Secretory vesicle exocytosis is a highly regulated process involving vesicle targeting, priming, and membrane fusion. Rabs and SNAREs play a central role in executing these processes. We have shown recently that Rab27a and its effector, granuphilin, are involved in the exocytosis of insulin-containing secretory granules through a direct interaction with the plasma membrane syntaxin 1a in pancreatic beta cells. Here, we demonstrate that fluorescence-labeled insulin granules are peripherally accumulated in cells overexpressing granuphilin. The peripheral location of granules is well overlapped with both localizations of granuphilin and syntaxin 1a. The plasma membrane targeting of secretory granules is promoted by wild-type granuphilin but not by granuphilin mutants that are defective in binding to either Rab27a or syntaxin 1a. Granuphilin directly binds to the H3 domain of syntaxin 1a containing its SNARE motif. Moreover, introduction of the H3 domain into beta cells induces a dissociation of the native granuphilin-syntaxin complex and a marked reduction of newly docked granules. These results indicate that granuphilin plays a role in tethering insulin granules to the plasma membrane by an interaction with both Rab27a and syntaxin 1a. The complex formation of these three proteins may contribute to the specificity of the targeting process during the exocytosis of insulin granules.

Intracellular fusion of vesicles or organelles with their target membranes is a common reaction of the compartmental structure of eukaryotic cells that is mediated by dynamic molecular assemblies involving conserved protein complexes. Central to these reactions are Rab GTpases and soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), which are conserved from yeast to man (1). The membrane-anchoring SNAREs form a four-helix bundle in a ternary protein complex that is thought to drive a fusion reaction. By contrast, many Rabs act on a more upstream process in the initial physical contact between a vesicle and its target (2). This process, known as tethering or docking, may provide specificity to membrane fusion.

Both yeast and mammalian systems have revealed various tethering factors. Some of them are large multisubunit complexes and others are long coiled-coil proteins (3). The former include the exocyst (the Sec6/8 complex), the conserved oligomeric Golgi complex (the Sec34/35 complex), the transport protein particle complexes (TRAPP-I and -II), and the Class C Vps complex (the homotypic fusion and vacuole protein sorting complex), and the latter are represented by p115 (yeast Uso1p) and EEA1. The monomeric tethering factor directly binds to Rab and functions as an effector protein, whereas the large complexes are associated with events upstream or downstream of the Rab function. These tethering factors are commonly expressed in almost all cells and function in constitutive processes depending on the accumulation of cargo. Compared with these pathways, the regulated secretory pathways that have developed in neuroendocrine cells have a unique property, which is first to store specific cargo and then to release it only in the presence of secretagogues. Because fusion frequencies and vesicle demands continuously change as a function of the external stimuli in regulated secretory pathways, releasable vesicles must be replenished in concert with fusion events. Therefore, the tethering process in regulated secretory pathways needs additional regulation compared with that in other pathways.

As in neurons, pancreatic beta cells express many components of secretory machinery, including Rab3 and its effector RIM, the SNAREs (synaptobrevin2, syntaptosomal-associated protein 25, and syntaxin 1a), and SNARE-associated proteins (Munc13-1 and Munc18-1) (4, 5). They appear to function in the exocytosis of insulin-containing secretory granules, although the precise mechanism still is unclear. Although the central fusion players are shared in endocrine cells and neurons, proteins expressed in a specific cell need to be characterized. This is because each regulated secretory pathway, depending on the function of the specific cargo, has developed unique organelles in which the morphological appearance, release kinetics, and biogenesis are distinct. For example, synaptic vesicles in neurons are placed at the active zone opposite the synaptic cleft and undergo rapid cycles of exocytosis and endocytosis at the nerve terminals. Vesicle recycling does not require input from the biosynthetic pathway because the neurotransmitter is refilled by specific transporters on the vesicle membrane. By contrast, secretory granules in endocrine cells are exocytosed with a slower onset but for a longer duration and rely on de novo protein biogenesis.
synthesis of polypeptides in the endoplasmic reticulum and transport through the Golgi network to the plasma membrane, although they have no discrete docking sites. Even among secretory granules in endocrine cells, there are some differences in exocytotic characteristics. For instance, pancreatic beta cells contain a small number of insulin granules that are docked below the plasma membrane, whereas chromaffin cells contain a large number of morphologically docked vesicles (6–8). Thus, the tethering process in regulated secretory pathways likely is diverse.

