced or declined the formation of SM-SNARE complex, Munc18b-KR or Munc18b-E59K, the same products as the above mentioned results.

The residues of 314 and 315 in Munc18b are located in a solvent accessible loop at domain3a, forming a hydrogen bond to Glu-170 of Syn-3 (22). PKC-induced

phosphorylation of neighbor Ser-313 which is conserved in Munc18b, reduced binding to syntaxin1 (maybe closed syntaxin) for neurotransmitter release (32). It was reported that the 226-314 residues in Munc18a interact with Mint-1 (Munc18-interacting protein1, recruit Munc18a to the region with neurexin rich for exocytosis, however Mint-1 and Mint-2 bind to Munc18a but not to Munc18b (33,34,35). Munc18b K314L/R315L double mutant (Munc18b-KR), changed lysine and arginine to leucine brought about changing of the characterize in solvent loop to hydrophobic by Olkkonen et al., was demonstrated that it caused a markedly reduction of binary with Syn-3, an increment of SNAP23 binding to Syn-3, and a possibility of augmentation in secretion, in vitro IP and in vivo caco-2 cell, which is human epithelial colorectal adenocarcinoma cell line (22). Binding protein assay with each isoform of closed Syntaxin showed that Munc18b-WT bind to Syn, (Syn-2 > Syn-3 > Syn-1A), Munc18b-KR bind less than half of WT (Syn-2 > Syn1 > Syn-3), in accordance with the IP result that showed weaker binding of *Myc*-Munc18b-KR to Syn-2 and no binding of *Myc*-Munc18b-E59K to Syn-2 and Syn-3 in CHO cells, Chinese Hamster Ovary cells, which has Syn-2 and Syn-3 endogenously (36).

The residue E59 of Munc18a, whish interact with arginine-114 in Habc domain of Syn-1A by salt bridge (37,38). Munc18b-E59K was produced by changing glutamic

acid to lysine, which cannot interact with Syn because of changing the charge from negative to positive. Binding protein assay with each isoform of closed Syntaxin showed that Munc18b-E59K almost deplete (22). A recent result of Munc18a-EK showed the reduction of affinity to SNARE complex and severe defect in neuronal release, consistently similar mutant Munc18a-E59R by changing charge represented a reduction of the fusion rate in SNARE mediated liposome-fusion (18). The mutant of arginine-114 in Syn-1A for unavailable interaction to Munc18a (or Munc18b) was not affected the fusion rate, therefore it supposed that the interaction between Munc18a (or Munc18b) and SNARE complex is necessary for SNARE mediated fusion through E59 of Munc18b, not via Habc domain of Syntaxin (13, 39). These residues are well conserved in both Munc18a and Munc18b. It is crucial that membrane fusion need an interaction between Munc18 and SNARE 4 herix bundle (19,40,41,42). Our results from Munc18-SNARE complex assembly after stimulation were consistent with above previous conclusions. Thus I used Munc18b-KR mutant as a mediator of the formation of activated SM-SNARE complexes, which intend to fusion strongly. Overexpressed Munc18b-E59K was used for suppressed function system. However it did not have an enough effect on insulin secretion due to endogenous Munc18b. Next I examined by shRNA to deplete endogenous Munc18b.

To examine how Munc18b modulates the dynamics of insulin vesicle exocytosis, I used two-photon microscopic imaging of exocytosis in β-cells of rat islets in which Munc18b had been overexpressed. A gain-of-function expression of Munc18b-WT or the Munc18b-KR mutant, which promotes SNARE complex assembly (22), greatly increased the two kinds of exocytotic events, as wells as the number of SGs involved in sequential SG-SG fusion. In contrast, I found that Munc18b contributed to 40% of primary exocytosis events and to all sequential SG-SG fusion events from depletion of endogenous Munc18b experiments. These results suggest that Munc18b plays an important role in both primary exocytosis and sequential exocytosis, and they are consistent with the idea that Munc18b facilitates insulin exocytosis through the post-stimulated formation of Munc18b/Syn-2 and Munc18b/Syn-3 SM/SNARE complexes. In addition, my results also indicate that Munc18b promotes the sequential SG-SG fusion of three or more insulin vesicles in warm environment by glucose stimulation with accelerants, especially including of “compound exocytosis”.

MATERIAL AND METHODS

Rat islet isolation for two-photon microscopy

Islets from Sprague Dawley rats (275–300 g) were isolated by collagenase digestion as described previously (43). The intact islets were infected with lentivirus carrying Munc18b-shRNA or *Ad*Munc18b constructs. For two-photon microscopy assays, the preparations were cultured under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium supplemented with 10% FBS, 100 μU/ml penicillin, and 100 mg/ml streptomycin for 48 or 24 h before the assay. The cells were stimulated with glucose in the presence of 10 nM GLP-1 (Sigma-Aldrich, Shinagawa, Tokyo, Japan) and 150 μM IBMX (Sigma-Aldrich), as indicated. The experiments were approved by the Animal Care Committees of the University of Tokyo.

Silencing Munc18b expression and adenovirus construction

Endogenous Munc18b was silenced by lenti-shRNA in islets. For construction of lentiviruses, the pLKO-Munc18b-eCFP plasmid was created by modifying the previously described parental pLKO-Munc18b-puro plasmid (15). The puromycin-resistance gene was replaced with eCFP. The knockdown plasmid was co-transfected with psPAX2 and pMDG2 into HEK-293FT cells for the generation of recombinant lentiviruses, lenti-shRNA/Munc18b-eCFP and lenti-eCFP (control).

For overexpressed experiment, I used amplified adenoviruses including Munc18b-WT or Munc18b mutants tagged with GFP in a separate transcription cassette (22), given by Dr. Gaisano, my collaborator.

Two-photon microscopy

Exocytosis in rat islets was imaged with an inverted microscope (IX81; Olympus, Tokyo, Japan) and a laser-scanning microscope (FV1000; Olympus, Tokyo, Japan) equipped with a ×60 (NA = 1.2) water-immersion objective lens and a femtosecond laser (MaiTai; Spectra Physics, Mountain View, CA, USA), as previously described (28). GFP, CFP, and Alexa Fluor® 594 Hydrazide (Alexa 594, a polar fluorescent tracer; Molecular Probes, Life Technologies, Tokyo, Japan) were excited at 830 nm. The emitted fluorescence of GFP, CFP, and Alexa594 was collected through 525/25BP, 490SP+570SP, and 605/35BP filters, respectively, because the emitted fluorescence of the fluorophores peaks at 509 nm, 477 nm, and 617 nm, respectively.

