The function of soluble N-ethylmaleimide-sensitive attachment protein-α (α-SNAP) in exocytosis still remains obscure. This study was conducted to determine the physiological role of α-SNAP in the secretion of insulin and γ-aminobutyric acid (GABA) from pancreatic β cells. Reverse transcriptase-polymerase chain reaction analysis of total RNA isolated from rat islets disclosed α-SNAP, but not β-SNAP, mRNA expression, and an immunofluorescence study of rat pancreas showed that α-SNAP was present predominantly in the cytoplasm of the islets of Langerhans. α-SNAP overexpression in rat islets enhanced insulin release relative to the control levels. An in vitro binding study showed that both wild-type α-SNAP and C-terminal-deleted α-SNAP mutant (1–285) can bind to syntaxin 1A. α-SNAP mutant (1–285) was overexpressed to evaluate its activity as dominant-negative effector on insulin release. Overexpression of α-SNAP mutant (1–285) in rat islets and MIN6 cells decreased glucose-stimulated insulin release to about 50% of the control levels. Suppression of endogenous α-SNAP in MIN6 cells by treatment with an antisense phosphorothioate oligonucleotide resulted in inhibition of insulin release. In order to examine if α-SNAP functions in exocytosis from synaptic-like microvesicles in pancreatic β cells, the functional role of α-SNAP in GABA release from MIN6 cells was studied. The data showed no effect of α-SNAP mutant (1–285) overexpression on GABA release. We conclude that 1) α-SNAP plays a crucial role in insulin exocytosis via large dense core vesicles, but not GABA released via synaptic-like microvesicles, in pancreatic β cells; and 2) the interaction of α-SNAP and syntaxin 1A may play an important role in the insulin exocytotic process.

The ambient glucose level plays a central role in the regulation of insulin secretion by pancreatic β cells. Although insulin release is controlled by a complex array of nervous, nutritional, and hormonal actions, glucose is the most important regulatory signal for insulin secretion. The process of insulin release by pancreatic β cells differs from most secretory mechanisms in other cell types in that a nutrient rather than a hormone acts as the first messenger. A large amount of evidence supports a hypothesis that glucose metabolism, in part, inhibits ATP-sensitive K⁺ (KᵦT) channels that are expressed in pancreatic β cells, resulting in membrane depolarization and activation of voltage-sensitive L-type Ca⁺⁺ channels. Finally, elevated intracellular Ca⁺⁺ concentration [Ca²⁺],1 evokes insulin exocytosis (1–4). Thus, although the sensor system for insulin release in pancreatic β cells differs from those in other types of secretory cells (5), insulin exocytosis by pancreatic β cells is strictly controlled by Ca²⁺ ions (3, 6), as in other secretory cells, including those of neuron origin (7). Therefore, the later stages of the process of exocytic insulin release by pancreatic β cells after [Ca²⁺] elevation have been assumed to resemble, although not exactly, exocytic mechanisms in other secretory cell types, such as neurons.

It is now widely accepted that the fundamental components of the machinery required for membrane fusion have been conserved evolutionally and that they are used in both constitutive and regulated membrane trafficking pathways (8, 9). These components include N-ethylmaleimide-sensitive factor (NSF) (10), the soluble NSF attachment proteins (α-, β-, and γ-SNAPs) (11), and membrane-associated SNAP receptors (SNAREs) (12). SNAREs are further divided into two classes of proteins: v-SNAREs (vesicular SNAP receptors) including syntaxin, vesicle-associated membrane protein, and related proteins, and t-SNAREs (target SNAP receptors) including syntaxin, SNAP-25, and related proteins. Recent experiments using purified recombinant proteins have led to further characterization of the protein-protein interactions of the SNAREs with each other (13–16). The findings suggest that syntaxin is the major receptor for α-SNAP that is able to stimulate the ATPase activity of NSF (17). The role of NSF may be to act as a molecular chaperon to modify the conformation of syntaxin (18). According to the original SNARE hypothesis (19, 20), v-SNAREs bind t-SNAREs tightly to form a 7 S complex, which is able to recruit α-SNAP and NSF to form a larger 20 S complex, and finally ATP hydrolysis by NSF then results in SNARE complex disassembly, a late event in the steps leading to membrane fusion during exocytosis. However, recent evi-

