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α-SNAP Functions in Insulin Exocytosis from Mature, but Not Immature Secretory Granules in Pancreatic β Cells

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To explore α-SNAP function in insulin exocytosis from either immature or mature secretory granules in pancreatic β cells, we studied the effects of overexpression of adenovirus-mediated wild-type α-SNAP and C-terminally deleted α-SNAP mutant (1–285) on newly synthesized proinsulin and insulin release by rat islets and MIN6 cells. Rat islets overexpressing α-SNAP and mutant α-SNAP were pulse-chased. Exocytosis from immature and mature insulin secretory granules was measured as fractional (%) labeled-proinsulin release immediately after the pulse-labeling and percentage labeled-insulin release after a 3-h chase period, respectively. There was no difference in percentage labeled-proinsulin release between the control and α-SNAP or mutant α-SNAP-overexpressed islets. Although percentage labeled-insulin release after a 3-h chase period was significantly increased in α-SNAP-overexpressed islets, it was decreased in mutant α-SNAP-overexpressed islets. Thus, the results demonstrated that α-SNAP overexpression in rat islets primarily increased exocytosis from mature, but not immature insulin secretory granules. On the other hand, in MIN6 cells, α-SNAP overexpression scarcely affected glucose-stimulated insulin release; therefore, we examined the effect of mutant α-SNAP overexpression as the dominant-negative inhibitor on the newly synthesized proinsulin/insulin release using the same protocol as in the rat islet experiments. α-SNAP mutant (1–285) overexpression in MIN6 cells decreased the percentage labeled insulin release from mature secretory granules, but not percentage labeled proinsulin release from immature secretory granules. Thus, our data demonstrate that α-SNAP functions mainly in the mature insulin secretory granules in pancreatic β cells.

Key Words: **exocytosis; secretory granule; SNAP; β cell; SNARE.**

The insulin release process from pancreatic β cells is divided into at least three stages, namely, biosynthesis, storage and finally exocytosis, each of which controls insulin release. Glucose not only stimulates insulin biosynthesis both transcriptionally (1–3) and translationally (4, 5), but also insulin release (6, 7); the biosynthetic rate is, however, not correlated with the insulin secretion rate. On the other hand, it appears that insulin secretory granule formation may be associated with final insulin exocytosis, however, there are few reports concerning this association because the details of the mechanisms underlying granule formation and proinsulin/insulin sorting have not been available. Recently, Arvan *et al.* extensively studied the process involved in the sorting of proteins and secretory granule formation within the secretory pathway in pancreatic β cells and demonstrated the physiological role of immature and mature granules in the regulated secretory pathway (8, 9). Immature granules are the major site of final proteolytic cleavage (10) that results in the formation of equimolar amounts of insulin and C-peptide. During the maturation of insulin secretory granules, the clathrin coat disappeared and insulin was crystallized in the cores of maturing granules (10). Thus, pancreatic β cells have at least two types of insulin secretory granules, exocytosis from each of which may have different characteristics. Glucose stimulates exocytosis from both immature and mature granules (11), however, details of the molecular mechanism underlying this stimulation are still unknown.

It is now accepted that the fundamental components of the exocytotic machinery are used in both constitutive and regulated membrane trafficking pathways (12,

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Abbreviations used: SNAP, soluble NSF attachment protein; NSF, *N*-ethylmaleimide sensitive factor; SNARE, SNAP receptor; SNAP-25, synaptosomal associated protein of 25 kDa; VAMP, vesicle associated membrane protein.

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13). These components include NSF, SNAP and SNAREs. We have previously demonstrated that the SNARE hypothesis also could be applied to the mechanism of insulin exocytosis (14, 15). Indeed, there are several reports of the implication of VAMP-2 (16), synaptotagmins (17, 18), syntaxin (14, 19), SNAP-25 (20), α-SNAP and NSF (21, 22) in Ca^{2+}-evoked insulin exocytosis. We have recently reported that α-SNAP plays an important role in glucose-stimulated insulin release by pancreatic β cells (15), though the detailed role of α-SNAP in the insulin exocytotic process of the different types of secretory granules is still undetermined. In the present study, we examined the physiological role of α-SNAP in the exocytotic process in immature and mature insulin secretory granules using pulse-chase experiments. The results demonstrate that α-SNAP functions in exocytosis from mature, but not immature insulin secretory granules of pancreatic β cells.