Two-photon extracellular polar-tracer (TEP) imaging

TEP imaging was performed to visualize the spatiotemporal patterns of exocytosis, as previously described **(**44,45**)**. In short, glucose-stimulated insulin exocytosis was

imaged with a solution containing Alexa 594 (0.3 mmol/L) (7) in the presence of 10 nM GLP-1 and 150 μM IBMX. The temperature of the preparation was maintained at ~32°C using a heating stage incubator (Tokai Hit Co, Ltd. Shizuoka, Japan). The frequencies of exocytosis were normalized to the value per 800 μm^2 (≈ per cell). The structures of the islets were determined by placing Alexa 594 in the extracellular medium. The probe rapidly diffused into the intercellular spaces, but did not readily permeate the plasma membrane. Fluorescent puncta represented individual exocytosis events where the Alexa 594 tracer rapidly diffused into the internal cavity of a SG upon opening of the fusion pore, which has a diameter of 1.4 nm (8), thus allowing passage of Alexa 594, which has a minimum length of 1.4 nm (46). The vesicles were confirmed to be positive with anti-insulin antibody in a previous study (8).

Detection of sequential exocytosis in TEP imaging

Sequential exocytosis occurred far more frequently than predicted from coincidental full fusion of a vesicle followed by another vesicle. Such coincidental events, occurring within 1 min and within 0.5 μm laterally *(b)* and 1 μm axially (T = 2 μm) from the initial event, can be estimated from the frequency of exocytosis per membrane area *(a)* using Poisson statistics, as previously described (7). Given exocytosis frequencies of 7.7,

14.1, 26.4, and 5.4 events·cell^{-1}·min^{-1} in adenovirus (*Ad*)GFP-, *Ad*Munc18b-WT-, *Ad*Munc18b-KR-, and *Ad*Munc18b-E59K-expressing islets, respectively, the proportions of random simultaneous events were estimated as 0.12–0.51, 0.22–0.92, 0.42–1.73, and 0.086–0.36%, respectively, using the following equations (7):

$$\lambda = \pi a b^2 \quad \ldots (1.1)$$

Considering the slope of the plasma membrane relative to the focal plane, λ is predicted to be between two data from (1.1) and (1.2).

$$\lambda = \pi a b \sqrt{b^2 + (T/2)^2} \quad \ldots (1.2)$$

Then, the proportion of random simultaneous events was estimated as below.

$$p = \left(\frac{1}{2}\lambda^2 e^{-\lambda}\right)/\lambda \quad \ldots (1.3)$$

Frequency distribution of SG diameters estimated by ΔV-TEP-IQ (image-based quantification) analysis of TEP imaging

Using the ΔV-TEPIQ method, the fluorescence intensity of the vesicle (F_V) was divided by F_E, representing the fluorescence intensity per unit area in an xy-image of a solution with an infinite depth, as $V = F_V/F_E/P_{xy}(0)$, where $P_{xy}(0)$ is the efficiency of focal illumination (0.56 in my setup). The diameters of the SGs were calculated from the volumes of the SGs (47).

Statistical analysis

All data were presented as the mean ± SEM. Student's *t*-test was used to compare two variables, and ANOVA followed by Scheffé's test or Tukey's test was used for multiple parametric comparisons. The Mann-Whitney's U test and Steel-Dwass test were applied for nonparametric comparisons. Significance was set at $P < 0.05$.

RESULTS

Munc18b gain-of-function promotes sequential SG-SG fusion and primary exocytosis

Although Munc18b may activate SNARE complex assembly, little is known about how these proteins function in GSIS in rat pancreatic islets or about their differential involvement in primary and secondary exocytosis. Sequential insulin SG-SG fusion has been visualized with two-photon imaging in β-cells (8). The technique is more suitable for the spatial and temporal imaging of sequential exocytosis and the analysis of its dynamics than other imaging tools such as electron microscopy (EM), confocal laser scanning microscopy (CLSM), and total internal reflection fluorescence microscopy (TIRFM) because it can capture the dynamics of insulin exocytosis in intact live islets. The process of sequential exocytosis might be mediated by the diffusion of SNAP25 from the PM into sequentially fused SGs after the assembly of SNARE complexes (7). I postulated that Munc18b-activated SNARE complexes containing Syn-2 or -3 might increase sequential SG-SG fusion. To test this idea, I used two-photon imaging analysis to examine whether overexpression of Munc18b mutants affected primary exocytosis and SG-SG fusion. To maximally potentiate 20 mM glucose-stimulated insulin exocytosis, I used GLP-1 plus IBMX, which also potentiates sequential SG-SG

exocytosis (43). After confirming that no morphological abnormalities were present in adenoviral vector-transfected islets by extracellular polar-tracer Alexa, I examined GSIS with TEP imaging.

First, I visualized single SG fusion and sequential SG-SG exocytosis described at “TEPimaging” in “Materials and Methods.” The imaging was taken by a fluorescence intensity of Alexa fluorescence from intercellular spaces and innercellular puncta which was now collapsing exocytic SG. Fig. 1 shows the full-fusion exocytosis of three single SGs (Fig. 1Ba, b, c from inset in Fig. 1Aa, b, c) in *Ad*GFP-transduced rat islets. In Fig. 1C, the vertical lines indicate the times when the images in Fig. 7B were acquired. The fusion of an insulin SG occurred at 1.2 s (white arrow in the second image of Fig. 7B indicates the direction of fusion), discharge of contents occurred at 2.4–4.9 s (dotted circle in Fig. 1B, right), and the SG collapsed into the PM for full fusion, which is schematically illustrated in Fig. 1D. Each islet transduced with Munc18b or a Munc18b mutant exhibited this type of single SG exocytosis, a universal style of insulin exocytosis in murine islets transfected with GFP or *Ad*GFP. The most frequent mode of sequential exocytosis observed involved two SGs. An example is shown in Fig. 2B in an *Ad*Munc18b-KR-transduced islet (Fig. 2B, inset in Fig. 2A). Here, the first SG fused with the PM at 1.3 s (arrow 1 indicates the direction of fusion) and then partially

emptied (dashed circle at 4.4 s). A second SG, coming from another direction (arrow 2) and from deeper in the cytosol than the first SG, fused <2 s later, at 5.6 s. The area of fluorescence intensity was larger and brighter (temporal analysis in Fig. 2C) because it emanated from the fusion of two SGs, and the decline in fluorescence was slower than that shown in Fig. 1C, reflecting insulin cargo emptying and the eventual collapse of the compound granule into the PM (Fig. 2D). Two-granule sequential exocytosis was identified in islets expressing Munc18b or Munc18b mutants as well as in control cells expressing *Ad*GFP.