---

The abbreviations used are: [Ca²⁺], intracellular calcium; RT-PCR, reverse transcriptase-polymerase chain reaction; NSF, N-ethylmaleimide-sensitive factor; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; SNAP-25, SNAP-25, syntaxosomal-associated protein of 25 kDa; GABA, γ-aminobutyric acid; LDLV, large dense core vesicle; SLMV, synaptic-like microvesicle; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; IRI, immunoreactive insulin.

---

* This work was supported by a Grant-in-Aid for Scientific Research (B) 08457057 from the Japanese Ministry of Education, Science and Culture, a grant from Research for the Future Program, The Japan Society for the Promotion of Science Grant JSPS-RFTF97100201, and by a grant from the Japan Private School Promotion Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biochemistry, Kyorin University School of Medicine, Mitaka, Tokyo 181-8611, Japan. Tel.: 81-422-47-5511 (ext. 3476); Fax: 81-422-47-5538; E-mail: shinya@kyorin-u.ac.jp.

§ This paper is available on line at http://www.jbc.org
α-SNAP Involvement in Insulin Exocytosis

dence in the yeast vacuole system had shown that membrane fusion can occur in the presence of v- and t-SNAREs alone (21, 22); thus, the physiological function of α-SNAP and NSF in exocytosis is now controversial.

Although the molecular machinery that triggers insulin exocytosis by endocrine pancreatic β cells is not fully characterized, in situ hybridization, immunoblotting, and Northern blotting studies have revealed that: 1) pancreatic β cells express v-SNAREs, vesicle membrane-associated protein-2, cellulobrein, and synaptotagmins (23–26); 2) t-SNAREs, SNAP-25, and syntaxin are localized in pancreatic β cells, mainly on plasma membranes (27, 28); and 3) ATPase NSF and α-SNAP are also expressed in insulinoma cells (29). Therefore, the SNAP hypothesis for neurotransmitter release may be applicable, at least in part, to insulin exocytosis. However, the two processes are not identical. Synaptic vesicles are recycled and neurotransmitter is transported into them after the endocytosis of fused granules (30), whereas there is no obvious evidence for a similar recycling of insulin secretory granules. Also, endocrine pancreatic β cells, which are analogous to neuron, contain two large dense core insulin secretory vesicles (LDCVs) (31) and synaptic-like microvesicles (SLMVs) (32) which makes a useful model to study the function of individual SNARE proteins in possibly different forms of exocytosis. In this study, we utilized a recombinant adeno virus-mediated gene transduction system, which enabled expression of high levels of α-SNAP and mutant form in β cells in normal islets or insulinoma cells, and exploited this methodology to evaluate the physiological role of α-SNAP in insulin release via LDCVs and GABA release via SLMVs.

**EXPERIMENTAL PROCEDURES**

**Antibody—**An affinity-purified anti-α- and β-SNAP antibody, which was raised against synthetic peptide corresponding to amino acid residues HYEQSAADYYKGEEL of rat α- and β-SNAP, was a generous gift from Dr. M. Takahashi (Mitsubishi Kasei Institute of Life Sciences, Tokyo, Japan). The characterization of this antibody and its specificity have been described (33).

**Islet Isolation and Cell Culture—**Pancreatic islets were isolated from male Wistar rats (200–250 g) by collagenase digestion and Ficoll gradient centrifugation, as described previously (34). Isolated islets were placed in 1.5-ml Eppendorf tubes and cultured in RPMI 1640 medium containing 11 mM glucose (Life Technologies, Inc., Rockville, MD), supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 200 units/ml penicillin, and 200 µg/ml streptomycin at 37 °C in an atmosphere of 5% CO2 at 37 °C. The media were removed, and the cells were fixed with 4% paraformaldehyde, and then permeabilized with Triton X-100. Then they were incubated with the primary antibody (diluted 1:50) in phosphate-buffered saline at 4 °C for 2 h, washed with 2 mM glycine in phosphate-buffered saline, incubated with fluorescence-labeled secondary antibody at room temperature for 4 h and finally, the cells on the coverslips were examined using fluorescence microscopy.

