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Interaction of ATP Sensor, cAMP Sensor, Ca^{2+} Sensor, and Voltage-dependent Ca^{2+} Channel in Insulin Granule Exocytosis*

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ATP, cAMP, and Ca^{2+} are the major signals in the regulation of insulin granule exocytosis in pancreatic β cells. The sensors and regulators of these signals have been characterized individually. The ATP-sensitive K^+ channel, acting as the ATP sensor, couples cell metabolism to membrane potential. cAMP-GEFII, acting as a cAMP sensor, mediates cAMP-dependent, protein kinase A-independent exocytosis, which requires interaction with both Piccolo as a Ca^{2+} sensor and Rim2 as a Rab3 effector. L-type voltage-dependent Ca^{2+} channels (VDCCs) regulate Ca^{2+} influx. In the present study, we demonstrate interactions of these molecules. Sulfonylurea receptor 1, a subunit of ATP-sensitive K^+ channels, interacts specifically with cAMP-GEFII through nucleotide-binding fold 1, and the interaction is decreased by a high concentration of cAMP. Localization of cAMP-GEFII overlaps with that of Rim2 in plasma membrane of insulin-secreting MIN6 cells. Localization of Rab3 coincides with that of Rim2. Rim2 mutant lacking the Rab3 binding region, when overexpressed in MIN6 cells, is localized exclusively in cytoplasm, and impairs cAMP-dependent exocytosis in MIN6 cells. In addition, Rim2 and Piccolo bind directly to the $\alpha_1$1.2-subunit of VDCC. These results indicate that ATP sensor, cAMP sensor, Ca^{2+} sensor, and VDCC interact with each other, which further suggests that ATP, cAMP, and Ca^{2+} signals in insulin granule exocytosis are integrated in a specialized domain of pancreatic β cells to facilitate stimulus-secretion coupling.

Stimulus-secretion coupling is a specialized feature of secretory cells in which regulated exocytosis occurs, including neuronal, neuroendocrine, endocrine, and exocrine cells (1). These cells possess various molecules that constitute the exocytotic machinery, including soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)[1] proteins (VAMP/synaptobrevin, soluble *N*-ethylmaleimide-sensitive factor attachment protein, and syntaxin), synaptotagmins, and Rab proteins (2, 3). In most secretory cells, the exocytotic process is triggered by an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). In neurons, elevation of $[Ca^{2+}]_i$ results from opening the VDCCs localized in the so-called active zone, a specialized region where synaptic vesicles dock and fuse (4, 5). SNARE proteins have been shown to interact with P/Q-type and N-type VDCC in neurons (6, 7). In addition, recent studies have suggested that the CAZ (cytoskeletal matrix associated with the active zone) proteins, including Piccolo/Aczonin (8, 9), Bassoon (10), Rim1 (11), Munc13–1 (12), and CAST (13), define and organize the site of neurotransmitter release. Piccolo/Aczonin, a 500-kDa protein with zinc fingers, PDZ (PSD-95, Dlg, and ZO-1) domain, and two C_2 domains, binds to PRA1 (14) and profilin (9). Rim1, which is structurally related to Piccolo/Aczonin, is a 180-kDa protein, which interacts with Rab3A (11), RIM-BPs (15), Munc13–1 (16), synaptotagmin I (17), N- and L-type VDCCs (17), and α-liprin (18).

The pancreatic β cell is a typical endocrine cell, in which exocytosis of insulin-containing vesicles is regulated by various intracellular signals. ATP, cAMP, and Ca^{2+} are the major intracellular signals in the regulation of insulin secretion (19). Physiologically, the glucose concentration is the most important determinant of insulin secretion. Glucose stimulation increases the ATP concentration, which closes the K_{ATP} channels, depolarizing the β cell membrane and opening L-type VDCCs, allowing Ca^{2+} influx and triggering exocytosis of insulin granules. The pancreatic β cell K_{ATP} channel comprises two subunits, pore-forming Kir6.2, a member of the inward rectifier K^+ channel family, and the regulatory subunit SUR1, a receptor of the sulfonylureas widely used in the treatment of type 2 diabetes mellitus (20). Studies of Kir6.2 and SUR1-knockout mice demonstrate that the K_{ATP} channel, acting as an ATP sensor, acts as a key molecule by coupling cell metabolism to membrane potential in glucose-induced insulin secretion (21, 22). On the other hand, incretins such as glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide strongly potentiate glucose-induced insulin secretion by cAMP signaling (23, 24). We recently found that cAMP-GEFII/Epac2 (thereafter, cAMP-GEFII) (25–27), acting as a cAMP sensor, mediates cAMP-dependent, protein kinase A-independent insulin secretion, and that this requires interaction with both Rim2 and Piccolo (27–29). We also found that Piccolo forms homodimers or heterodimers with Rim2 in a Ca^{2+}-dependent

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[1] The abbreviations used are: SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; VAMP, vesicle-associated membrane protein; K_{ATP} channel, ATP-sensitive K^+ channel; VDCC, voltage-dependent Ca^{2+} channel; NBF-1, nucleotide-binding fold 1; CAZ, cytoskeletal matrix associated with the active zone; PDZ, PSD-95, Dlg, and ZO-1; SUR, sulfonylurea receptor; Epac, exchange protein directly activated by cAMP; GEF, guanine nucleotide exchange factor; MDR, multidrug resistance receptor; MBP, maltose-binding protein; GST, glutathione S-transferase; 8-bromo-cAMP, 8-bromoadenosine 3′, 5′ cyclic monophosphate; ABC, ATP-binding cassette.

manner and that Piccolo rather than Rim2 may function as a Ca^{2+} sensor (29). In addition, L-type VDCCs are known to regulate Ca^{2+} influx into pancreatic β cells. Although the sensors and regulators of ATP, cAMP, and Ca^{2+} signals have been characterized individually, the interactions of these molecules have not been demonstrated directly.