Using this analysis, I counted the total number of exocytotic events as determined by number of omega figures (exocytotic events·cell^{-1}·min^{-1} during 10 min [Fig. 3A]). Islets maintained the secretion of insulin after glucose stimulation with or without accelerants. Stable secretion over time was similar in each transfected group (Fig. 3A). Exocytosis within 2, 4, 5, and 7–10 minutes was higher in cells expressing WT Munc18b than in cells expressing *Ad*GFP. Exocytosis in cells expressing Munc18b-KR was higher than in cells expressing *Ad*GFP at all times and higher than in Munc18b-WT-expressing cells at most times. Exocytosis in Munc18b-E59K-expressing cells was lower than in Munc18b-WT or -KR transfected cells at all times, except for 9 minutes in WT transfected cells, however exocytosis in E59K was similar to in *Ad*GFP.

Primary events sustained exocytosis (Fig. 3B). Sequential exocytosis occurred anytime (Fig. 3C).

Analysis of TEP imaging (Fig. 4A) showed that *Ad*Munc18b-KR induced the most exocytotic events within 10 minutes (26.39 ± 2.89, 58 cells/6 islets), followed by *Ad*Munc18b-WT (14.06 ± 1.39, 44 cells/7 islets), *Ad*GFP (7.71 ± 0.89, 68 cells/8 islets), and *Ad*Munc18b-E59K (5.40 ± 0.53, 75 cells/5 islets). I then examined how many of these exocytotic events involved sequential exocytosis (Fig. 4B). The rate of sequential exocytosis induced by the expression of *Ad*Munc18b-KR (11.2% ± 1.4%) and *Ad*Munc18b-WT (7.1% ± 1.7%) was ~3.2 and 2.0 times higher, respectively, than that induced by *Ad*GFP expression (3.5% ± 1.0%; $P < 0.05$). Sequential exocytosis constituted only 1.6% ± 0.6% of the total exocytotic events in *Ad*Munc18b-E59K-expressing β-cells. I assessed the percentage of cells undergoing sequential exocytosis (versus no sequential exocytosis). The percentage was higher in *Ad*Munc18b-KR-transduced (74.1%) and *Ad*Munc18b-WT-transduced (54.5%) cells than in *Ad*GFP-transduced (30.9%) and *Ad*Munc18b-E59K-transduced (13.3%) cells. These results indicate that the number of β-cells in which sequential exocytosis occurred increased upon expression of Munc18b-KR and Munc18-WT (Fig. 4C).

In *Ad*Munc18b-KR-infected β-cells, sequential SG-SG fusion accounted for

11.2% of the exocytotic events. Thus, primary exocytosis accounted for the majority, ~89%, of the increase in total exocytotic events observed upon expression of *Ad*Munc18b-KR (Fig. 4A). With *Ad*Munc18b-WT expression, primary exocytosis accounted for 93% of the total exocytotic events. Therefore, it appears that a large increase in primary exocytosis and SG-SG fusion accounts for the overall increase in biphasic GSIS (Appendix Fig. 5A) resulting from Munc18b-KR and Munc18b-WT overexpression. Consistent with the results in Fig. 4A and B, Munc18b-E59K expression did not have a significant dominant-negative effect on exocytosis by competing with endogenous Munc18b and thus did not cause a detectable reduction in GSIS (Appendix Fig. 5A, B) and sequential fusion events.

Munc18b mediates long-chain sequential SG fusion

Long-chain sequential exocytosis is the mode of exocytosis in highly efficient secretory cells such as mast cells (48), eosinophils (5), and pancreatic acinar cells, but not in less efficient cells such as β-cells. The molecular mechanism underlying long-chain sequential exocytosis is unknown. I postulated that upregulation of Munc18b expression or activity would increase the number of SGs participating in long-chain sequential SG-SG fusion. Therefore, I assessed the proportion of sequential SG fusion events with respect to chain length, as determined by the number of omega figures (Fig. 5). Higher

order long-chain sequential SG-SG fusion of up to five SGs was detected in *Ad*Munc18b-KR-transduced and *Ad*Munc18b-WT-transduced cells. In *Ad*GFP- and *Ad*Munc18b-E59K-expressing cells, sequential SG-SG fusion involved no more than two SGs.

I occasionally detected very-long-chain sequential SG fusion in *Ad*Munc18b-KR-expressing β-cells, with sequential fusion of seven insulin SGs (positions indicated by dashed circles in Fig. 6A). The time course of this exocytotic event showed an initial delay in the fusion of the second SG (Fig. 6B, numbers correspond to those in Fig. 6A), followed by rapid successive fusions through the fifth SG. The diameters of the puncta increased with each successive fusion. Events 2, 3, and 4 were associated with abrupt changes in the shapes of the compound SGs and with increases in fluorescence. The direction of exocytosis (Fig. 6A, indicated by arrows) of the individual SGs varied from event to event. The focal plane did not change during the acquisition of images (see Fig. 6C): low magnification images demonstrated that the landmarks did not change. The rapid successive fusion might be attributable to “primed” insulin SGs (third to fifth SGs), and the resulting sustained plateau of fluorescence intensity (Fig. 6B) might reflect a slower flattening of compound SGs. The positions of the compound SGs (point of highest fluorescent intensity) tended to shift at each

sequential SG-SG fusion (dashed circles, Fig. 6A). This suggests that the fusion pores of primary SGs were stably maintained even though the positions of the SGs were altered by subsequent sequential fusion events.

I investigated whether the average diameter of the SGs undergoing sequential exocytosis was same as that of SGs undergoing primary exocytosis. In islet cells transduced with Munc18b-WT, primary and sequential SGs were almost same size. Similarly, primary SGs and secondary sequential SGs were almost same size in *Ad*Munc18b-KR-trancduced cells, *Ad*Munc18b--E59K-transduced cells, and control *Ad*GFP-transduced cells. However tertiary and subsequent SGs were larger in a diameter than primary and secondary SGs. Nevertheless, such long-order exocytosis was detected only in Munc18b-WT- or Munc18b-KR-expressing cells. The diameter of the SGs was about 0.3 μm, when estimated from the distinct increase in the fluorescence profile of the omega figures, as shown in Fig. 7. This value is consistent with values determined from the analysis of insulin SG size with EM (49) and TEP imaging (7), supporting the interpretation that increases in fluorescence in the present two-photon assay genuinely reflected exocytosis of insulin SGs. Tertiary and subsequent SGs were combined for analysis because of their low frequency of detection. The mean diameter of these SGs in Munc18b-WT- or Munc18b-KR-transduced cells was 361 nm and 377

nm, respectively, larger than the diameter of primary and secondary SGs. The estimation (Fig. 8) showed that the volume of tertiary and subsequent SGs was about 1.7 times greater than that of primary and secondary SGs. This suggests that tertiary and later SG fusion events involve fusion of vesicles generated by the fusion of two or more SGs.