From the analysis of genes preferentially expressed in pancreatic beta cells, we have identified previously a novel set of Rab and its effector, Rab27a and granuphilin, respectively, both of which are specifically localized on insulin-containing mature granules (9, 10). Overexpression of Rab27a in beta cells enhanced depolarization-induced insulin secretion without affecting basal secretion, suggesting that granules in a pool primed for fusion are accumulated by the action of Rab27a. Granuphilin directly binds to the plasma membrane-anchored SNARe, syntaxin 1a, and Rab27a regulates this interaction (11). Our data, estimated by the universal function of Rab effectors, suggest that granuphilin has a function in tethering insulin granules with the plasma membrane at the docking stage of exocytosis. In the present study, we provide both morphological and biochemical evidence that granuphilin efficiently induces the translocation of granules to the plasma membrane. The tethering function of granuphilin requires its interaction with both Rab27a and syntaxin 1a. We propose that granuphilin regulates accurate targeting of insulin granules to the exocytic site by a specific interaction with syntaxin 1a.

**EXPERIMENTAL PROCEDURES**

**DNA Construction—** Partial cDNA fragments of mouse phogrin were amplified from the MIN6 cDNA library by PCR using primers 5'-CCA-TGGACTGACCGCCACC-3' and 5'-TTGTTAGGGCTCGACGATC-3', 5'-GCCAAGCTTACCTGATAC-3' and 5'-GATGATTGCGGAAGCTT-3', and 5'-GAATGACCGACAAAGGCTT-3' and 5'-CGAGCTTGATATCATGTC-3'. The amplified cDNAs were ligated to construct full-length phogrin cDNA. The resultant phogrin cDNA and the enhanced green fluorescent protein (EGFP) cDNA cut from the pEGFP-N2 vector (Clontech, Palo Alto, CA) were simultaneously subcloned into the HindIII and NotI sites of the pcDNA3 vector. Mouse Munc18-1 cDNA similarly was amplified using primers 5'-GAAGACTGACCGCCACC-3' and 5'-TGTGGCTGGTTGAC-3', and was cloned into the pcDNA3-HA vector (11). Truncated fragments of syntaxin 1a were constructed by PCR using pGEX-KG-syntaxin 1a (1–264) (11) as a template and cloned into the same vector. The granuphilin-a mutants W118S and L43A and a recombinant adenovirus bearing each form of granuphilin cDNA were described and characterized previously (12). Cell Culture and Transfection—MIN6 and AtT20 cell lines were grown in high glucose (25 mM) Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum except where indicated otherwise. Transfections were performed with LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer’s instructions. MIN6 cells were transfected with the phogrin-EGFP plasmid, and stable clones were selected in the presence of 1 mg/ml G418. Individual clones were evaluated by fluorescence microscopy. Phogrin-EGFP was communally localized with anti-insulin antibodies (60–90%) (see Fig. 1) as described previously (12).

**Antibodies—** The rabbit anti-granuphilin antibodies, α-Grp-aC that recognizes granuphilin-a (9), α-Grp-bC that recognizes granuphilin-b (11), and α-Grp-N that recognizes both granuphilin-a and -b (10), were described and characterized previously. The rabbit anti-secretogranin III antibodies (13) were gifts from Drs. M. Hossack (Gunn University) and T. Watanabe (Asahikawa University School of Medicine). The guinea pig anti-porcine insulin serum was a gift from Dr. T. Matozaki and H. Kobayashi (Gunma University). Anti-syntaxin 1a (HPC-1) mouse, anti-Rab27a mouse, anti-ACTH mouse, and anti-hemagglutinin (anti-HA) clone 3F10 rat monoclonal antibodies were purchased from Sigma, Pharmingen, Biogenesis (Poole, UK), and Roche Diagnostics (Mannheim, Germany), respectively.