MATERIALS AND METHODS

Antibody. An affinity-purified anti-α- and β-SNAP antibody, which was raised against synthetic peptide corresponding to amino acid residues HYEQSADYYKGEE 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 has been as described (23).

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 (14). 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 (FBS, Life Technologies, Inc.), 200 units/ml penicillin and 200 μg/ml streptomycin at 37°C, in an atmosphere of 5% CO_2. MIN6 cells (a gift from Dr. J-i. Miyazaki, Osaka University, Osaka, Japan) at passage 15–25 were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS in an atmosphere of 5% CO_2 at 37°C.

Preparation of recombinant adenoviruses. A 1.2-kb cDNA fragment containing the entire coding sequence of bovine α-SNAP (a generous gift from Dr. Rothman, Sloan-Kettering Inst. NY) was ligated into the pAdex1CA cosmid vector (24), which contains the modified chicken β-actin promoter with Cytomegalovirus-IE enhancer (CAG promoter) (a generous gift from Dr. Izumi Saito, Tokyo University Inst. of Med. Sci. Tokyo, Japan). Then, the recombinant adenovirus Adex1CA α-SNAP was prepared by homologous recombination of the expression cosmid cassette and parental viral genome (25, 26), and amplified to achieve a stock with a titer of approximately 10^9 plaque-forming units (pfu)/ml. For the construction of α-SNAP mutant, C-terminally 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′-GCTATGGACAACTCCGGGA-3′; antisense, 5′-CTCGTCACCCTGGATTTACTTCTTGATGCGCAG-3′. The PCR products were confirmed to be correct by automated sequencing, ligated to the pAdex 1CA cosmid vector, and the recombinant adenovirus was constructed as described above.

Adenovirus-mediated gene transduction. Rat islets and MIN6 cells were incubated with DMEM containing 5% FBS and the required adenovirus for 2 h at 37°C, after which, RPMI 1640 medium containing 11 mM glucose was added and two days later, the experiments were performed. As described previously (15), about 70–100% of pancreatic β cells was infected with this procedure. Insulin biosynthesis and secretion by rat islets and MIN6 cells infected with Adex1w, which contain no foreign genes, were almost the same as those by non-infected cells on postinfection day 2 (data not shown). Therefore, in this study, rat islets and MIN6 cells infected with Adex1w were used as controls.

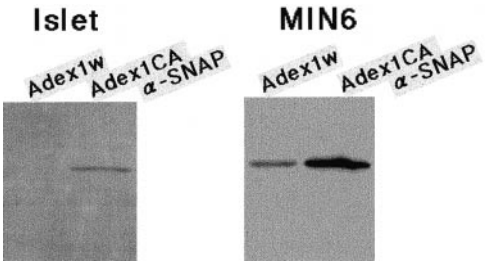

FIG. 1. Immunoblot analysis of recombinant adenovirus-mediated overexpression of α-SNAP in isolated rat islets and MIN6 cells. Rat islets and MIN6 cell proteins were extracted 2 days after treatment with the indicated adenovirus, subjected to SDS–PAGE and immunoblotted with anti-α/β-SNAP antibody. The α-SNAP protein band was visualized by chemiluminescence detection.

Immunoblotting. Rat islets and MIN6 cells were disrupted by sonication, boiled in SDS sample buffer with 10 mM dithiothreitol (DTT), 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 Laboratories, Hercules, CA). The filters were incubated with the required primary antibody, followed by the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody and the bands were visualized using a chemiluminescence detection system (NEN, Boston, MA).