With regard to the latency time between sequential exocytotic events (Fig. 9A), there was no difference in the latency between the first and second sequential exocytotic events in *Ad*Munc18b-KR- and *Ad*Munc18b-WT-expressing cells vs. control and *Ad*Munc18b-E59K-expressing cells. In *Ad*Munc18b-KR- and *Ad*Munc18b-WT-expressing cells, the latency between the second and third sequential exocytotic evens (Fig. 9B) and between subsequent sequential exocytotic events (Fig. 9C) was approximately 40 s, similar to the latency between the first two sequential exocytotic events, suggesting that primary exocytosis and secondary exocytosis are induced by the same mechanisms: the assembly of SNARE proteins, which are not assembled before stimulation.

Depletion of endogenous Munc18b by shRNA reduces primary exocytosis by 50% and abrogates sequential SG-SG fusion

I used Munc18b-E59K as a negative control because the E59K mutation is thought to

disrupt the interaction with the SNARE complex (Appendix Fig. 7), but it did not sufficiently suppress insulin exocytosis (Fig. 4).

Finally, I examined the effect of endogenous Munc18b depletion on primary and sequential exocytosis in β-cells. A decrease in exocytosis upon depletion of endogenous Munc18b with lenti-Munc18b-shRNA (50) would support a role for Munc18b in potentiating exocytosis. I used a construct in which shRNA-Munc18b was linked to the fluorescent protein eCFP. Forty-eight hours after shRNA transfection of islets, we compared exocytotic events in eCFP-expressing/Munc18b-depleted β-cells and control lenti-eCFP-expressing β-cells. The total number of exocytotic events (Fig. 10A) in Munc18b-depleted (67 cells/8 islets) and control β-cells (48 cells/8 islets) over a 10-min period of stimulation was 6.10 ±0.50 and 9.47 ± 0.65, respectively, representing a reduction of ~40% ($P < 0.001$). With depletion of Munc18b, the number of exocytotic events/cell (Fig. 10B) decreased by 35% in the first 5 min (49.4 ± 7.9 vs. 31.9 ± 4.2 for control and Munc18b shRNA, respectively; $P < 0.05$) and by 41% in the second 5 min (44.2 ± 8.8 vs. 26.0 ± 5.1; $P < 0.05$) (Fig. 10B, *right panel*). The extent and pattern of the sustained reduction in exocytosis presented a corresponding image of GSIS in *Ad*Munc18b-WT- or *Ad*Munc18b-KR-expressing islets (Fig. 3A). I determined the number of exocytotic that involved sequential exocytosis (Fig. 10C). Sequential

exocytotic events in control β-cells constituted only 3.57% ± 0.59% of the total number of exocytotic events, consistent with a previous report (7). In contrast, Munc18b depletion reduced the percentage to 0.64% ± 0.22%, an 83% reduction ($P < 0.001$), indicating that sequential exocytosis was almost totally abrogated.

DISCUSSION

This study demonstrated a role for Munc18b in insulin SG exocytosis as an accelerator and a prerequisite for one-half of biphasic GSIS. Furthermore, the study showed that Munc18b influences several modes of insulin secretion, including the SG-SG fusion mode, in rat β-cells.

Secretion is essential for the transportation of proteins, peptides, minerals, and membrane components. Accordingly, it occurs in all tissues. Insulin secretion is required to accelerate glucose uptake in muscles and adipose tissues, to repress gluconeogenesis and accelerate glucose uptake in the liver for glucose homeostasis, and to relay messages that maintain fundamental biological processes, such as cell growth, in many target cells. The classification of the dynamics of SG exocytosis into “primary exocytosis” and “sequential exocytosis” was determined by electrophysiology experiments in eosinophils, neutrophils, mast cells, and the parotid gland (51). In primary exocytosis, only one SG fuses to the PM. In secondary exocytosis (sequential exocytosis in a broad sense), two or more SGs in the cytosol fuse with the SG at the PM, and/or one following SG fuse with the membrane of a former collapsing SG, the later in narrow sense. In recent years, tools such as two-photon microscopy have demonstrated that sequential exocytosis occurs in insulin-secreting cells (8) and adrenal medullary

cells (45). TEP imaging has shown that sequential exocytosis occurs in pancreatic acini (6), the nasal gland (44), and the salivary gland (52), consistent with the results obtained with static imaging of pituitary prolactin-secreting cells (53). Multigranular exocytosis (compound exocytosis) is another form of sequential exocytosis, in which one vesicle undergoes primary exocytosis, followed by the fusion of the primary vesicle with cytosolic vesicles generated by the fusion of multiple SGs. This multigranular exocytosis is particularly suited for the large amount of secretion by mast cells and eosinophils (54,55). In addition, a study using two-photon microscopy showed that sequential exocytosis occurred in insulin-secreting cells. However, the frequency was low, only 2%–5%, and the main secretion modes were primary exocytosis and another was sequential exocytosis of two SGs (7). Furthermore, long-chain sequential exocytosis or multigranular exocytosis has not been reported in glucose stimulated situations.

In the present study, I used high-glucose stimulation with GLP-1 and IBMX to increase the frequency of exocytosis in order to study the exocytotic process (40,41). Under glucose stimulation with accelerants, the frequency of sequential exocytosis was 3.5% (*Ad*GFP-transfected controls), similar to the effect of accelerant observed in previous studies (7,8). In GSIS experiments with accelerants, the amount of total

exocytosis and sequential exocytosis was 1.8 and 2 times higher, respectively, in *Ad*Munc18b-WT expressing cells than in control cells, consistent with the results in mouse insulin-secreting Min6 cells transduced with Munc18b (58). In cells transfected with dominant-positive Munc18b-KR, total exocytosis and sequential exocytosis were 3.4 times and 3.2 times higher, respectively, than in control cells. Total exocytosis and sequential exocytosis were 0.7 times and 0.5 times lower, respectively, in Munc18b-E59K cells than in control cells. Upon depletion of Munc18b with Munc18b-shRNA, total exocytosis and sequential exocytosis were 0.6 times and 0.2 times lower than in control cells. The suppressive effects of Munc18b-E59K overexpression on total and sequential exocytosis were weaker than those of Munc18b-shRNA, possibly because endogenous Munc18b was sufficient for maintaining exocytosis.

Munc18b-KR enhanced exocytosis, increased the number of SGs in sequential exocytosis, and increased the frequency of cells exhibiting sequential exocytosis to a greater extent than did Munc18b-WT. These results provide insight into the function of Munc18b and suggest that the KR mutant promoted the reaction to insulin secretion. Munc18b might help the SNARE complex assemble tightly (Appendix Fig. 7; (22,59)). However, sequential exocytosis increased only 11% in Munc18b-KR-expressing cells.

The frequency of sequential exocytosis was higher than that of accidental exocytosis at overlap areas because insulin reduces serum glucose levels, even if inappropriate insulin discharge leads to hyperinsulinemia, which induces hypoglycemia and coma. These phenomena suggest a mechanism for the suppression of insulin secretion. Suppression might be influenced by binary binding between Munc18b and Syn-1 or by the relative amounts of Munc18b and the cognate SNARE complex. Other factors, such as Rabs, actin filaments, and phosphorylation, might positively and negatively affect insulin exocytosis.