**Indirect Immunofluorescence Microscopy—** Indirect immunofluorescence analysis was performed as described previously (11). Briefly, MIN6/phogrin-EGFP cells (clone 41) cultured on 8-well Lab-Tek chamber slides were fixed with 4% paraformaldehyde and permeabilized with either 0.1% Triton X-100 or ice-cold methanol. The cells then were incubated with primary antibodies followed by indocarbocyanine (Cy3)-conjugated, species-specific anti-IgG secondary antibodies (Jackson Immunoresearch, West Grove, PA). Intrinsic EGFP and antibody staining signals were observed with confocal microscopes TCS-SP2 (Leica Microsystems Vertrieb GmbH, Bensheim, Germany), LSM5 PASCAL (Carl Zeiss, Jena, Germany), and MRC-1024 (Bio-Rad) or with an epi-fluorescence microscope (BX-50; Olympus Optical Co., Tokyo, Japan) equipped with a SenSys™ charge-coupled device camera (Photometrics, Tucson, AZ).

A peripheral pattern of EGFP signals was quantified under a fluorescence microscope as follows. MIN6/phogrin-EGFP cells were transiently transfected with 0.1 μg of an expression plasmid encoding HA-granuphilin. The cells were incubated under a low glucose condition (2 mM) for 24 h. The cells then were fixed with 4% paraformaldehyde, permeabilized with 0.05% Triton X-100, and incubated with anti-HA rat monoclonal antibodies followed by Cy3-labeled anti-rat IgG. Cells that revealed a linear distribution along a part of the plasma membrane were judged as positive, whereas those that showed only a punctate granular pattern were judged as negative. Cells that showed ambiguous patterns, which constituted ~10% of the cells, were excluded from the counting. Although only Cy3-positive, HA-granuphilin expressing cells were judged in the assay, ~20% of untransfected cells normally show the peripheral pattern of EGFP signals. For each experiment, a total of 100 cells were unambiguously assessed.

**Immunoprecipitation and in Vitro Binding Analysis—** Immunoprecipitation and immuno blot analyses were performed as described previously (11). In vitro translation of HA-tagged proteins was performed using the Tnt-coupled reticulocyte lysate system (Promega, Madison, WI). Purified glutathione S-transferase (GST)-fused proteins (1 μg) immobilized on 10 μl of glutathione-Sepharose beads were incubated with equal amounts of in vitro translated proteins (6–10 μl) in binding buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM MgCl2, 1 mM EGTA, 0.1% Nonidet P-40) at 4 °C for 3 h. The beads were washed three times, and the bound proteins were subjected to SDS-PAGE and immunoblotting.

**Subcellular Fractionation Cells** were suspended in buffer containing 250 mM sucrose, 20 mM HEPS (pH 7.4), 2 mM MgCl2, 2 mM EGTA, and the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride and 5 μg each of aprotinin, pepstatin A, and leupeptin per ml. The cells were homogenized for 40 strokes by the tight fitting Dounce homogenizer and the cell condition was monitored under a microscope. The total homogenate was centrifuged at 700 × g for 10 min to precipitate the nuclear and intact plasma membranes. The resultant supernatant then was centrifuged at 12,000 × g for 20 min to separate the heavy organelle fraction including the secretory granules from the cytoplasmic materials. Fractions were lysed in buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM MgCl2, 1 mM EDTA, 1% Triton X-100), and the following protease inhibitors described above, and protein that each lysate were subjected to SDS-PAGE and immunoblotting analysis.

**Transduction of MIN6 Cells with Tat Fusion Proteins—** Isolation of Tat-EGFP and Tat-H3 was performed as described previously (14). MIN6 cells were transduced for 50 min with 70 μg/ml of Tat fusion proteins, and the cell extracts were prepared as described for the immunoprecipitation experiments.