**Preparation of Recombinant Adenoviruses—**A 1.2-kilobase cDNA fragment containing the entire coding sequence of bovine α-SNAP (a generous gift from Dr. Rothman, Sloan-Kettering Inst., New York) was ligated into the pcAdex1CA cosmide vector (36), which contains the modified chicken β-actin promoter with cytomagalovirus-IE enhancer (CAG promoter) (a generous gift from Dr. Izumi Saito, Tokyo University Institute of Medical Science, Tokyo, Japan). Then, the recombinant adenovirus Adex1CA α-SNAP was prepared by homologous recombination of the expression cosm id cassette and parental viral genome (37, 38), and amplified to achieve a stock with a titer of approximately 10° plaque-forming units/ml. For the construction of α-SNAP mutant, C-terminal truncated α-SNAP mutant (1–285 amino acid residue) was amplified by PCR from a plasmid encoding full-length α-SNAP using the following primers: sense, 5′-GGCTATGCAACTCTCGGGA-3′; antisense, 5′-CTCGTCCACCTGATGTTCTTCTTGGTCGCAGC-3′. The PCR products were confirmed to be correct by automated sequencing, ligated to the pcAdex1CA cosmid vector, and the recombinant adeno virus was constructed as described above.

**Adeno virus-mediated Gene Transduction—**Rat islets, βTC3 and MIN6 cells were cultured with Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum and the required adenosin for 2 h at 37 °C, after which, RPMI 1640 medium containing 11 mM glucose was added and 2 days later, the experiments were performed. When βTC3 and MIN6 cells were infected with the adenovirus Adex1CA Lac Z, Lac Z gene expression was observed in nearly 100% of these cells (data not shown). In agreement with report from another laboratory (30), rat islets infected with Adex1CA mutant and stained with toluidine blue, 3-indolyl-β-d-galactopyranoside were a clear blue color in more than 70% of infected cells (data not shown). Furthermore, insulin biosynthesis and secretion by rat islets infected with Adex1Lw, which contains no foreign genes, were almost the same as those by non-infected islets on post-infection day 2 (data not shown). Therefore, in this study, rat islets and cultured cells infected with Adex1Lw were used as controls.

**Insulin Release—**Two days after infection, the incubation media were removed, the islets or cultured cells were washed three times with RPMI 1640 medium and preincubated with 2.2 mM glucose for 2 h. Tissues were then challenged with 2.2 or 22 mM glucose alone, or 22 mM glucose + 20 mM forskolin for 1 h. The forskolin was added to evoke a near-maximal response. The media were collected at the end of the challenge period, the cells were disrupted by sonication, and aliquots of media and cell extracts were analyzed for immunoreactive insulin (IRI) by radioimmunoassay or enzyme-linked immunosorbent assay (Medical Biology Laboratory [MBL], Nagoa, Japan). In some experiments, insulin release was expressed as the fractional secretion rate per hour, derived from the following equation: total IRI secreted/final IRI in islets + total IRI secreted/final IRI in islets + IRI secreted.

**GABA HPLC Determinations—**Bioanalytical systems HPLC (System Gold; Beckman instruments, Inc., Fullerton, CA) was used to analyze GABA in MIN6-conditioned Hank’s media by a modified isocratic procedure with electrochemical detection (40). Briefly, 30 µl of sample was mixed with 10 µl of derivatization reagent (4 mM o-phthalaldehyde and 2-mercaptoethanol) 2.5 min before injection onto a 4.6 × 150-mm MA-50S column (Eicom Co. Ltd., Kyoto, Japan). Mobile phase (pH 3.5)
was 0.05 M sodium phosphate buffer with 50% methanol delivered at a flow rate of 1.0 ml/min. Quantitation was by electrochemical detection, using a glass carbon electrode set at 0.60 V.