The present study showed that sequential exocytosis, including unusual chains of three or more SGs, occurred in Munc18b-transduced cells. A maximum of 8 sequential SGs was detected in Munc18b-KR-transduced cells. The number of β-cells exhibiting sequential exocytosis was greatest after Munc18b-KR expression. The volume of sequential SGs was about twice that of primary SGs. The former might have been compound SGs or immature SGs (60). Compound SG exocytosis is not usually detected in GSIS, even with accelerants, but has been detected in cholinergic stimulation (61).

SM protein/Syn complex has been known to inhibit binding to cognate SNARE partners (62). Binding of SM proteins to the SNARE complex is thought to induce a

state favorable for the tight assembly of the SNARE complex, leading to exocytosis (10). The relationship between SM proteins and cognate SNARE proteins was shown in IP studies and in vitro liposome fusion assays (19). Munc18a interacted mostly with Syn-1. Munc18b, which is 63% identical to Munc18a at the amino acid level, interacted mostly with Syn-2 and Syn-3. Munc18c, which is 45% identical to Munc18b at the amino acid level, interacted with Syn-4. The relationship of Munc18 proteins to SNAP25 and VAMPs depends on the identity of the syntaxin (63).

How do Munc18b and SNARE proteins participate in primary and sequential exocytosis in insulin-secreting cells? We showed that Munc18a, b, and c and SNARE proteins were expressed in β cells and INS-1 cells (Figs. 1–3). Syn-1 -2, and -3 and SNAP25 mostly localized on the PM. Syn-2, -3, and -4 and SNAP23, SNAP25, and VAMP2 localized on the membrane of SGs. Moreover, the majority of Munc18a localized on the PM, while Munc18b localized on the PM and on SGs. In insulin-secreting cells, like in neuronal cells, Syx-1/SNAP25/VAMP2 appear to play a role in exocytosis, specifically primary exocytosis (64,7,65). SNAP25 plays a role in sequential exocytosis. This implies that SNAP25 diffuses on lipid membranes from the primary SG to subsequent fusing SGs during sequential exocytosis. (7,13). In other cells, such as mast cells and acinar cells, Syn-2 and Syn-3 are abundant and are known to

function in sequential exocytosis. Moreover, a recent study found that VAMP8 and Syn-3 mediated the primary exocytosis of newcomer insulin SGs and that Syn-3 mediated insulin SG-SG fusion (25,66).

Munc18b-KR, which showed weaker bind to Syn-1, augmented primary exocytosis. Munc18b bound to Syn-1 (competitively with Munc18a) and to Syn-2 at the PM to induce SNARE complex assembly. Preferential binding of Munc18b to Syn-2 and Syn-3 rather than Syn-1 has been reported (22), which could account for the increase in sequential exocytosis mediated by Munc18b in this study. Consistently, a relationship between Syn-3 and primary/sequential exocytosis has been demonstrated (66). After stimulation of cells with glucose plus an accelerator, the amount of SNAP25/VAMP2 that coimmunoprecipitated with Syn-2 and Syn-3 increased. Munc18b and, to a greater extent, Munc18b-KR induced a tight assembly of proteins in the SNARE complex. A schema of the main SM and SNARE proteins localized in the insulin exocytotic machinery is shown in Fig. 11.

I showed that Munc18b-mediated pathways in β-cells accounted for half of the biphasic GSIS and that Munc18b mediated the glucose-induced assembly of SNAREs for primary exocytosis and secondary exocytosis.

Enhancing the activity or expression of Munc18b to activate SNARE

complexes and mobilize more insulin SGs (newcomer SGs) to the PM for sequential SG-SG fusion and primary exocytosis might compensate for the exocytotic defects of diabetic β-cells. Such an approach could be used in the treatment of diabetes to attain normoglycemic control (67).

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FIGURE LEGENDS

FIG. 1. TEP imaging of primary exocytosis in rat β-cells.

(***A***) TEP imaging of rat islets in a solution containing 0.3 mM Alexa 594 hydrazide. The fluorescence distinguishes several adjacent β-cells in the islet, along with the intercellular space and blood vessels. Scale bar, 10 μm. On a representative cell, the white circle (*right panel* (***a***)–(***c***)) indicates the location of three exocytotic events (***B***). Scale bars, *left panel*: 10 μm; *right panel*: 5 μm.

(***B***) Three examples of TEP imaging of single insulin SG exocytosis (single granule) in an *Ad*GFP-transduced rat islet stimulated with 20 mmol/L glucose plus 10 nmol/L GLP-1 and 150 μmol/L IBMX. The imaging interval was 1.2 sec. An omega construction appeared when the SG(s) coalesced with the PM. The numbers below each panel indicate the time after the onset of exocytosis. Dashed white circles show the position of the omega construction in a former panel. Arrows point in the direction of SG fusion toward the cell interior. (***a***) and (***b***) show exocytotic events on the edge of a β-cell, and (***c***) shows an exocytotic event at the β-cell surface. Scale bar, 1 μm.

(***C***) Time course of fluorescence in the area containing the exocytotic events. Vertical bars indicate the times when the images in ***B*** were acquired. The fluorescence value before exocytosis was set to zero. A.U., arbitrary units.

(***D***) The cartoon illustrates primary exocytosis (single SG fusion) in a cross-sectional image. Initially, intracellular, unfused insulin granules are not detected because the Alexa 594 probe is hydrophilic. Once the SG fused to the PM, the fusion pore opened permitting the probe influx into the interior of the SG. The membranes of the SG and PM merge during membrane concrescence. The fluorescence intensity decreases as the volume of internal space in the SG decreases and then returns to zero as the membrane flattens at the end of exocytosis. Most insulin granule exocytosis involves full fusion (68).

FIG. 2. Munc18b mediates primary exocytosis and sequential SG-SG fusion in rat β-cells.

(***A***) The Alexa 594 fluorescence distinguishes several adjacent β-cells in the islet, along with small blood vessels (white arrowheads) and major blood vessels (white arrows). The white square shows the location of the exocytotic images shown in ***B***. Scale bar, 10 μm.

(***B***) Example of sequential exocytosis (two granules) involving two SGs in an *Ad*Munc18b-KR-transduced rat islet stimulated as described in **Fig. 1**. The imaging interval was 0.6 sec. The numbers below each panel indicate the time after the onset

of exocytosis. Dashed circles show the position of the omega construction in a former panel. Arrows point in the direction of SG fusion towards the cell interior. Scale bar, 1 μm.

(***C***) Time course of fluorescence in the area containing the exocytotic events. Vertical bars indicate the times when the images in ***B*** were acquired. The fluorescence value before exocytosis was set to zero. Panels A–C were reproduced from *Diabetes*, vol. 62 p. 2416–2428 (2013).