**RESULTS**

**Granuphilin Promotes Insulin Granule Targeting to the Plasma Membrane—** We demonstrated recently that Rab27a and its effector granuphilin are peripherally localized on the membrane of mature dense core granules in pancreatic beta cells and regulate insulin secretion through interaction with the plasma membrane t-SNARE, syntaxin 1a (10, 11). These biochemical studies suggest that granuphilin plays a role in tethering two membranes, secretory granules and the plasma membrane, at the event of exocytosis. To explore this possibility, we morphologically assessed the function of granuphilin by tracing EGFP-labeled granules. A MIN6 cell line first was established that expresses phogrin-EGFP fusion protein, which was shown previously to be properly targeted to insulin granules in pancreatic beta cells (12, 15). Immunostaining analysis confirmed that phogrin-EGFP is almost entirely colocalized with insulin on secretory granules but not on the perinuclear Golgi region (Fig. 1). Using these cells, the motion of insulin granules can be followed.
by tracking the EGFP fluorescence. When HA-tagged granuphilin-a was transiently overexpressed, EGFP signals clearly were redistributed at the peripheral region exactly where granuphilin was colocalized (Fig. 2A, arrows). In contrast, neighboring untransfected cells showed only punctate EGFP signals mainly distributed around the Golgi/endosomal region (Fig. 2A, asterisks), although some of these normally display a peripheral pattern in this assay condition. These observations suggest that overexpression of granuphilin induces translocation of insulin granules toward the plasma membrane.

Next we used a recombinant adenovirus to express granuphilin more efficiently in MIN6/phogrin-EGFP cells. In our study, almost all cells were infected and expressed exogenous protein (11). Cells infected with the adenovirus that expressed granuphilin-a and -b uniformly exhibited a prominent peripheral redistribution of labeled granules compared with those expressing control β-galactosidase protein (Fig. 2B). Granuphilin-b was more effective for the targeting of granules to the plasma membrane. Furthermore, electron microscopic analysis revealed an accumulation of dense core granules near the cell-cell contact region in granuphilin-overexpressing MIN6 cells.2

To see these phenomena biochemically, a conventional fractionation procedure was employed. Total MIN6 cell homogenates (T) in sucrose buffer were centrifuged at low speed (700 × g) to precipitate the plasma membrane sheets as well as the nucleus (P1). The resultant supernatant then was centrifuged at high speed (12,000 × g) to separate the heavy organelles including dense core granules (P2) and other components containing cytosol (S). In control cells expressing β-galactosidase protein, the plasma membrane-associated syntaxin 1a mainly was present in the P1 fraction, whereas peripherally granule-associated Rab27a (10) and the granule content, secretogranin III (SgIII) (13), were distributed in the P2 fraction (Fig. 3, upper panels). Overexpression of granuphilin-a by the adenovirus induced significant redistributions of Rab27a and SgIII from P2 to P1, suggesting that insulin granules were moved into the heavier fraction containing the plasma membrane (Fig. 3, lower panels). The localization of phogrin, an integral granule-membrane protein, was similar to that of SgIII, and the immunoreactivity of insulin was correlated with these changes (data not shown). Thus, granuphilin overexpression changed the subcellular localization of granule marker proteins. This biochemical finding indicates that the redistributed granules are engaged in distinct molecular interaction rather than simply forced against the membrane. These results, obtained using the adenovirus expression system, strongly suggest that granuphilin facilitates the docking of insulin granules to the plasma membrane.