Immunoblotting—Rat islets, βTC3 and MIN6 cells were disrupted by sonication, boiled in SDS sample buffer with 10 mM dithiothreitol, subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and then transferred onto nitrocellulose filters. The protein concentrations were determined using a protein assay kit (Bio-Rad). The filters were incubated with the required primary antibody, followed by the appropriate horseradish peroxidase-conjugated secondary antibody and the bands were visualized using a chemiluminescence detection system (NEN Life Science Products Inc., Boston, MA).

Syntaxin Binding Assay—All the syntaxin binding assays were run in duplicate. Maltose-binding protein-fused syntaxin 1A was attached to amosoy-resin beads as described previously (28). The beads were washed three times with binding buffer (10 mM Hepes-NaOH (pH 7.4), 0.15 mM NaCl, 2 mM MgCl₂, 0.5% Triton X-100) and resuspended in 400 μl of binding buffer containing protease inhibitors (50 μg/ml pepstatin and 1 μM phenylmethylsulfonyl fluoride) and approximately 50–150 ng α-SNAP and/or α-SNAP mutant proteins and rotated (head over hand) overnight at 4°C. The beads in the pellet fraction were washed four times with 1 ml of binding buffer, the bound proteins were eluted in SDS sample buffer and analyzed by SDS-PAGE followed by immunoblotting with anti-α/β-SNAP antibody.

Antisense Phosphorothioate Oligonucleotide—The antisense phosphorothioate oligonucleotide (5′-TCTCATGAGCTGGCCGAGTCTG-3′) complementary to the mouse α-SNAP sequence surrounding the initiation codon and the corresponding sense phosphorothioate oligonucleotide were designed based on the α-SNAP cDNA sequence. They were synthesized on a DNA synthesizer (Applied Biosystem Instruments) and purified by reverse phase high-pressure liquid column chromatography. For antisense experiments, MIN6 cells were treated with a mixture of FuGene® (Boehringer-Mannheim) and 50 μM of each oligonucleotide for 24 h. The medium was renewed and 20 μM of each oligonucleotide was added to the renewed medium every 24 h. After 7 days, the treated MIN6 cells were challenged by 2.2 or 22 mM glucose for 1 h and subjected to IRI and immunoblot analysis.

Statistical Analysis—Results are presented as mean ± S.E. from at least three different experiments performed independently on at least three different cell preparations, unless stated otherwise. Statistical analysis was performed using Student’s t test and ANOVA in multiple comparisons.

RESULTS

α-SNAP Expression and Localization in Pancreatic β-Cells—We initially studied the expression of α/β-SNAP mRNA in the islets of Langerhans, insulinoma βTC3 and MIN6 cells using RT-PCR analysis. The cDNAs reverse-transcribed from the total RNAs isolated from rat islets, βTC3 and MIN6 cells were amplified with degenerate oligonucleotide primers, which were designed to bind to α- and β-SNAP cDNAs simultaneously, and the PCR products were hybridized with the rat α- or β-SNAP cDNA probe. As shown in Fig. 1A, the insulinoma βTC3 and MIN6 cells expressed both α- and β-SNAP mRNAs, whereas rat islets expressed only α-SNAP mRNA. Although the EtBr-stained gel showed a single band in all lanes (data not shown), the autoradiogram from the islets lane showed 2 bands. The upper band above the 627-base pair DNA fragment may have been an unknown isoform of SNAP or an alternatively spliced form, although Northern blot analysis of total RNA extracted from 500 islets showed a weak single band (data not shown). To verify whether rat islets express β-SNAP, we utilized specific oligonucleotide primers based on the rat β-SNAP cDNA sequence obtained by RT-PCR analysis. This method circumvents the lack of information on SNAP in mice. The α-SNAP/β-SNAP protein levels in the islets and insulinoma cell lines were evaluated by performing immunoblot analysis of total cellular protein. Fig. 1B shows that MIN6 cells (10⁴ cells) expressed an appreciable amount of α-SNAP protein, although, βTC3 (10⁵ cells) and rat islets (≈ 10⁶ cells/100 islets) expressed relatively low levels of this protein. A single band of α-SNAP protein with a molecular mass of ≈ 35 kDa was detected in all the cell lysates, but the β-SNAP band was barely detectable in βTC3 and MIN6 cell lysates. Thus, pancreatic β cells predominantly expressed α-SNAP. In order to determine the localization of α-SNAP protein in rat pancreas, we performed an immunofluorescence study. Immunohistochemical staining of rat pancreas sections revealed the presence of α-SNAP in the cytoplasm of the islets of Langerhans (Fig. 2). There was little positive staining of α-SNAP in the exocrine cells.