Pulse-chase studies of rat pancreatic islets and insulinoma MIN6 cells. Two days after infection of rat islets and MIN6 cells with the indicated recombinant adenoviruses, they were preincubated with 2.2 mM glucose for 3h, and washed twice with leucine-free RPMI 1640 before pulse-labeling for 30 min at 37°C in the same medium containing 22 mM glucose and 1 mCi L-[4.5-^{3}H]leucine and [^{35}S]methionine and cystein (NEN). Pulse-labeling of cells was stopped by washing in ice cold RPMI 1640, 2.2 mM glucose, and the cells were split into two groups. One was challenged with a basal glucose concentration (2.2 mM) and the other with a stimulatory glucose concentration (22 mM) for 1 h. In some experiments, after stopping the pulse-labeling, 3 h-chase incubation was conducted to permit newly synthesized proinsulin to reach to the mature secretory granules and to be converted to insulin (10, 11). This was followed by a 1h-challenge period with 2.2 or 22 mM glucose. 20 μM forskolin was added to evoke the maximal response in islet experiments. The supernatants were then collected and the labeled cells were lysed by sonication as described previously (23). All samples were spun in a microfuge for 5 min at 4°C to remove debris, and immunoprecipitated with guinea pig anti-insulin serum (DAKO) using protein A–Sepharose beads as described (23). Proinsulin and insulin were separated on a Superdex Peptide column by FPLC system (System Gold; Beckman instruments, Inc., Fullerton, CA), and the radioactivity was measured by a liquid scintillation counter. Immunoreactive insulin (IRI) in the media and cells were measured by ELISA [Medical Biology Laboratory (MBL), Nagoya, Japan] or radioimmunoassay.

Statistical analysis. Results are presented as means ± SEM from at least three different experiments performed independently on at least three different cell preparations, unless stated otherwise. Statistical analysis was performed using ANOVA in multiple comparisons.