Granuphilin Promotes ACTH Granule Targeting to the Plasma Membrane—Previous studies have shown that overexpression of granuphilin significantly inhibits high K+-induced insulin secretion but enhances basal insulin secretion (11, 16). Thus, the peripheral translocation of granules by granuphilin seen in beta cell line MIN6 could be a mere reflection of accumulated granules resulting from the inhibition of secretion, although the phenomenon was observed in both nonstimulatory (Fig. 2A) and stimulatory glucose concentrations (Fig. 2B). To exclude this possibility, we examined the effect of granuphi-

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2 H. Yokota-Hashimoto, S. Torii, and T. Izumi, unpublished observations.
lin overexpression in pituitary corticotroph cell line AtT20. This cell line also physiologically expresses Rab27a/b and granuphilin (17) and does not stimulate hormone secretion in response to a high glucose level in custom medium, which is in contrast to MIN6 cells. In addition, a relatively lower density of granules in the peripheral cytoplasm of AtT20 cells enabled us to distinguish each ACTH-positive spot. Cells infected with adenovirus expressing granuphilin-b again induced a redistribution of granules along the plasma membrane (Fig. 4A). Furthermore, as with MIN6 cells, biochemical fractionation analysis showed that granule marker proteins such as SgIII are significantly redistributed in these cells (Fig. 4B). Taken together, these results indicated that the peripheral redistribution of granules by granuphilin overexpression does not simply reflect its inhibitory effect on secretion.

Granuphilin Co-works with Syntaxin 1a on the Peripheral Targeting of Insulin Granules—We demonstrated previously that granuphilin is partially colocalized with and directly binds to syntaxin 1a in pancreatic beta cells (11). Because syntaxin 1a is one of the SNARE proteins on the plasma membrane that mediates the membrane fusion reaction, granuphilin may tether insulin granules to the exocytic site by an association with syntaxin 1a. To explore this possibility, first we examined the intracellular localization of syntaxin 1a and EGFP-labeled granules that are redistributed to the plasma membrane in the granuphilin-overexpressing cells. The peripheral localization of labeled granules overlapped well with the local distribution of syntaxin 1a in cells overexpressing granuphilin-b (Fig. 5A).

Next we examined the targeting activities of granuphilin mutants that are defective in binding to either Rab27a or syntaxin 1a. We demonstrated previously that the W118S and L43A mutations specifically disrupt interactions of granuphilin with Rab27a and syntaxin 1a, respectively (11). Overexpression of neither W118S nor L43A altered the distribution of granules labeled by phogrin-EGFP in contrast to wild-type granuphilin-a (Fig. 5B). Some of the W118S-expressing cells showed a more compact, Golgi-like EGFP fluorescence pattern compared with control cells. Immunostaining of the W118S mutant revealed a diffuse cytosolic distribution, which may reflect the inert binding activity of this mutant to Rab27a (data not shown). Although it is difficult to evaluate the targeting activities quantitatively, cells with peripheral distribution of EGFP signals were counted in conventional transfection assays (under “Experimental Procedures”). Wild-type granuphilin-a and granuphilin-b showed 59 ± 5% and 76 ± 4% (mean ± S.E., n = 4) targeting activity, respectively. In contrast, W118S and L43A mutants displayed much lower activity, 21 ± 3% and 27 ± 6%, respectively, which is comparable with levels found in control cells (20 ± 2%). These observations suggest that interactions with both Rab27a and syntaxin 1a are required for the targeting function of granuphilin.

Transduction of MIN6 Cells with TAT-fused H3 Fragment of Syntaxin 1a Inhibits the Plasma Membrane Docking of Insulin Granules and the Formation of Granuphilin-Syntaxin 1a Complex—Ohara-Imaizumi et al. (14) recently examined the effect of a TAT-fused H3 fragment of syntaxin 1a on the exocytosis of insulin granules in MIN6 cells using an evanescent wave microscopic technique. Human immunodeficiency virus-1 TAT protein can cross a biological membrane efficiently and promote delivery of fused peptides into cells. Ohara-Imaizumi et al. (14) showed that the introduction of TAT-H3 inhibits the plasma membrane docking of newly arrived insulin granules as well as the membrane fusion of predockded granules. These findings suggest that syntaxin 1a is required for replenishment of insulin granules into the docked pool. Combined with the...
Fig. 5. Granuphilin targets secretory granules to the plasma membrane by the interaction with syntaxin 1a. A, MIN6/phogrin-EGFP cells were infected with recombinant adenovirus bearing granuphilin-b and fixed after 12 h. The cells were permeabilized with methanol and immunostained with anti-syntaxin 1a and Cy3-labeled anti-mouse IgG antibodies. Intrinsic EGFP fluorescence (a), Cy3 fluorescence (b), and merged fluorescence (c) are shown. B, MIN6/phogrin-EGFP cells were infected with a recombinant adenovirus encoding wild-type granuphilin-a (a) or its point mutants, W118S (b) and L43A (c), and were examined for the plasma membrane targeting of EGFP signals.