In the present study, we have investigated interactions of the K_{ATP} channel, cAMP-GEFII, Rim2, Piccolo, and L-type VDCC. We found that these proteins interact with each other, suggesting the integration of ATP, cAMP, and Ca^{2+} signals in a specialized domain of pancreatic β cells to facilitate stimulus-secretion coupling.

EXPERIMENTAL PROCEDURES

Recombinant Fusion Proteins—Partial SUR1 (amino acid residues 598–1003) (SUR1-NBF-1), C terminus of rat SUR1 (1299–1581) (SUR1-NBF-2), rat SUR2A (589–963) (SUR2A-NBF-1), and human MDR1 (347–710) (MDR1-NBF-1) were expressed as maltose-binding protein (MBP)-fused proteins in BL21 and purified by affinity chromatography with amylose-resin (New England Biolabs). Rim2 (538–863), Rim2 (198–830) (Rim2ΔA), Rim2 (198–649), Rim2 (650–797) (Rim2PDZ), Rim2 (762–970) (Rim2-C_2A), Rim2 (1334–1531) (Rim2-C_2B), Piccolo (4505–4758), Piccolo (4704–5010) (Piccolo-C_2A), and Piccolo (4955–5165) (Piccolo-C_2B) were expressed as a GST (glutathione *S*-transferase)-fused protein in BL21 and purified by affinity chromatography with glutathione-resin (Amersham Biosciences). Site-directed mutagenesis of the PDZ domain in Rim2 was performed by the PCR-based method. The mutant was expressed as a GST-fused protein. A fragment containing the $\alpha_1$1.2-subunit (745–892) or $\alpha_1$1.3-subunit (773–907) of L-type VDCC was subcloned in pGBKT7 vector (Clontech) as Myc-tagged protein.

Pull-down Assays—For cosedimentation assays, COS-1 cells were transfected with each plasmid using LipofectAMINE (Invitrogen). Transfected COS-1 cells or mouse insulinoma MIN6 cells were sonicated in buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, and 0.5% Nonidet P-40). The cellular lysates were incubated with 1 μg of MBP or GST-fused protein immobilized on amylose- or glutathione-resin for 90 min at 4 °C. The washed complexes were separated by SDS-PAGE, and subjected to immunoblot analysis with the IgG-purified anti-cAMP-GEFII antibody (27), anti-FLAG M2 antibody (Sigma), or anti-Myc antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

GST-fused Piccolo-C_2A, Piccolo-C_2B, Rim2-C_2A, and Rim2C_2B proteins immobilized on glutathione-resin were incubated with fragments of $\alpha_1$1.2-subunit (745–892) or $\alpha_1$1.3-subunit (773–907) produced by *in vitro* translation (Promega Corp., Madison, WI) in binding buffer (20 mM HEPES, pH 7.5, 150 mM KCl, 1 mM dithiothreitol, 5% glycerol, 0.05% Tween 20, 1 mg/ml bovine serum albumin). The washed complexes were separated by SDS-PAGE, and subjected to immunoblot analysis with anti-Myc antibody.

Subcellular Fractionation—MIN6 cells lysed with hypotonic buffer containing 10 mM HEPES, pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride were used. The lysate was incubated on ice for 30 min and centrifuged at 800 × *g* for 5 min to remove nuclear fractions. The supernatant was centrifuged at 200,000 × *g* for 1 h and collected as cytosolic fractions. The pellet (membrane fraction) was suspended with 10 mM HEPES, pH 7.4, 1.5 mM $MgCl_2$, 1 mM EDTA, 150 mM NaCl, 10% glycerol, and 0.5 mM dithiothreitol. The mixture was sonicated for 30 s twice on ice and centrifuged at 200,000 × *g* for 1 h. The supernatant was collected as a membrane extract fraction. Each fraction was subjected to immunoblot analysis using anti-cAMP-GEFII antibody and anti-Rim2 antibody raised against Rim2 peptide (amino acid residues 348–365).

Sucrose gradient fractionation of MIN6 cells was performed as described previously (27). Briefly, MIN6 cells were harvested by homogenization buffer containing 200 mM sucrose, 50 mM NaCl, 2 mM EGTA, 10 mM HEPES at pH 7.2, and 1 mM phenylmethylsulfonyl fluoride and homogenized. The homogenate was centrifuged at 1770 × *g* for 6 min at 4 °C. The resulting postnuclear supernatant was applied to the top of gradients of sucrose steps (0.4, 0.6, 0.8, 1.0, 1.4, or 1.8 M sucrose in 10 mM HEPES, pH 7.2, and 2 mM EGTA), and centrifuged at 55,000 × *g* for 2 h at 4 °C. The fractions were collected from the top to the bottom, precipitated with 15% trichloroacetic acid, and subjected to immunoblot analysis using anti-Rim2 antibody, anti Na^+-K^+-ATPase α-1 antibody (Upstate Biotechnology, Lake Placid, NY), anti-Rab3 antibody (Transduction Laboratories, Lexington, KY), and anti-VAMP-2 antibody (Calbiochem-Novabiochem Corp.).