Adenovirus-mediated α-SNAP Overexpression in Isolated Islets—Overexpression of α-SNAP in rat islets was achieved utilizing an adenovirus-mediated gene transduction system, as demonstrated by immunoblotting with antibodies against α/β-SNAP (Fig. 3). Infecting islets with the Adex1CA α-SNAP recombinant adenovirus resulted in a 10–20-fold increase in the level of the protein relative to that of control islets infected with the Adex1w control adenovirus. The efficiency of the recombinant adenovirus system for gene transfer into mammalian pancreatic islet cells was evaluated with an adenovirus system for gene transfer into mammalian islet cells. In order to determine the localization of α-SNAP protein in rat pancreas, we performed an immunofluorescence study. Immunohistochemical staining of rat pancreas sections revealed the presence of α-SNAP in the cytoplasm of the islets of Langerhans (Fig. 2). There was little positive staining of α-SNAP in the exocrine cells.

α-SNAP was observed in Adex1CA α-SNAP-infected islets. Phase-contrast studies showed similar results. To show that adenovirus-mediated overexpression does not affect protein targeting or sorting, we compared the immunofluorescence of endogeneous α-SNAP protein to that of α-SNAP overexpressed in βTC3 cells. As shown in Fig. 5, in cells infected with Adex1CA α-SNAP, the
immunoreactivity for α-SNAP was observed only in the cytoplasm similar to the distribution of endogenous α-SNAP in uninfected cells.

Effect of α-SNAP Overexpression on Insulin Release by Rat Islets and Insulinoma Cells—Two days post-infection, islets overexpressing α-SNAP were incubated with 2.2 mM glucose or 22 mM glucose alone, or 22 mM glucose + 20 μM forskolin for 1 h. Basal insulin release in the presence of 2.2 mM glucose by islets infected with Adex1CA α-SNAP almost doubled that of the Adex1w-infected control (1.9 ± 0.5 versus 3.9 ± 0.8%/h) (Fig. 6). Insulin release by islets infected with Adex1CA α-SNAP in response to 22 mM glucose alone or 22 mM glucose plus 20 μM forskolin increased by approximately 128 or 144% Adex1w-infected control levels, respectively, (22 mM glucose, 6.7 ± 2.0 versus 8.6 ± 1.6%/h; 22 mM glucose + 20 μM forskolin, 9.4 ± 1.6 versus 13.5 ± 1.7%/h) (Fig. 6). The insulin content of islets infected with Adex1CA α-SNAP tended to decrease, but did not differ significantly from the control value (data not shown). We also examined the effect of α-SNAP overexpression on insulin release by insulinoma MIN6 cells. MIN6 cells infected with Adex1CA α-SNAP showed about a 5–10-fold increase in the level of α-SNAP protein detected by immunoblot analysis, relative to the control level (data not shown). Since MIN6 cells responded to glucose within the range of physiological concentrations during a short incubation period, these cells were challenged with 2.2, 5.5, 11, and 22 mM glucose for 1 h. As shown in Fig. 7, insulin release by MIN6 cells was stimulated by glucose, but was not affected by α-SNAP overexpression.