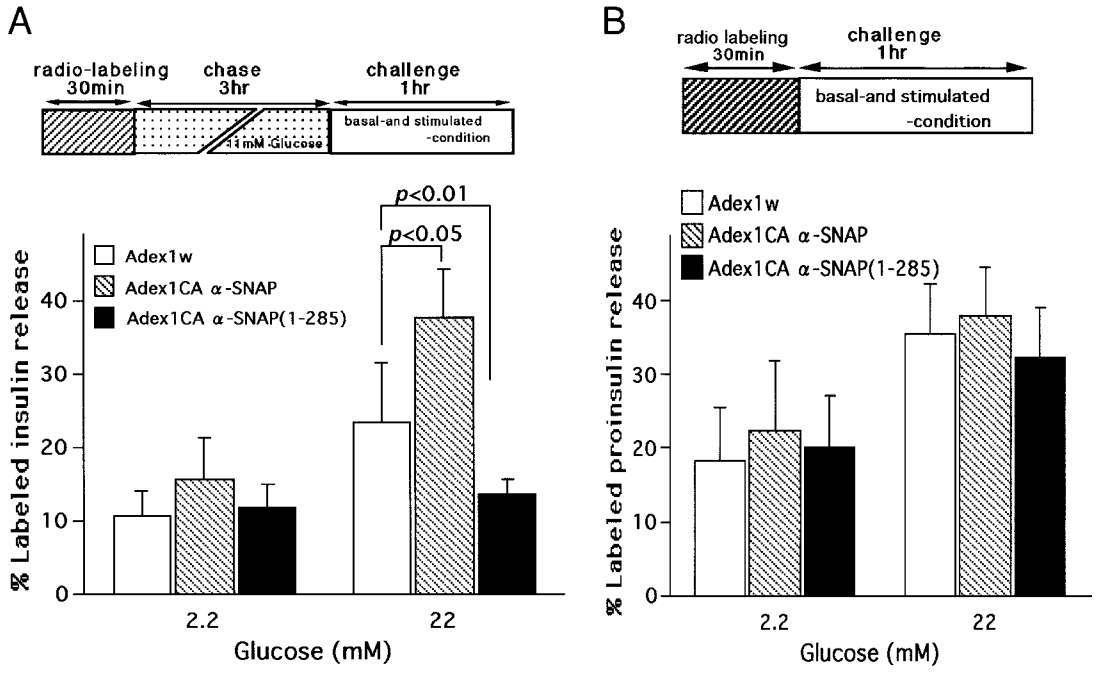

FIG. 2. Percentage labeled-proinsulin and insulin release by rat islets overexpressing α-SNAP and mutant α-SNAP (1-285). Rat islets were infected with Adex1w, Adex1CA α-SNAP, or Adex1CA α-SNAP (1-285), then 2 days later labeled with [^{3}H]leucine and [^{35}S]methionine/cystein for 30 min. Immediately after washing the labeling solution (A), and after the 3-h chase period (B), islets were challenged with 2.2 or 22 mM glucose for 1 h. 20 μM forskolin was added to evoke the maximal response. Proinsulin and insulin from cells and media were immunoprecipitated and separated on a Superdex Peptide Column using the FPLC system as described under Materials and Methods. Labeled-proinsulin and insulin release were expressed as the fractional (%) secretion rate per hour. (A) percentage labeled-proinsulin release during the challenge period immediately after pulse-labeling, and B percentage labeled-insulin release after the 3-h chase period after pulse-labeling.

RESULTS AND DISCUSSION

To examine the role of α-SNAP function in the exocytosis of insulin secretory granules, we utilized the adenovirus-mediated gene transduction system, which could efficiently transfer foreign genes into intact rat islets and insulinoma cell lines as shown previously (15). Infection of rat islets and MIN6 cells with Adex1CA α-SNAP recombinant adenovirus resulted in 5- to 15-fold increases in the level of protein relative to those in controls as determined by immunoblot analysis (Fig. 1).

To investigate the function of -SNAP in the exocytosis from immature and mature insulin secretory granules, we first performed pulse-chase experiments using rat islets 2 days postinfection according to the experimental protocol shown in Fig. 2. The islets were pulse-labeled with [^{3}H]leucine for 30 min, then stimulated by 2.2 or 22 mM glucose for 1 h immediately after pulse-labeling. Because most of newly-synthesized proinsulin enters the immature insulin secretory granules (27) and the conversion of proinsulin to insulin occurs in the immature granules (10), labeled proinsulin release during the challenge period of 1 h immediately after pulse-labeling reflects exocytosis from immature secretory granules. As shown in Fig. 2A, the percentage labeled-proinsulin release from islets overexpressing α-SNAP did not differ from that from control islets, whereas α-SNAP overexpression increased the percentage IRI secretion into the media (Table 1). For measurement of exocytosis from mature insulin secretory granules, after the islets were pulse-labeled with [^{3}H]leucine and [^{35}S]methionine/cystein for 30 min, they were chased for 3 h in 11 mM glucose, then stimulated by glucose as shown in Fig. 2B. Since newly

TABLE 1

	Insulin secretion (IRI) (as % secretion)	
	2.2 mM glucose	22 mM glucose
Control islets	2.15 ± 0.3	9.3 ± 0.4
α-SNAP overexpressed islets	3.5 ± 0.4	11.8 ± 0.7*

Note. Rat islets were infected with Adex1w or Adex1CA α-SNAP, then 2 days later they were preincubated with 0 mM glucose prior to the challenge with 2.2 mM glucose or 22 mM glucose for 1 h. Forskolin (20 μM) was added to evoke a maximal response. The amounts of IRI in the medium and cells were measured and fractional (%) insulin release was calculated. (*$P < 0.01$ vs control values).