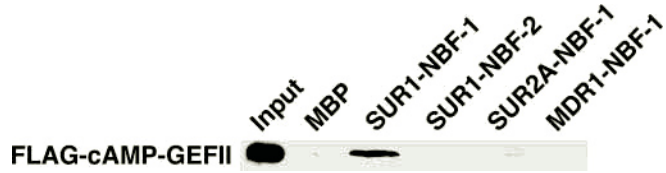

FIG. 1. **Binding of full-length cAMP-GEFII to ABC proteins.** FLAG-tagged full-length cAMP-GEFII was transfected into COS-1 cells. The cell lysates were incubated with MBP-fused proteins.

Measurement of C-peptide Secretion—MIN6 cells were cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose, 10% (v/v) fetal bovine serum under a humidified condition of 95% air and 5% CO_2. MIN6 cells were transfected with human preproinsulin expression vector plus pCMV-luciferase, pCMV-Myc-Rim2ΔN, or pCMV-Myc-Rim2ΔC_2. As a control, luciferase was used. Three days after transfection, the C-peptide secretory response to 1 mM of 8-bromo-cAMP (8-bromoadenosine 3′, 5′ cyclic monophosphate) (Sigma) in the presence of glucose (16.7 mM) for 60 min was evaluated by human C-peptide released into medium. Human C-peptide was measured by human C-peptide RIA kit (Linco Research Inc., St. Charles, MO).

Immunocytochemistry—MIN6 cells were transfected with pCMV-Myc-full-length Rim2 and Rim2ΔN. The cultured cells were fixed with 3.7% formaldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 min at room temperature and thoroughly rinsed with 0.1 M phosphate-buffered saline. After the samples were pretreated with 0.2% Triton X-100 and 10% normal donkey serum, they were incubated with rabbit anti-Myc antibody and mouse anti-Rab3 antibody, followed by Cy3-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and fluorescein isothiocyanate-labeled donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories), respectively. The sections were mounted with coverslips using PourmaFlour (Immunon, Pittsburgh, PA) and observed by confocal laser scanning microscopy (LSM410, Carl Zeiss, Tokyo).

RESULTS

Specific Interaction of SUR1 and cAMP-GEFII—In our search for a molecule directly modulating K_{ATP} channel activity, we previously performed a yeast two-hybrid screen of the MIN6 cDNA library using a partial SUR1 as bait and found that cAMP-GEFII (amino acid residues 186–729) bound to NBF-1 of SUR1 (598–1003) (27). Because the NBF is conserved in the ATP-binding cassette (ABC) protein family, we examined cAMP-GEFII binding to other ABC proteins. MBP-fused proteins with SUR1-NBF-2 (1299–1581), SUR2A-NBF-1 (589–963) (30), and MDR1-NBF-1 (347–710) (31) were constructed. As shown in Fig. 1, FLAG-tagged full-length cAMP-GEFII expressed in COS-1 cells did not bind SUR1-NBF-2, SUR2A-NBF-1, or MDR1-NBF-1, indicating that cAMP-GEFII specifically binds to NBF-1 of SUR1.

Binding of cAMP-GEFII and SUR1 Is Regulated by cAMP—Because cAMP induces conformational change of the protein kinase A regulatory subunit and cAMP-GEFII upon binding (32, 33), we investigated whether cAMP binding affects the interaction of SUR1 and cAMP-GEFII. MBP-SUR1-NBF-1 was incubated with lysate of MIN6 cells in 1 or 100 μM 8-bromo-cAMP to determine whether endogenous cAMP-GEFII in MIN6 cells interacts with SUR1-NBF-1 and if such an interaction is regulated by cAMP. Interaction between cAMP-GEFII and SUR1 is decreased by a high concentration (100 μM) of 8-bromo-cAMP (Fig. 2*A*). We also examined whether cAMP regulates the interaction of cAMP-GEFII with Rim2 or Piccolo. MIN6 cells treated with 1 mM 8-bromo-cAMP were subjected to *in vitro* binding assay with GST-Rim2 (538–863) (27) or GST-Piccolo (4505–4758) (29) immobilized on glutathione resins. cAMP-GEFII formed a complex with both Rim2 (538–863) and Piccolo (4505–4758) both in the presence and in the absence of 1 mM 8-bromo-cAMP (Fig. 2*B*). These results show that endogenous cAMP-GEFII binds to SUR1 and suggests that cAMP

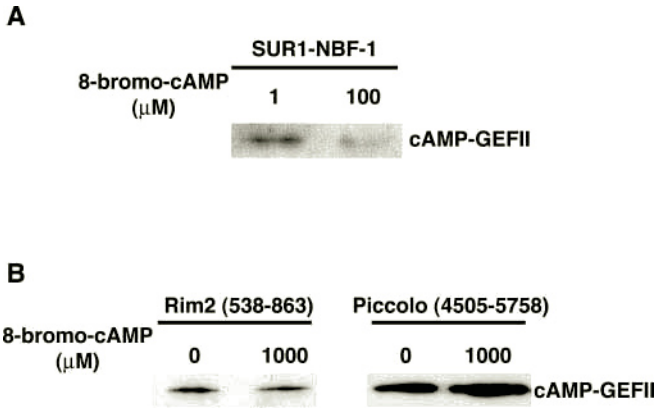

FIG. 2. **Effects of cAMP upon binding of cAMP-GEFII to SUR1, Rim2, or Piccolo.** The lysates of MIN6 cells were incubated with MBP-SUR1-NBF-1, GST-Rim2 (538–863), or GST-Piccolo (4505–4758) in the absence or presence of 8-bromo-cAMP. The samples were analyzed by immunoblotting with anti-cAMP-GEFII antibody. *A*, binding of cAMP-GEFII to SUR1. *B*, binding of cAMP-GEFII to Rim2 (538–863) or Piccolo (4505–4758).

inhibits interaction of SUR1 and cAMP-GEFII and does not affect the interaction of cAMP-GEFII and Rim2 or Piccolo.