Binding of Truncated α-SNAP Mutant (1–285) to Syntaxin 1A—In order to further clarify the function of α-SNAP in insulin exocytosis, we attempted to produce and express dominant-negative mutant forms of α-SNAP. For this purpose, we pro-

![Fig. 2. Immunofluorescence photomicrograph of rat pancreas immunostained with anti-α/β-SNAP antibody. Sections of rat pancreas were incubated with rabbit preimmune serum, or affinity-purified anti-α/β-SNAP antibody, followed by fluorescence-conjugated bovine anti-rabbit immunoglobulin. A, anti-α/β-SNAP antibody; B, preimmune serum.](image)

![Fig. 3. Immunoblot analysis of recombinant adenovirus-mediated overexpression of α-SNAP in isolated rat islets. Islet proteins (20–30 islets/lane for experiment 1 and 2, 80 islets/lane for experiment 3) were extracted 2 days after treatment with the indicated adenovirus, subjected to SDS-PAGE, and immunoblotted with anti-α/β-SNAP antibody. The α-SNAP protein band in the lane of Adex1w-treated islets from experiments 1 and 2 was too weak to be visualized by this chemiluminescence detection.](image)

![Fig. 4. Immunofluorescence photomicrograph of adenovirus-infected isolated rat islets stained with anti-α/β-SNAP antibody. Two days after infection with adenovirus, rat islets were processed for immunofluorescence with anti-α/β-SNAP antibody as described under "Experimental Procedures." A, infected with Adex1w; B, infected with Adex1CA α-SNAP. Left panels, immunofluorescence; right panels, phase-contrast.](image)
duced C-terminal deletion mutant α-SNAP (1–285), which has reduced ability to stimulate NSF ATPase activity to less than 30% that of wild-type α-SNAP in vitro (41). The binding of wild-type α-SNAP and truncated α-SNAP mutant (1–285) to syntaxin 1A immobilized on agarose bead was first examined. As shown in Fig. 8, both wild-type α-SNAP and α-SNAP mutant (1–285) proteins bound to syntaxin 1A, although the binding capacity of mutant protein was slightly decreased, in agreement with previous results (41), indicating that overexpression of this mutant may competitively inhibit the cellular binding of wild-type α-SNAP to syntaxin 1A.

Effects of Overexpressed α-SNAP Mutant (1–285) on Insulin Release by Rat Islets and MIN6 Cells—As shown in Fig. 9, immunoblot analysis revealed that rat islets infected with Adex1CA α-SNAP mutant (1–285) expressed high levels of α-SNAP mutant protein. Figs. 9 and 10 show the diminished insulin release by α-SNAP mutant overexpression in rat islets and MIN6 cells, respectively. In particular, a marked reduction in glucose-stimulated insulin release (approximately 50% of control levels) was observed with this mutant in both islets and MIN6 cells (islets, 8.4 ± 2.0 versus 4.6 ± 1.4 ng/islet/h; MIN6, 1785 ± 310 versus 950 ± 220 ng/ml/10⁵ cells). By contrast, the change in insulin release at low glucose was small in any condition.

Involvement of α-SNAP in Insulin Exocytosis—To further examine the physiological function of α-SNAP, we attempted to suppress the expression of endogenous α-SNAP in MIN6 cells by treatment with its antisense oligonucleotide. An antisense phosphorothioate oligonucleotide complementary to the α-SNAP Involvement in Insulin Exocytosis 8057
a-SNAP cDNA sequence surrounding the initiation codon was added to the serum-free medium at a concentration of 40 μM and again at 20 μM every 24 h in medium containing 5% fetal bovine serum. The corresponding sense oligonucleotide was used in parallel as the control. When MIN6 cells were treated with the antisense oligonucleotide for 7 days, the expression of a-SNAP was reduced as observed by immunoblot analysis (Fig. 11). However, treatment of MIN6 cells with the sense oligonucleotide did not affect the expression level of a-SNAP protein.

Antisense oligonucleotide treatment decreased the insulin release to less than 50% the level of oligonucleotide-untreated control values in both basal and stimulated conditions (basal 40 ± 13%, and stimulated 48 ± 16% versus oligonucleotide-untreated control values).