Next we introduced TAT-H3 peptides into MIN6 cells and confirmed that they significantly reduce the docking of newly recruited granules as shown previously (14) (data not shown). On this condition, we examined whether TAT-H3 treatment causes dissociation of the endogenous granuphilin/syntaxin 1a complex. After incubating cells with TAT proteins for 50 min, cell extracts were prepared for coimmunoprecipitation experiments. When TAT-H3 was introduced, the amount of syntaxin 1a coprecipitated with anti-granuphilin-a or anti-granuphilin-b antibodies was significantly decreased as compared with the case of control TAT-GFP (Fig. 6B). The expression levels of granuphilins and syntaxin 1a were not influenced in this condition. We could detect only a small amount of complex between the TAT-H3 peptide and endogenous granuphilin using transduced MIN6 cell lysate. It is, however, difficult to use metal-binding beads for sedimentation of the His6-tagged TAT peptide because efficient interaction of these proteins requires a chelator.3 Taken together, these data suggest that both granuphilin and syntaxin 1a are involved in tethering of insulin granules to the exocytotic site in the plasma membrane, possibly through a direct interaction.

DISCUSSION

The slow and biphasic kinetics of insulin secretion from pancreatic beta cells often is explained by the small population of docked granules and the subsequent steps in which a number of granules are moved and targeted to the plasma membrane (4, 19). The targeting process of secretory granules, however, has not been characterized in part because of the absence of obvious vesicle docking sites such as the active zone in neural cells. In the present study, we suggest that granuphilin is a regulator for the plasma membrane targeting of insulin granules in cooperation with Rab27a and syntaxin 1a. Granuphilin efficiently promotes a peripheral redistribution of insulin granules, which likely represents a physiological targetting event because the same peripheral distribution was observed in some cells without overexpression of granuphilin. Our previous demonstration that granuphilin directly and specifically binds to Rab27a and syntaxin 1a suggests that granuphilin tethers insulin granules and the plasma membrane through these interactions (10, 11, 20). Consistently, two kinds of granuphilin mutants that have a defect in binding to

3 S. Torii and T. Izumi, unpublished observations.
either Rab27a or syntaxin 1a lose the targeting activity. The importance of syntaxin 1a in the tethering or docking process of insulin granules also is supported by recent findings from evanescent wave microscopy. Ohara-Imaizumi et al. (14) have shown that introduction of the H3 domain of syntaxin 1a into MIN6 cells inhibits new docking events of insulin granules during the glucose stimulation. We have found that the H3 fragment of syntaxin 1a directly binds to granuphilin in vitro and that its introduction into MIN6 cells induces a concomitant decrease in newly docked granules and the amount of endogenous granuphilin-syntaxin 1a complex. These results further support the finding that the targeting process of insulin granules is mediated in part by the interaction between granuphilin and syntaxin 1a.

The targeting activity of granuphilin with syntaxin 1a is consistent with the function of other Rab effectors that act as tethering factors in various transport pathways. Two types of tethering factors are proposed: a group of proteins containing coiled-coil structures and several multisubunit complexes (3). Granuphilin is considered to be relatively similar to the former, as its family proteins, exophilins, possess a putative coiled-coil structure at their N termini (21). In terms of its functional role, it is like Rab5 effector EEA1 that is involved in an endosome tethering reaction through the interaction with the target SNARE syntaxin 6 (22). Similarly, the Rab1 effector p115 at the cis-Golgi directly binds to syntaxin 5 and GOS28 and stimulates the SNARE complex formation (23). These previous findings along with our own suggest that the tethering/docking process, especially that executed by monomeric Rab effector proteins, may be mediated in part by an interaction with syntaxins.