Protein Domains Responsible for Binding Rim2 and cAMP-GEFII—Both Rim1 and Rim2 have an amino (N)-terminal zinc finger domain, a PDZ domain, and two C_2 domains. It has been suggested that Rim1 functions as a scaffolding protein, which interacts physically through these domains with Rab3, Munc13–1, Ca^{2+} channels, synaptotagmin I, and α-liprin (11, 16–19). Assuming that Rim2 also functions as a scaffolding protein in determining which region of Rim2 interacts with cAMP-GEFII, we constructed the various Rim2 deletion mutants shown in Fig. 3*A* and performed a GST pull-down assay. GST-fused proteins were incubated with lysates of MIN6 cells. Fig. 3*B* shows the interaction of endogenous cAMP-GEFII in MIN6 cells and Rim2 mutants. Although the Rim2 mutant (198–649) lacking a PDZ domain did not bind to cAMP-GEFII, the mutant having a PDZ domain (Rim2PDZ) bound to cAMP-GEFII, indicating that the PDZ domain contains the cAMP-GEFII binding region. The Rim2 mutant, in which the well conserved amino acids (Arg^{682}, Leu^{688}, and Gly^{689}) critical for binding to PDZ-interacting protein (34) are replaced with alanines, did not bind to endogenous cAMP-GEFII in MIN6 cells (Fig. 3*C*). This further confirms that the PDZ domain of Rim2 is responsible for binding to cAMP-GEFII, using its mutants shown in Fig. 3*D*. All cAMP-GEFII mutants lacking the carboxyl (C)-terminal region bound to Rim2PDZ, but the cAMP-GEFII mutant lacking the N-terminal region (residues 291–1011) did not (Fig. 3*E*). These results show that cAMP-GEFII, through its N-terminal region, binds to the PDZ domain of Rim2.

Subcellular Localization of cAMP-GEFII and Rim2—Localization of cAMP-GEFII and Rim2 in MIN6 cells was examined using subcellular fractionation (Fig. 4*A*). cAMP-GEFII is localized both in cytosolic and membrane fractions, whereas Rim2 is localized almost exclusively in membrane fractions. Rim2 was also fractionated by sucrose gradient centrifugation from MIN6 cells (Fig. 4*B*). Rim2 is present in plasma membrane fractions (fractions 2 and 3; Na^+-K^+-ATPase α-1 as a marker) and large dense-core granule fractions (fractions 7 and 8; VAMP-2 is a marker). These results show that although the intracellular localizations of cAMP-GEFII and Rim2 differ, they overlap in the membrane fraction.

The N Terminus of Rim2 Is Important for both Subcellular Localization and cAMP-dependent Exocytosis—The N-terminal regions of Rim1 and Rim2 have been shown to bind Rab3 (11,