Lack of a-SNAP Function in GABA Release—Although pancreatic β cells are known to release GABA from synaptic-like microvesicles (32), it is still controversial if the release is regulated by glucose (42, 43). Fig. 12 shows that GABA release by MIN6 cells was not affected by glucose, although insulin release was markedly stimulated by glucose as shown in Fig. 7. Thus, our data indicate that GABA is released via a constitutive pathway in pancreatic β cells, in agreement with the report.
represent the mean in vivo with the final fusion step. MIN6 cells. Glucose-stimulated conditions (Fig. 12), although insulin MIN6 cells also did not affect GABA release under both basal and glucose-stimulated conditions (Fig. 12), although insulin release (Fig. 7). Overexpression of mutant a-SNAP (1–285) in MIN6 cells also did not affect GABA release under both basal and glucose-stimulated conditions (Fig. 12), although insulin release was markedly inhibited (Fig. 10).

**DISCUSSION**

In the docking/fusion of secretory vesicles, the v- and t-SNAREs bind each other in a pairwise, cognate fashion (44–46), followed by binding of SNAPs and NSF to the SNARE complex and resulting in membrane fusion (19, 20). However, recent evidence has indicated that in at least some systems, SNAPs and NSF are not essential for fusion (21, 22). Lin and Scheller (47) have recently proposed a new SNARE model, suggesting that the major function of SNARE proteins is in the endocytic reuptake of fused granules (47). On the other hand, there are several reports that a-SNAP function is associated with the final fusion step in vivo (48–51), but most of the evidence has been obtained from experiments with permeabilized cells, which may not fully reproduce the normal localization and/or physiological function of this protein. Function of a-SNAP may appear to differ in various systems, probably because the components themselves, their biochemical characterization, and/or the nature and size of secretory granules in the cells are heterogeneous, but details remain obscure. In the present study, we explored the function of a-SNAP in exocytosis of both insulin and GABA from pancreatic β cells. We analyzed the effect of overexpression of wild-type and mutant a-SNAP on the release of insulin and GABA via LDCVs and SLMVs, respectively, using pancreatic islets and insulinoma β cells.

Since endocytic reuptake and recycling of insulin secretory granules is not likely to occur in pancreatic β cells, our results showing enhanced insulin release by a-SNAP overexpression indicate that a-SNAP functions in the initial docking and fusion of insulin secretory granules consistent with the originally proposed SNARE hypothesis. The mechanism underlying a-SNAP induced increase in insulin release may be as follows. As a-SNAP stimulates the ATPase activity of NSF in vitro (17), a-SNAP overexpression may cause efficient disassembly of the SNARE complex, resulting in efficient membrane fusion. Since the amount of endogeneous a-SNAP in MIN6 cells may already be at saturating levels for the disassembly reaction, a-SNAP overexpression in MIN6 cells is thought to induce no significant changes in insulin release. Although NSF protein expression in rat islets was found to be extremely low based on immunofluorescence and immunoblotting studies,2 it appears from in vitro studies that a-SNAP and NSF functions are coordinated in islets. Indeed, in permeabilized HIT T15 cells, the addition of these proteins into the medium restored the Ca2+-dependent insulin release (51). In the present study, we could not resolve the question as to why the expression of a-SNAP is so low in islets relative to insulinoma cell lines, despite the evidence that βTC3 and MIN6 cells contain much lower insulin and a much smaller population of secretory granules (52–54). Although the other type of SNAP may mainly function in islets, one possible explanation is that since a-SNAP and NSF may be rate-limiting factors for the docking/fusion process, lower expression levels are more adequate for the tightly controlled regulation of secretion characteristics of the islets.