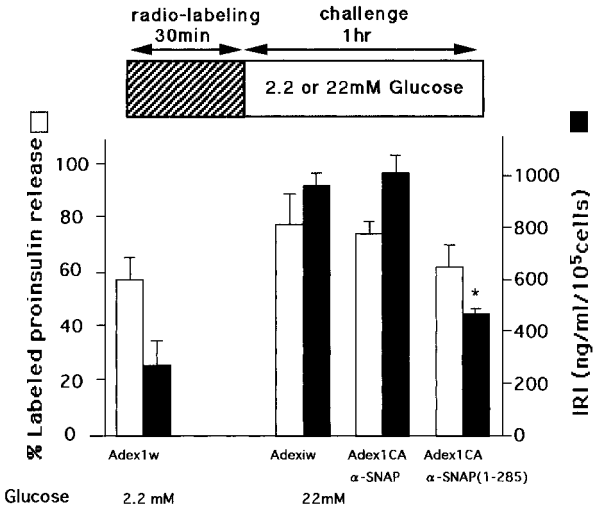

FIG. 3. Effects of α-SNAP and/or α-SNAP mutant (1-285) overexpression on percentage labeled-proinsulin release from MIN6 cells. Two days after infection of MIN6 cells with the indicated recombinant adenoviruses, they were pulse-labeled with [^{3}H]leucine and [^{35}S]methionine/cystein for 30 min. Immediately after washing, cells were challenged with 2.2 or 22 mM glucose for 1 h. The amount of IRI in the media was measured, proinsulin was immunoprecipitated, separated, and the percentage labeled-proinsulin release was calculated as described in the legend to Fig. 2. *$P < 0.001$ (versus Adex1w control values).

synthesized proinsulin and insulin reach the mature secretory granules during the 3 h-chase period and most of the newly synthesized labeled-proinsulin has at least been converted to labeled-insulin during this period (10, 11), the fractional (%) labeled-insulin release during the 1 h challenge period after the 3 h-chase period reflects the exocytosis from mature secretory granules. The percentage labeled-insulin release from α-SNAP-overexpressed islets was 2-fold that from Adex1w-infected control islets under glucose-stimulated conditions (Fig. 2B). On the other hand, percentage labeled-insulin release from mutant α-SNAP (1–285) overexpressed islets was less than that from control islets, because of the dominant negative effect of this mutant. Under unstimulated conditions, there was no observed difference in labeled-insulin release between α-SNAP and mutant α-SNAP overexpressing islets, and control islets indicating that α-SNAP mainly functions in the regulated pathway, in agreement with our previous result (15). Thus, the data indicate that α-SNAP functions in the exocytosis from mature granules but not that from immature granules in rat islets. In contrast, both labeled-proinsulin release and labeled-insulin release were stimulated by glucose (Fig. 2), indicating that glucose stimulates the exocytosis from not only immature secretory granules but also mature secretory granules, in agreement with the results reported previously by Arvans *et al.* (11). In the present study, 22 mM glucose stimulated labeled-insulin and proinsulin secretion by only 2-fold in control islets, probably because islets were desensitized to glucose during 2-days culture period.

To further confirm the data obtained from the rat islet experiments, we studied the functions of α-SNAP in the different type of secretory granules using insulinoma MIN6 cells. As reported previously (15), overexpression of wild-type α-SNAP does not have any effect on MIN6 cells, whereas overexpression of mutant α-SNAP(1–285) with a C-terminal deletion markedly inhibited immunoreactive insulin release (IRI) as the dominant negative inhibitor. Using the same protocol as in the rat islet experiments, the pulse-chase experiments were performed in MIN6 cells infected with recombinant adenoviruses. Two days after infection of MIN6 cells with Adex1CA mutant α-SNAP (1–285), MIN6 cells were challenged with 22 mM glucose for 1 h immediately after 30-min pulse-labeling. The percentage labeled-proinsulin release during this period was not affected by overexpression of the α-SNAP mutant, whereas the IRI release into the media was markedly inhibited by overexpression of the α-SNAP mutant (1–285) (Fig. 3). While the IRI secretion was stimulated about 4-fold by 22 mM glucose, the stimulation of percentage labeled-proinsulin release by glucose was low. This may be due to the reason that insulinoma cells release the newly synthesized proinsulin via both constitutive and regulatory pathway. On the other hand, as shown in Fig. 4, the percentage labeled insulin release after a 3-h chase period was decreased by the overexpression of the α-SNAP mutant (1–285). Thus, the data from MIN6 cell experiments agree with the results obtained from rat islet experiments, indicating that a-SNAP functions mainly in the mature insulin secretory granules of pancreatic β cells.