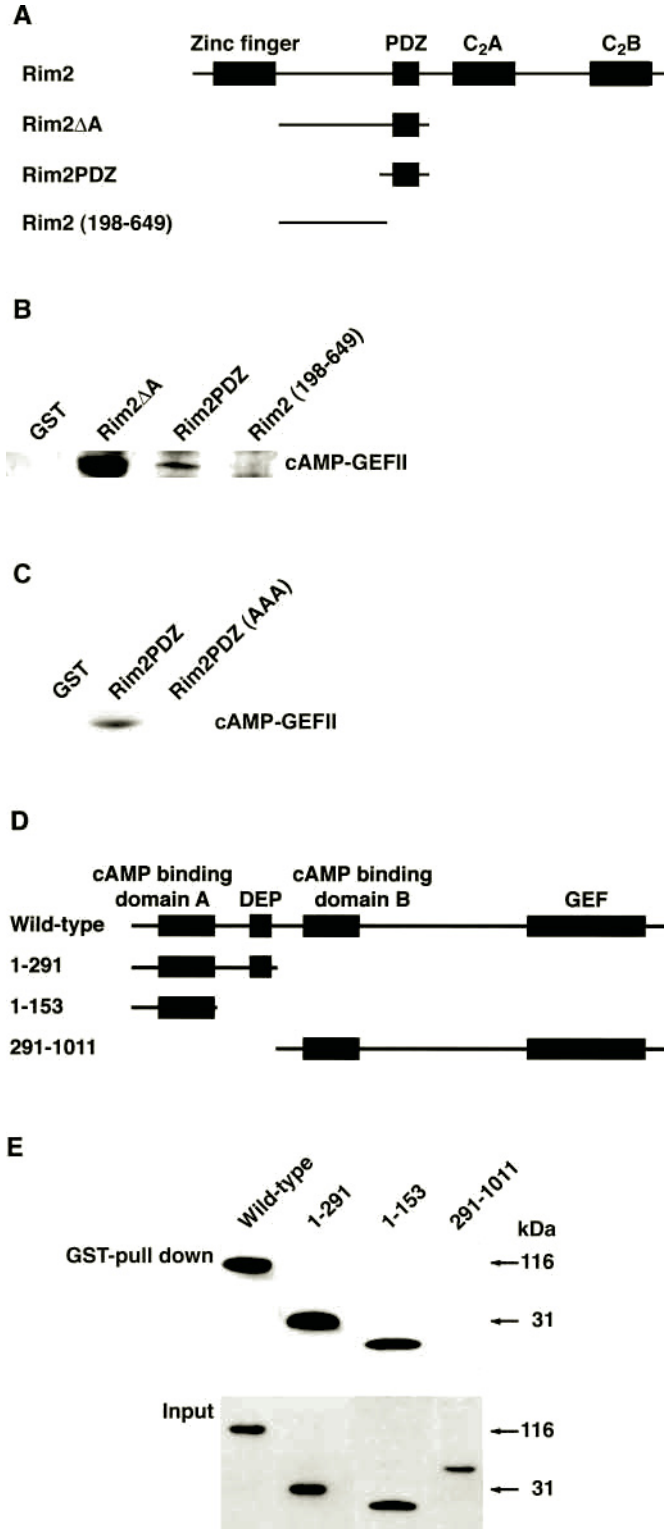

FIG. 3. **Protein domains responsible for binding cAMP-GEFII and Rim2.** *A*, schematic representation of full-length Rim2 and its mutants. *B*, binding of Rim2 mutants to cAMP-GEFII. The lysates of MIN6 cells were incubated with GST-Rim2 mutants. The samples were analyzed by immunoblotting with anti-cAMP-GEFII antibody. *C*, binding of Rim2PDZ to cAMP-GEFII. The Arg^{682}, Leu^{688}, and Gly^{689} in the PDZ domain of Rim2 respectively were replaced with alanines. The GST-Rim2PDZ(AAA) was subjected to binding assay with endogenous cAMP-GEFII in MIN6 cells. *D*, schematic representation of full-length cAMP-GEFII and its mutants. *E*, Myc-tagged full-length cAMP-GEFII or cAMP-GEFII mutant was transfected into COS-1 cells. The lysates of transfected cells were incubated with GST-Rim2PDZ protein. The samples were analyzed by immunoblotting with anti-Myc antibody.

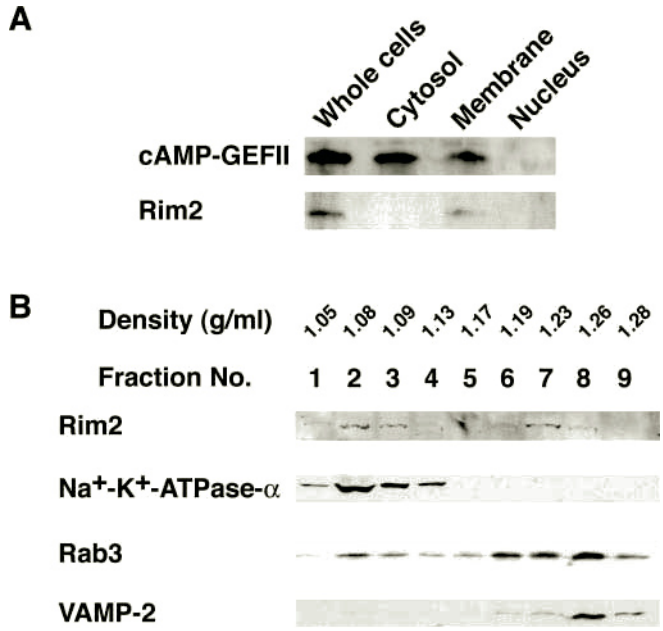

FIG. 4. **Localization of cAMP-GEFII and Rim2 in MIN6 cells.** *A*, immunoblot analysis of cAMP-GEFII and Rim2 in MIN6 cells. Cytosolic, membrane, and nuclear fractions from MIN6 cells were separated and analyzed by immunoblotting with anti-cAMP-GEFII antibody and anti-Rim2 antibody. *B*, immunoblot analysis of sucrose gradient fractions of MIN6 cells. Postnuclear supernatants from MIN6 cells were separated on sucrose step gradients. The fractions were analyzed by immunoblotting with anti-Rim2 antibody, anti-Na^+-K^+-ATPase α-1 antibody, anti-Rab3 antibody, and anti-VAMP-2 antibody.

27). We examined the role of this region in subcellular localization using the N-terminal deletion mutant (Rim2ΔN) (Fig. 5*A*). Wild-type Rim2 transfected into MIN6 cells is localized with Rab3 in both insulin granules and plasma membrane. In contrast, Rim2ΔN is localized exclusively in the cytoplasm. Localization of Rim2ΔN is quite different from that of wild-type Rim2, indicating that the N terminus of Rim2 is critical in localization with the granules (Fig. 5*B*). We next examined the effect of Rim2ΔN on cAMP-dependent insulin secretion (Fig. 5*C*). Insulin secretion was monitored indirectly by measuring C-peptide secretion from MIN6 cells, and transfected with human preproinsulin cDNA as reported previously (27). cAMP-dependent C-peptide secretion from Rim2ΔN-transfected MIN6 cells was reduced significantly, compared with that from luciferase-transfected MIN6 cells (control). However, Rim2 mutant lacking C_2 domains (Rim2ΔC_2) did not inhibit cAMP-dependent C-peptide secretion. These results show that the interaction of Rim2 and Rab3 is required in cAMP-dependent insulin secretion.