(***D***) A schematic illustration of primary and sequential exocytosis. An insulin granule from exocytosis to full membrane fusion is shown. The first single granule fuses with the PM as in primary exocytosis (**Fig. 1*D***), and subsequent SGs with distinct directionality sequentially fuse to the previous collapsing SG.

FIG. 3. Munc18b-WT or Munc18b-KR changes total and sequential exocytosis in rat β-cells.

The graphs display exocytosis data captured every 1 min during 10 min of stimulation with high glucose in the presence of an accelerator.

(***A***) Total number of exocytotic events was calculated as the average number of exocytotic events·cell^{-1}·min^{-1} in β-cells from islets expressing *Ad*GFP (8 islets, 68 cells), *Ad*Munc18b-WT (7 islets, 44 cells), *Ad*Munc18b-KR (6 islets, 58 cells), and *Ad*Munc18b-E59K (5 islets, 75 cells), as described above (**Figs. 1, 2**).

(***B***) The fraction of total exocytotic events occurring through primary exocytosis.

(***C***) The fraction of total exocytotic events occurring through sequential exocytosis.

FIG. 4. Munc18b or Munc18b-KR accelerates GSIS in rat β-cells.

(***A***) Total number of exocytotic events was calculated as the number of exocytotic events·cell^{-1}·min^{-1} in β-cells from islets expressing *Ad*GFP, *Ad*Munc18b-WT, *Ad*Munc18b-KR, and *Ad*Munc18b-E59K during a 10-min period of stimulation, as in **Fig. 3*A*.**

(***B***) The fraction of total exocytotic events occurring through sequential exocytosis**.** The number of secondary exocytotic events considered to be sequential exocytosis was expressed as the percentage of the total number of exocytotic events determined in (***A***).

(***C***) Proportion of cells showing sequential exocytosis among all cells undergoing primary and/or sequential exocytosis.

The results in (***A***), (***B***), and (***C***) represent the mean ± SEM. The data was analyzed using the Steel-Dwass test for multiple comparisons with *Ad*GFP as the control, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

FIG. 5. Munc18b induces long-chain sequential SG fusion involving three or more SGs.

The ordinate represents the ratio for increasing chain length of sequential SG fusion (the abscissa) events relative to total number of sequential exocytotic events in β-cells transfected with *Ad*GFP (8 islets, 68 cells), *Ad*Munc18b-WT (7 islets, 44 cells), *Ad*Munc18b-KR (6 islets, 58 cells), or *Ad*Munc18b-E59K (6 islets, 88 cells).

FIG. 6. Munc18b mediates long-chain sequential SG fusion.

(***A***) TEP imaging of long-chain sequential exocytosis of insulin SGs in a rat β-cell treated expressing *Ad*Munc18b-KR and stimulated with 20 mmol/L glucose plus 10 nmol/L GLP-1 and 150 μmol/L IBMX. Dashed circles show the positions of an omega construction in a former panel. Arrows indicate the direction of an oncoming SG undergoing sequential fusion.

(***B***) Time course of the fluorescence intensity within the region containing all the exocytotic events shown in (***A***). *Independent single exocytotic event, occasionally seen close to compound SGs (data not shown). Scale bars, 1 μm. A.U., arbitrary units.

(***C***) The low magnification images correspond to those shown in **Fig. 4*B*** and **Fig. 6*A***. The large oblique areas (red space surrounded by a white dotted line) showed high fluorescence intensity that did not change. These represent intercellular spaces, which served as landmarks to indicate that the focal plane did not deviate during the acquisition of successive images. An example of exocytosis is shown in the upper middle area of each panel, demonstrating the changes in fluorescence intensity and pattern that reflect sequential SG-SG fusion, indicated by the numbered arrows. Note the other scattered, independent exocytosing vesicles that appear in the right lower regions between the two oblique fluorescent areas in the later panels.

The figure was reproduced from *Diabetes*, vol. 62 p. 2416–2428 (2013) and supplementary data.

FIG. 7. Frequency distribution of SG diameters estimated by ΔV-TEPIQ (image-based quantification) analysis of TEP imaging.

Using the ΔV-TEPIQ method, the fluorescence intensity of the vesicle (F_V) was divided by F_E, which represents the fluorescence intensity per unit area in an xy-image of a solution with an infinite depth, as $\Delta V = F_V/F_E/P_{xy}(0)$, where $P_{xy}(0)$ is the efficiency of focal illumination (0.56 in our setup). Using this analysis, I found no significant difference in the diameters of single, first, and second SGs in each adenovirus-infected group (the significance of the differences was evaluated using Scheffé's method for multiple comparisons; data not shown). The black dotted lines

represent the Gaussian distribution of the means and the standard deviation of single granule fusion. The analysis was performed using β-cells transfected with *Ad*GFP (56, 10, and 10 omega constructions for single, first, and second granules, respectively), *Ad*Munc18b-WT (76, 22, 25, and 17 for single, first, second, and third and later granules, respectively), *Ad*Munc18b-KR (37, 29, 29, and 25 for single, first, second, and third and later granules, respectively), and *Ad*Munc18b-E59K (38, 8, and 8 for single, first, and second granules, respectively). "Single" means only a single SG fusion in primary exocytosis and excludes the first SG fusion in sequential exocytosis. "First," "second," and "third or later" mean the first SG, the second SG, and third and the subsequent SGs that exocytosed via sequential exocytosis, respectively.

The figure was reproduced from *Diabetes*, vol. 62 p. 2416–2428 (2013) and supplementary data.

FIG. 8. Estimated volume of fused SGs.

As described in **Figure 7,** using the ΔV-TEPIQ method, volumes were estimated for the single SG in primary exocytosis and for first, second, and third or later SGs in sequential exocytosis. The volumes of primary and second exocytotic SGs were not significantly different. On the other hand, the volume of third or later exocytotic SGs was larger than the volume of the other types of SGs. The results shown represent the mean ± SEM. The data was analyzed by Scheffé's test for multiple comparisons ***$P < 0.001$.

FIG. 9. Latency between SG-SG fusion.

I defined the lag-time for SG fusion with the preceding fused SG.

(***A***) The time from the first exocytotic event to the second exocytotic event was determined for islets transfected with *Ad*GFP, *Ad*Munc18b-WT, *Ad*Munc18b-KR, or *Ad*Munc18b-E59K, as in **Fig. 3**. There was no significant difference between cells transfected with *Ad*GFP (counted exocytosis n = 24), *Ad*Munc18b-WT (n = 51), *Ad*Munc18b-KR (n = 188), and *Ad*Munc18b-E59K (n = 17) when the data was assessed with Scheffé's test for multiple comparisons.