Why does α-SNAP function in exocytosis from mature insulin secretory granules, but not that from immature insulin secretory granules? So far, there is no clear explanation, but it may be described as follows. Completely selective sorting of soluble trans Golgi network (TGN) proteins appears not to be required at the entry point into forming granules, so that immature insulin secretory granules may contain both regulated and unregulated secretory proteins, as proposed by Arvan (8, 9). Indeed, a sorting pathway out of maturing granules has been directly demonstrated, involving mannose phosphate receptors (MPRs), adaptor protein-1 (AP-1), clathrin, and syntaxin 6 in pancreatic β cells (28). Accordingly, interaction between v- and t-SNAREs may be inefficient in immature granules. Then, granule maturation progresses, and surface components may be replaced, exposed or activated so as to facilitate interactions between v- and t-SNAREs resulting in more efficient functioning of α-SNAP in the final fusion step. Alternatively, immature secretory

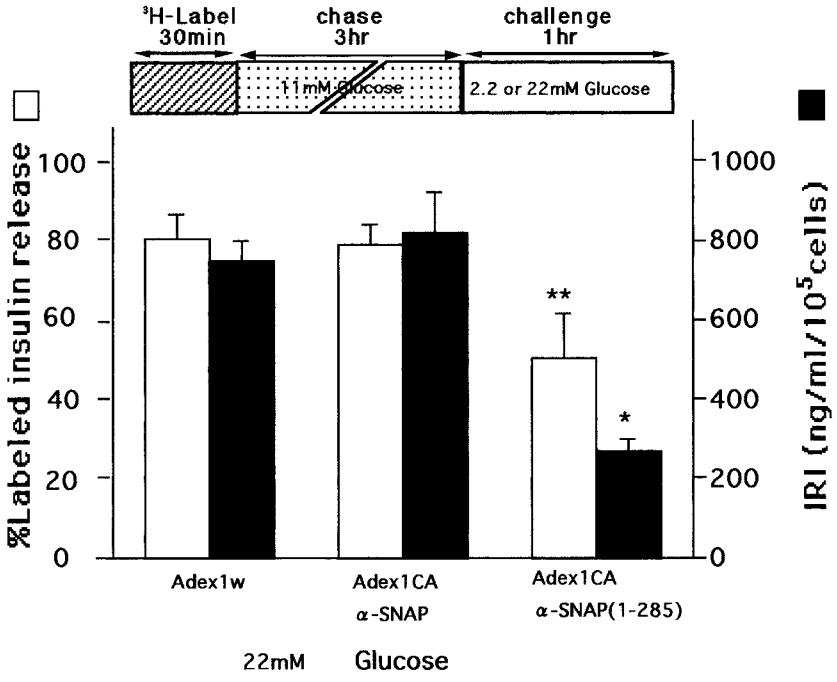

FIG. 4. Effects of α-SNAP and/or α-SNAP mutant (1-285) overexpression on percentage labeled-insulin release post 3-h chase period after pulse-labeling. After MIN6 cells were infected with the indicated recombinant adenoviruses, they were pulse-labeled. Cells were washed, and chased for 3 h in 11 mM glucose, then challenged with 22 mM glucose for 1 h. IRI and percentage labeled-insulin release were processed as described in the legend to Fig. 3. $*P < 0.01$ and $**P < 0.001$ (versus Adex1w control values).

granules may be prevented from approaching the plasma membranes by other mechanisms. *In vitro* binding experiments using immature granule membranes, SNARE proteins and α-SNAP protein would be of interest but are not technically feasible at present.

In conclusion, α-SNAP may play a physiological role in the exocytosis from mature, but not immature insulin secretory granules.

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