Both Piccolo and Rim2 Interact Directly with L-type VDCC—Because synaptotagmin I, which has a C_2 domain, is known to interact with α-subunits of VDCC through their cytoplasmic loops which connect domains II and III, we examined whether Piccolo and Rim2, both of which have C_2 domains, also interact with the α-subunit. The C_2 domains of Piccolo bind directly to the cytoplasmic loop of the $\alpha_1$1.2-subunit but not the $\alpha_1$1.3-subunit of L-type VDCC (Fig. 6). Similarly, C_2 domains of Rim2 bind directly to the $\alpha_1$1.2-subunit but not to the $\alpha_1$1.3-subunit.

DISCUSSION

Stimulus-secretion coupling is a critical event in pancreatic β cells, where the K_{ATP} channel controls glucose-induced and sulfonylurea-induced insulin release. Pancreatic β cell K_{ATP} channels are composed of Kir6.2 and SUR1 subunits, the pore-forming subunit, and a regulatory subunit that primarily confers activation by MgADP, potassium channel openers, and inhibition by sulfonylureas, respectively. SUR1 also is required for the functional expression of the K_{ATP} channel at the cell surface (35). In our search for a molecule that interacted with NBF-1 of SUR1, we identified cAMP-GEFII (also referred to as Epac2) (27). Because the NBF is conserved among ABC proteins, cAMP-GEFII might well bind to other ABC proteins. However, cAMP-GEFII did not bind to SUR1-NBF-2, SUR2A-NBF-1, or MDR1-NBF-1, indicating that cAMP-GEFII binds specifically to NBF-1 of SUR. In addition, cAMP-GEFII had no effect upon K_{ATP} channel activity under various conditions (data not shown). Accordingly, it is possible that SUR1 functions as a scaffolding protein as well as the regulatory subunit of the K_{ATP} channel. cAMP-GEFII has been shown to bind to Rim2 and Piccolo (27–29), both of which are known as CAZ proteins (8, 9, 11). Although the PDZ domain of Piccolo was found to bind to cAMP-GEFII (29), the region of Rim2 responsible for binding to cAMP-GEFII is not known. Using various Rim2 deletion mutants, we found that the PDZ domain of Rim2 is necessary for binding. These findings indicate that CAZ proteins having PDZ domains can form a complex with cAMP-GEFII. It is generally thought that the PDZ domain binds to consensus sequence E/D/S/T/ϕ-X-ϕ (where ϕ is a hydrophobic amino acid and X is any amino acid) of the C-terminal region of PDZ-interacting proteins (34). However, we find that cAMP-GEFII, through its N-terminal region that does not have such a consensus sequence, binds to the PDZ domain of Rim2, suggesting that cAMP-GEFII binds uniquely to the PDZ domain. As deletion of the C-terminal region with GEF activity toward Rap1 does not affect binding of cAMP-GEFII to either Rim2 or Piccolo, Rap1 is not involved. cAMP-GEFII has been shown to be responsible in cAMP-dependent, protein kinase A-independent exocytosis (27, 28). Accordingly, we examined the effects of cAMP upon the binding of SUR1 and cAMP-GEFII and upon the binding of cAMP-GEFII and Rim2 or Piccolo. Interestingly, cAMP-GEFII and SUR1 binding is decreased by a high cAMP concentration. It has been shown also that cAMP binding to cAMP-GEFII induces its conformational change (33). Such an alteration might dissociate cAMP-GEFII from SUR1 and induce the interaction of cAMP-GEFII and Rim2. We found that Ca^{2+} does not affect the binding of cAMP-GEFII to Rim2 or Piccolo. These results indicate that neither cAMP nor Ca^{2+} are factors in the binding of cAMP-GEFII and Rim2 or Piccolo. Analyses of the subcellular localizations of cAMP-GEFII and Rim2 show that they overlap at least in the plasma membrane. Piccolo also has been shown to be located in the plasma membrane in neurons (8). Taken together, these findings suggest that cAMP-GEFII interacts with Rim2 and Piccolo in the plasma membrane.

The N-terminal region of Rim1 binds to Rab3, which is involved in Ca^{2+}-triggered exocytosis (11). We find that deletion of the N-terminal region of Rim2 (Rim2ΔN) results in the loss of binding ability to Rab3 and that, unlike wild-type Rim2, Rim2ΔN is distributed diffusely in the cytoplasm; localization is also quite different from that of Rab3. Overexpression of Rim2ΔN in MIN6 cells shows that cAMP-dependent, Ca^{2+}-triggered secretion is reduced significantly compared with control (wild-type Rim2-transfected MIN6 cells). This reduction probably occurs because Rim2ΔN cannot transduce the cAMP signal to Rab3-mediated exocytosis and/or cannot couple to the exocytotic machinery. We reported recently that Piccolo functions as a Ca^{2+} sensor in pancreatic β cells (29). The present finding that deletion of the C_2 domain of Rim2 does not affect cAMP-potentiated, Ca^{2+}-triggered secretion supports this report.