(***B***) The time from the second to the third exocytotic event is shown. There was no significant difference between cells transfected with *Ad*Munc18b-WT (n = 16) and *Ad*Munc18b-KR (n = 58) when the data was assessed with the Mann-Whitney U test.

(***C***) The time from the third (or later) exocytotic event to the subsequent exocytotic event. There was no significant difference between cells transfected with *Ad*Munc18b-WT (n = 8) and *Ad*Munc18b-KR (n = 50) when the data was assessed with the Mann-Whitney U test. Data for *Ad*GFP and *Ad*Munc18b-E59K are not

displayed because exocytosis involving more than two SGs was not detected in islets expressing these proteins.

FIG. 10. Munc18b depletion reduces primary and sequential exocytosis in rat β-cells.

TEP images of single SG exocytosis and SG-SG fusion in islets treated with lenti-shRNA/Munc18b-eCFP (67 cells, 8 islets) or lenti-eCFP (control; 48 cells, 8 islets) and stimulated for 10 min with 20 mmol/L glucose plus 10 nmol/L GLP-1 and 150 μmol/L IBMX. The number of exocytotic events was calculated.

(***A***) Total number of exocytotic events. (***B***) Number of exocytotic events within each indicated time interval during the 10-min recording. *Right panel*: summation of the first 5 min and second 5 min of recording. (***C***) Secondary exocytotic events considered to be sequential exocytosis were expressed as the percentage of the total number of exocytotic events determined in (***A***). The results represent the mean ± SEM, $*P < 0.05$, $***P < 0.001$. Data in ***A***, and the *right panels* of ***B*** and ***C*** were analyzed by *t*-test; data in the *left panel* of ***B*** was analyzed with Tukey's test (vs. control) for multiple comparisons.

The figure was reproduced from *Diabetes*, vol. 62 p. 2416–2428 (2013).

FIG. 11. Schema for the arrangement of exocytotic molecules and the exocytotic process in insulin-secreting cells.

In the insulin secretory pathway, stimulation with glucose and/or an incretin such as GLP-1 or gastric inhibitory peptide promotes SG motion or induces deeper SGs to move near the PM for exocytosis by increasing cAMP and the cytosolic concentration of free Ca^{2+}. The main molecules involved in insulin secretion are SNARE proteins and SM proteins. Those proteins localize on the lipid bilayer of the PM and SGs.

For primary and secondary exocytosis, working SM and SNARE proteins are shown in the *lower panel*. In primary exocytosis, Munc18b promotes the formation of the Syn-1, SNAP25, and VAMP2 complex, similar to Munc18a. In sequential exocytosis, Munc18b might activate SNARE complex formation by orchestrating Syn-2 or -3, VAMPs, and the transfer of SNAP25 from the cell membrane to the bilayer of primary fused SGs. Moreover, long-chain sequential exocytosis might involve complexes containing other SNAPs, such as SNAP23, other VAMPs, such as VAMP8, and Syn-2 or -3, whose formation is accelerated by Munc18b.

APPENDICES MATERIALS AND METHODS

The experiments mentioned below were performed by my collaborators, Professor Gaisano, Dr. Lam and his colleagues, Dr. Dolai, Dr. He, Dr. Qin, Dr. Liang, Dr. Zhu, Dr. Kang, Dr. Liu Dr. Xie, Dr. Wan, Dr. Bin, Professor Shuzo Sugita (University of Toronto, Toronto, Ontario, Canada).

Cell culture and rat islet perifusion assays

Islets from Sprague Dawley rats (275–300 g) were isolated by collagenase digestion as described previously (1). Islets and INS-1 cells (823/13; C. Newgard, Duke University, Durham, NC, USA) were cultured in RPMI 1640 medium. Batches of 30 rat islets (or INS-1 cells) were transduced with Munc18b lenti-shRNA (siRNA for INS-1) or *Ad*Munc18b constructs, loaded in perifusion chambers (~1.3-mL capacity), and perfused at a flow rate of ~1 mL/min (37°C). Cells were stimulated with glucose in the presence or absence of 10 nM GLP-1 (Sigma-Aldrich, St. Louis, MO, USA) and 150 μM IBMX (Sigma-Aldrich), as indicated. Insulin secretion was measured with RIAs (Linco Research, St. Louis, MO, USA). The experiments were approved by the Animal Care Committees of the University of Toronto.

Silencing Munc18b expression and adenovirus construction

Endogenous Munc18b was silenced using two strategies, siRNA in INS-1 cells and

lenti-shRNA in islets. For siRNA experiments, two 64-base pair siRNA duplex sequences targeting Munc18b were created with reference to rat Munc18b cDNA (GenBank Accession Number AF263346). Sense, antisense, and control scrambled sequences were used. INS-1 cells were transfected using Lipofectamine2000 (Invitrogen, Burlington, ONT, Canada).

Munc18b-WT and Munc18b mutants tagged with GFP in a separate transcription cassette (2) were subcloned into a pAdTrack shuttle vector and inserted into a pAdeasy-1 backbone. Positive clones were used to transfect HEK293 cells. Viruses released from single plaques were then amplified to high titers.

Confocal immunofluorescence microscopy

As previously described (3), dispersed islet β-cells were plated on glass coverslips, fixed with 2% paraformaldehyde, and rinsed in PBS for 5 min. The preparations were then blocked with 5% normal goat serum/0.1% saponin for 0.5 h at 25°C. The cells were then washed, incubated with the indicated primary antibodies for 1 h at a 1:100 dilution, and stained with the appropriate fluorochrome (fluorescein isothiocyanate)-conjugated secondary antiserum (1 h, 25°C). The coverslips were then mounted on slides in a fading retarder, 0.1% *p*-phenylenediamine in glycerol, and examined using a Leica DMIRE2 inverted confocal microscope system (Leica

Microsystems GmbH, Wetzlar, Germany) equipped with a Hamamatsu Back-Thinned EM-CCD camera (Hamamatsu Photonics, Shizuoka, Japan) and spinning disk confocal scan head. The unit was equipped with four separate diode-pumped solid state laser lines (Spectral Applied Research Inc., Concord, Ontario, Canada) at 405 nm, 491 nm, 561nm, 638 nm; an ASI motorized XY stage; and an Improvision Piezo Focus Drive (for Z-scans, 0.1-μm steps) (PerkinElmer, Waltham, MA). The equipment was driven by Volocity Acquisition software (PerkinElmer) and powered by an Apple Power Mac G5 computer. Cells were visualized with a 63× objective lens, and lasers at 491 nm, 561 nm, and 638 nm were used to excite FITC, Texas Red, and Cy5 fluorophores, respectively. A deconvolution algorithm was applied to the images to remove out-of-focus background noise.