To establish the physiological function of a-SNAP in insulin exocytosis, we overexpressed the a-SNAP mutant to induce a dominant-negative effect on insulin release. a-SNAP, which is composed of 295 amino acids (11), has at least 4 coiled-coil domains (55) and is involved in several protein-protein interactions (12). a-SNAP has the proposed syntaxin-binding domain in the N- and C-terminal portion and has the NSF-binding domain in the C-terminal region (55). Indeed, a-SNAP binds to syntaxin 1A, SNAP-25, and NSF (15). In an attempt to disrupt such interactions by introducing a-SNAP mutant into cells, we produced a C-terminal truncated a-SNAP mutant (1–285), which was effective as a dominant-negative inhibitor as shown in Figs. 9 and 10. Overexpression of a-SNAP mutant (1–285), which can bind to syntaxin 1A but lacks the ability to stimulate NSF ATPase activity (41, 55), decreased markedly insulin release, but, overexpression of the deletion mutant a-SNAP (1–199), which can neither bind to syntaxin 1A nor stimulate NSF ATPase activity (41), has only marginal effect on insulin release (data not shown). Thus, our data indicate that a-SNAP plays a key role in glucose-stimulated insulin exocytosis, probably via the interaction with syntaxin 1A, and suggest that a-SNAP functions in the docking/fusion of insulin secretory granules with the plasma membrane. It may also be speculated that a-SNAP acts as the priming for insulin secretory granules to be targeted to the docking site on the plasma membrane.

In additional studies, we performed experiments using the antisense oligonucleotide in order to suppress the endogeneous a-SNAP expression in MIN6 cells. Antisense-treated MIN6 cells exhibited a marked decrease in both basal and stimulated insulin release, while a dominant-negative effect of a-SNAP mutant (1–285) overexpression was mainly observed in the stimulated insulin release. It is conceivable that antisense oligonucleotide treatment of MIN6 cells inhibited the expression of not only a-SNAP, but also of b-SNAP since the sequence used in the antisense oligonucleotide for a-SNAP was identical to that of b-SNAP. However, a- and b-SNAPs have been re-

2 S. Nagamatsu, T. Watanabe, Y. Nakamichi, C. Yamamura, K. Tsuzuki, and S. Matsushima, unpublished results.
ported to have interchangeable roles in regulated exocytosis (50) and hence may function coordinately in insulinoma cells. Further studies on function of β-SNAP in exocytosis will be necessary to settle this issue.

The question was then addressed as to whether α-SNAP also functions in GABA release. GABA is believed to be released via the docking/fusion of SLMVs. Thus, α-SNAP appears to work differently in different types of secretory granules in even the same cell.

In conclusion, our data demonstrate that α-SNAP plays a key role in insulin release via LDCVs, but not in GABA release via SLMVs in pancreatic β cells. Thus, the functional role of α-SNAP in β cells depends on the nature and types of secretory granules.

Acknowledgments—We thank Dr. I. Saito for the generous gift of bovine Miyazaki for supplying insulinoma MIN6 cells; Dr. J. E. Rothman for critical reviewing the manuscript; and A. Nakahara for assistance in the preparation of the manuscript.

REFERENCES

1. Meglasson, M. D., and Matschinsky, F. M. (1986) Diabetes Metab. Rev. 2, 163–214
2. Newgard, C. B., and McCurdy, J. D. (1995) Annu. Rev. Biochem. 64, 689–719
3. Prentki, M., and Matschinsky, F. M. (1987) Physiol. Rev. 67, 1185–1248
4. Seino, S. (1996) Diabetes Rev. 4, 177–190
5. Malaisse, M. J., Sener, A., Herchuelz, A., and Hutton, J. C. (1979) Metabolism 28, 373–386
6. Wollheim, C. B., and Sharp, G. W. G. (1981) Physiol. Rev. 61, 914–973
7. Knight, D. E., von Graevenitz, H., and Ahidaye, C. M. (1989) Trends Neurosci. 12, 451–458
8. Ferro-Novick, S., and Jahn, R. (1994) Nature 370, 191–193
9. Rothman, J. E., and Warren, G. (1994) Curr. Biol. 4, 220–233
10. Block, M. R., Glick, B. S., Wilcox, C. A., Wieland, F. T., and Rothman, J. E. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7852–7856
11. Whiteheart, S. W., Griff, I. C., Brunner, M., Clary, D. O., Mayer, T., Buhrow, S. A., and Rothman, J. E. (1993) Nature 362, 353–355
12. Sollner, T., Brunner, M., Glick, B. S., Wieland, F. T., and Rothman, J. E. (1993) Nature 362, 318–324
13. Nakahara for assistance in the preparation of the manuscript.