Synaptotagmin I, a Ca^{2+} sensor in neurons, interacts with the $\alpha_1$2.1-subunit of P/Q-type VDCC (36) and $\alpha_1$2.2-subunit of

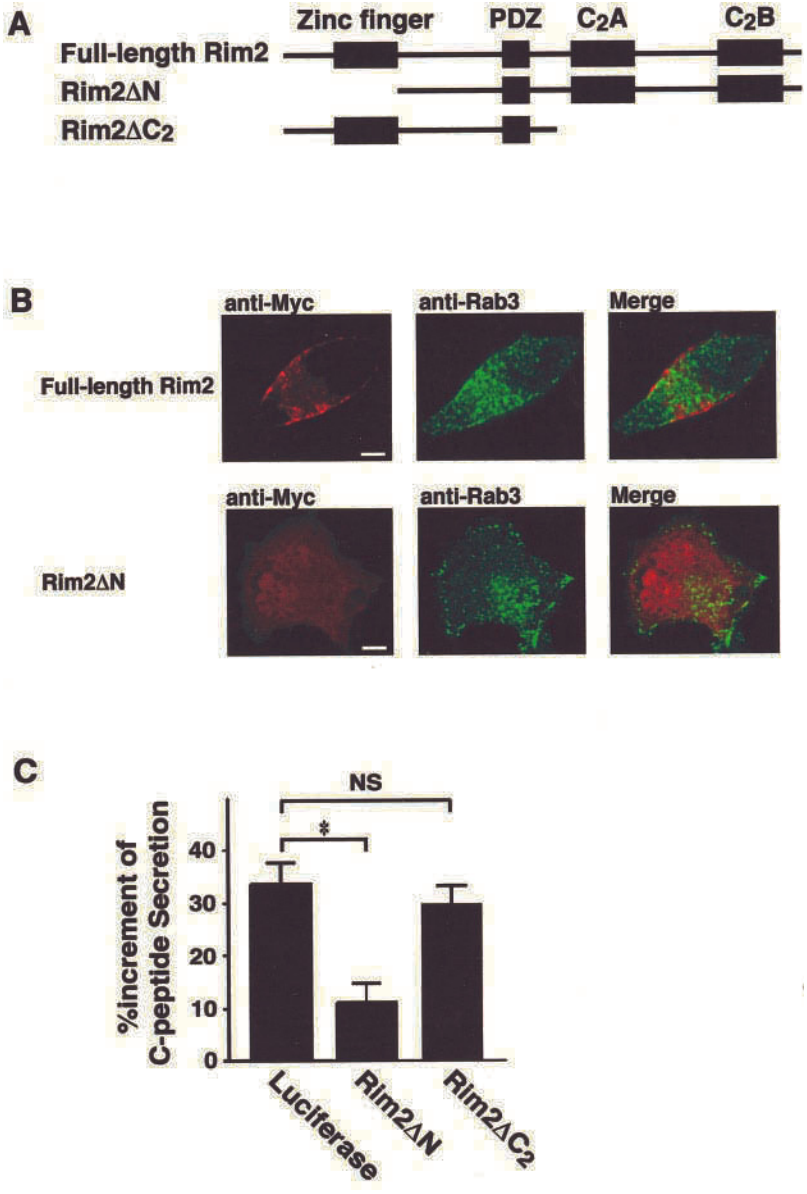

FIG. 5. **Subcellular localization of Rim2ΔN and cAMP-dependent exocytosis.** *A*, schematic representation of full-length Rim2 and Rim2 mutants. *B*, localization of Rim2 and Rab3 in MIN6 cells. MIN6 cells were transfected with Myc-tagged full-length Rim2 cDNA (*upper panels*) or Myc-tagged Rim2ΔN (*lower panels*). Myc-tagged full-length Rim2 and Myc-tagged Rim2ΔN visualized in Cy3 channel (*left two panels*). Endogenous Rab3 visualized in fluorescein isothiocyanate channel (*middle two panels*). *Bar*, 10 μm. *C*, human C-peptide secretion in response to 8-bromo-cAMP (1 mM) in MIN6 cells transfected with human preproinsulin together with luciferase, Rim2ΔN, or Rim2ΔC_2 (*, $p < 0.01$). C-peptide secretion is expressed as percent increment of secretion in the absence of 8-bromo-cAMP. Data were obtained from three independent experiments ($n = 14–16$). Values are expressed as mean ± S.E. Data are compared using Student's unpaired *t* test.

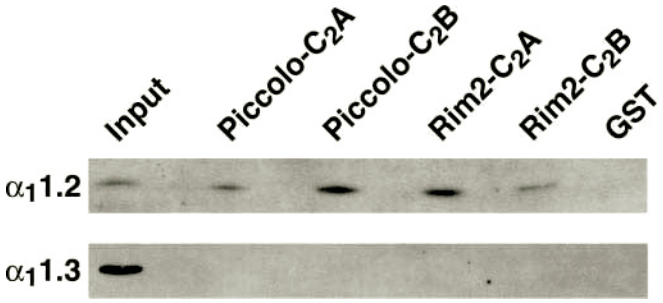

FIG. 6. **Binding of Piccolo or Rim2 and α_1-subunit of VDCC.** GST-C_2 domains of Piccolo and Rim2 were incubated with Myc-tagged $\alpha_1$1.2-subunit (745–892) or $\alpha_1$1.3-subunit (773–907) of L-type VDCC. The samples were analyzed by immunoblotting with anti-Myc antibody.

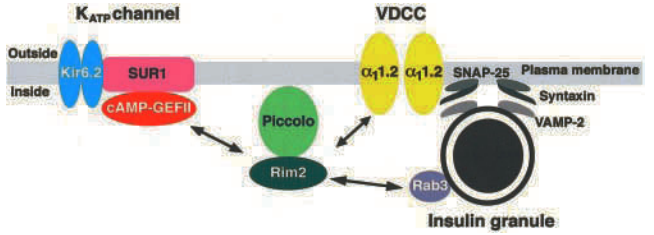

FIG. 7. **Model of interaction of K_{ATP} channel, cAMP-GEFII, Rim2, Piccolo, and VDCC in insulin granule exocytosis.** ATP, cAMP, and calcium signals are integrated in a specialized region of the pancreatic β cell. The K_{ATP} channel interacts specifically with cAMP-GEFII through NBF-1 of SUR1. A rise in cAMP concentration can dissociate cAMP-GEFII from SUR1. Rim2 and Piccolo form a heterodimer in a Ca^{2+}-dependent manner. Although not shown in the figure, formation of Piccolo homodimer is also possible. Rab3 links Rim2 to the insulin granule. How Rim2, Piccolo, cAMP-GEFII, and VDCC interactions are regulated is unclear at present.