Immunoprecipitation

Immunoprecipitation (IP) was performed as previously described (3). INS-1 cells (70%–75% confluent) were transduced with *1*) Munc18b siRNA or scrambled nonsense siRNA or *2*) wild-type or mutant *Ad*Munc18b or control *Ad*GFP. INS-1 cell lysates were immunoprecipitated with antibodies (1.2–2.0 μg) specific to Syn-1A, -2, or -3. Coprecipitated proteins were identified with western blotting using the indicated antibodies. All antibodies used were commercial antibodies except Munc18c (Y. Tamori,

Kobe University, Kobe, Japan), VAMP8 (W. Hong, Institute of Molecular and Cell Biology, Singapore), Syn-4 (J.E. Pessin, Albert Einstein College of Medicine, Bronx, NY, USA), and Munc18b (P.P.L. Lam, University of Toronto, Toronto, Canada) (2).

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APPENDICES FIGURE LEGENDS

Appendix FIG. 1. Munc18b and SNARE expression in rat islets.

Pancreatic β-cells (rat islets, INS-1 cells) expressed SM proteins and syntaxins. Rat pancreatic acini and brain tissues were used as controls for sequential (compound) exocytosis model in the absence of Munc18a and for basal exocytosis mediated by classical SNARE proteins, respectively. The figure was reproduced from **Fig. 1*A*** in *Diabetes*, **62** p. 2418 (2013) (4).

Appendix FIG. 2. Munc18b localizes to insulin granules and the plasma membrane in rat β-cells.

In immunofluorescence analysis, cognate Munc18b, Syn-2, and Syn-3 were abundant in insulin SGs in rat β-cells. Cognate Munc18a and Syn-1A were abundant in the PM. The images are representative of four independent experiments. Scale bars, 10 μm. The figure was reproduced from **Fig. 1*B*** in *Diabetes*, **62** p. 2418 (2013) (4).

Appendix FIG. 3. Depletion of Munc18b in islets reduces biphasic GSIS.

(***A***) Munc18b depletion in rat islets by lenti-Munc18b shRNA/eCFP vs. lenti-eCFP control. *Top panel*:Representative blots. *Bottom panel*: Analysis of three experiments, shown as means ± SEM. *P < 0.05, **P < 0.01.

(***B***) Islet perifusion assays of 48 h lenti-shRNAMunc18b-eCFP (vs. lenti-eCFP [control]) depletion of endogenous Munc18b in rat islets showing reduction of GSIS and GLP-1 (10 nM) plus IBMX (150 μM)-potentiated GSIS, shown as means ± SEM of three independent experiments, and AUC analysis; *P < 0.05, ***P < 0.001.

G, glucose. These figures were reproduced from **Fig. 1*C, D*** in *Diabetes*, **62** p. 2418 (2013) (4).

Appendix FIG. 4. Overexpression of Munc18b or Munc18b mutants in islets does not affect SM or SNARE proteins.

(***A***) Expression of *Ad*Munc18b proteins (-WT, -KR, -E59K) in rat islets did not affect the expression of SNARE proteins or other SM proteins. The results are representative of three independent experiments. The analysis of Munc18b protein expression is shown in the *bottom panel*. The level of Munc18b protein in cells overexpressing the WT or mutant proteins was higher than in control cells. *$P < 0.05$ by ANOVA.

(***B***) Confocal imaging showed that *Ad*Munc18b mutants did not affect the targeting of Syn-2 or Syn-3 to insulin SGs in rat β-cells. The results are representative of four independent experiments. Scale bars, 10 μm. The figure was reproduced from **Fig. 2*A*,**

B *Diabetes*, **62** p. 2419 (2013) (4).

Appendix FIG. 5. Munc18b is a mediator of GSIS in pancreatic islet β-cells.

(***A***) Islet perifusion assays showed that expression of *Ad*Munc18b mutants in rat islets affected GSIS. The results shown are the mean ± SEM from three or four sets of experiments, in which the four conditions were analyzed simultaneously in each experiment. *Bottom panels* show the quantification of the AUC analysis of first-phase (11–22 min) and second-phase (22–40 min) GSIS. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by one-way ANOVA. A symbol of asterisk without a black comparison bar showed a comparison with *Ad*GFP (contral).

(***B***) Islet perifusion assays showed that expression of *Ad*Munc18b mutants in rat islets affected GSIS potentiated by 10 nM GLP-1 plus 150 μM IBMX. The results shown are the mean ± SEM from three or four sets of experiments, in which the four conditions were analyzed simultaneously in each experiment. *Bottom panels* show the quantification of the AUC analysis of first-phase (11–22 min) and second-phase (22–40 min) GSIS. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by one-way ANOVA. A symbol of asterisk without a black comparison bar showed a comparison with *Ad*GFP (contral).

(***C***) Effects of *Ad*Munc18b mutants on GSIS and 10 nM GLP-1 plus 150 μM IBMX-potentiated GSIS in INS-1 cells. The results shown are the mean ± SEM from three independent experiments performed in duplicate or triplicate (*n* = 6–8). **P* < 0.05 by ANOVA and Scheffé's test. A symbol of asterisk without a black comparison bar showed a comparison with *Ad*GFP (contral).

The figure was reproduced from **Fig. 2*C-E*** *Diabetes*, **62** p. 2419 (2013) (4).

Appendix FIG. 6. Munc18b depletion in INS-1 cells disrupts SM-activated SNARE complex formation.

INS-1 cells transduced with Munc18b siRNA or control scrambled RNA were maintained under basal conditions (0.8 mM glucose) or stimulated with 16.7 mM glucose plus 10 nM GLP-1 with IBMX (150 μM). Cell lysates were coimmunoprecipitated (*left panels*) with antibodies against Syn-1A (A), Syn-2 (B), or Syn-3 (C). The corresponding *right panels*, showing the "Input" controls (25 μg protein, total INS-1 lysates), confirmed that Munc18b levels were reduced and that levels of the indicated SNARE proteins were unchanged. The results shown are representative of three independent experiments. Data from densitometry analyses are not shown**.**

The figure was reproduced from **Fig. 3** in *Diabetes*, **62** p. 2421 (2013) (4).

Appendix FIG. 7. A Munc18b gain-of-function mutant increases SM-activated SNARE complex formation.

INS-1 cells were transduced with AdMunc18 mutants, maintained under non-stimulated (*lanes 1–4*) or stimulated (*lanes 5–8*) conditions as in **Fig. 3**, and used for coimmunoprecipitation experiments (*left panels*) with antibodies against Syn-1A (***A***), Syn-2 (***B***), or Syn-3 (***C***). The expression of exogenous Munc18b (tagged with Myc) was detected with a Myc antibody. The corresponding *right panels*, showing input controls (25 μg protein, total INS-1 lysates), indicated that SNARE protein levels were unchanged. The results shown are representative of three independent experiments. Data from densitometry analyses are not shown.

The figure was reproduced from Fig. 4 in *Diabetes*, **62** p. 2422 (2013) (4).