In contrast to these findings, previous data from Drosophila and squid have indicated that syntaxin is not required for the docking of synaptic vesicles (24–26). Neuronal synapses, however, develop a structural and functional specialization of the active zone that imposes a spatial restriction on release, which is not seen in other secretory systems (27). Thus, the machinery for docking may be different between endocrine and neuronal cells. Furthermore, the docking step, in general, relies on relatively weak ultrastructural definition. Even if synaptic vesicles accumulated near the active zones in the absence of syntaxin, they may not be physically and properly docked to them. Without molecular definition, it is difficult to directly demonstrate the docking step, especially in endocrine cells that lack the specialized site. It also is likely that additional factors at the plasma membrane simultaneously play a role in the targeting process.

Recent progress in membrane fusion suggests that Rab proteins and tethering factors play crucial roles in regulating the loose attachment between vesicles and target membranes including initial recognition, whereas SNARE proteins facilitate a tight attachment of two membranes through the formation of a trans-SNARE complex (28). All of these factors appear to contribute to the fidelity of membrane fusion. In this context, granuphilin may play a role for the specific targeting of insulin granules to the exocytotic site. This is supported by the finding that granuphilin specifically binds to syntaxin 1a but not to other syntaxins (syntaxin 2 and syntaxin 3) expressed on the plasma membrane in beta cells (11). Recently it has been shown that peripheral syntaxin 1 is concentrated in lipid rafts or in cholesterol-dependent clusters, 200 nm in size, at which secretory vesicles preferentially dock and fuse in PC12 cells (29, 30). Furthermore, Ohara-Imaizumi et al. (31) recently have found by evanescent wave microscopic analysis that syntaxin 1 is distributed in numerous separate clusters in the intact plasma membrane of MIN6 cells, where insulin granules were preferentially docked. The targeting site of insulin granules may represent a functionally defined subcompartment of the plasma membrane.

Although the increment of basal insulin secretion caused by granuphilin overexpression may be explained by the peripheral translocation of granules, the profound inhibition of secretagogue-dependent secretion is not well understood. It is of note, however, that regulated secretory pathways should be equipped with machinery such that the activation of the pre-fusion process does not automatically lead to an increase in fusion events. It is possible that other molecular players interact with granuphilin and are involved in this phenomenon. In this context, the finding that Munc18-1 interacts with granuphilin in vitro and by the mammalian two-hybrid assay (16) is intriguing. Munc18-1 has been shown to act at a prefusion event promoting vesicle docking (32, 33). Furthermore, Munc18-1 exhibits an affinity exclusively to a closed form of syntaxin 1a that is not compatible with the SNARE assembly, and this mode of interaction seems to represent a special adaptation for regulated secretory pathways (34). Thus, the pre-fusion steps such as docking and priming likely proceed through multiple steps and are closely interconnected with the fusion machinery in regulated secretory pathways. Currently it is unclear whether granuphilin forms a complex with syntaxin 1a and Munc18-1 simultaneously or separately in vivo. In any case, granuphilin and Munc18-1 somehow must be dissociated from syntaxin 1a to allow syntaxin 1a to form an open conformation and thus a core complex with other SNARE proteins. In this regard, it should be noted that both syntaxin 1a and Munc18-1 may serve as negative regulators of exocytosis in beta cell lines (5, 35), although both proteins are known to mediate and promote SNARE complex formation. Further studies are required to clarify how the complex formation involving granuphilin, syntaxin 1a, and Munc18-1 is regulated. It also is necessary to identify all other components associated with granuphilin to elucidate the multiple functions of this unique molecule.

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Tethering of Insulin Granules by Granuphilin

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