N-type VDCC (37). In addition, synaptotagmin I binds to the $\alpha_1$1.2-subunit of L-type VDCC in pancreatic β cells (38). Rim1 has also been shown to bind to $\alpha_1$1.2-subunit through C_2 domains (17). However, whether or not Piccolo binds to VDCC was unknown. Our data indicate that Rim2 and Piccolo bind directly to $\alpha_1$1.2-subunits through their C_2 domains. Together, these findings clarify the link between Ca^{2+} sensors and L-type VDCCs in exocytosis of insulin granules. The L-type VDCCs are concentrated in the area of the pancreatic β cell that contains the insulin granules, suggesting that VDCCs and the granules are targeted to the same region in the pancreatic β cell and that

the molecular organization is critical in regulated exocytosis within a zone of voltage-dependent Ca^{2+} entry (39).

Based on the present *in vitro* findings, we propose interactions of the K_{ATP} channel as an ATP sensor, cAMP-GEFII as a cAMP sensor, Piccolo as a Ca^{2+} sensor, and the L-type VDCC (Fig. 7). The K_{ATP} channel interacts specifically with cAMP-GEFII through NBF-1 of SUR1. The increase in cAMP concentration dissociates cAMP-GEFII from SUR1, inducing dimerization of Rim2 or Piccolo in a Ca^{2+}-dependent manner. Rab3 links Rim2 to the insulin granules. Further studies are required to determine the spatial and temporal regulation of the interactions of these molecules *in vivo*.

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REFERENCES

1. Calakos, N., and Scheller, R. H. (1996) *Physiol. Rev.* **76,** 1–29
2. Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J. E. (1993) *Nature* **362,** 318–324
3. Fernandez-Chacon, R., and Sudhof, T. C. (1999) *Annu. Rev. Physiol.* **61,** 753–776
4. Landis, D. M. (1988) *J. Electron Microsc. Technol.* **10,** 129–151
5. Yorifuji, H., and Hirokawa, N. (1989) *J. Electron Microsc. Technol.* **12,** 160–171
6. Sheng, Z. H., Rettig, J., Takahashi, M., and Catterall, W. A. (1994) *Neuron* **13,** 1303–1313
7. Rettig, J., Sheng, Z. H., Kim, D. K., Hodson, C. D., Snutch, T. P., and Catterall, W. A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 7363–7368
8. Cases-Langhoff, C., Voss, B., Garner, A. M., Appeltauer, U., Takei, K., Kindler, S., Veh, R. W., De Camilli, P., Gundelfinger, E. D., and Garner, C. C. (1996) *Eur. J. Cell Biol.* **69,** 214–223
9. Wang, X., Kibschull, M., Laue, M. M., Lichte, B., Petrasch-Parwez, E., and Kilimann, M. W. (1999) *J. Cell Biol.* **147,** 151–162
10. tom Dieck, S., Sanmarti-Vila, L., Langnaese, K., Richter, K., Kindler, S., Soyke, A., Wex, H., Smalla, K. H., Kampf, U., Franzer, J. T., Stumm, M., Garner, C. C., and Gundelfinger, E. D. (1998) *J. Cell Biol.* **142,** 499–509
11. Wang, Y., Okamoto, M., Schmitz, F., Hofmann, K., and Sudhof, T. C. (1997) *Nature* **388,** 593–598
12. Brose, N., Hofmann, K., Hata, Y., and Sudhof, T. C. (1995) *J. Biol. Chem.* **270,** 25273–25280
13. Ohtsuka, T., Takao-Rikitsu, E., Inoue, E., Inoue, M., Takeuchi, M., Matsubara, K., Deguchi-Tawarada, M., Satoh, K., Morimoto, K., Nakanishi, H., and Takai, Y. (2002) *J. Cell Biol.* **158,** 577–590
14. Fenster, S. D., Chung, W. J., Zhai, R., Cases-Langhoff, C., Voss, B., Garner, A. M., Kaempf, U., Kindler, S., Gundelfinger, E. D., and Garner, C. C. (2000) *Neuron* **25,** 203–214
15. Wang, Y., Sugita, S., and Sudhof, T. C. (2000) *J. Biol. Chem.* **275,** 20033–20044
16. Betz, A., Thakur, P., Junge, H. J., Ashery, U., Rhee, J. S., Scheuss, V., Rosenmund, C., Rettig, J., and Brose, N. (2001) *Neuron* **30,** 183–196
17. Coppola, T., Magnin-Luthi, S., Perret-Menoud, V., Gattesco, S., Schiavo, G., and Regazzi, R. (2001) *J. Biol. Chem.* **276,** 32756–32762
18. Schoch, S., Castillo, P. E., Jo, T., Mukherjee, K., Geppert, M., Wang, Y., Schmitz, F., Malenka, R. C., and Sudhof, T. C. (2002) *Nature* **415,** 321–326
19. Zawalich, W. S., and Rasmussen, H. (1990) *Mol. Cell. Endocrinol.* **70,** 119–137
20. Seino, S. (1999) *Annu. Rev. Physiol.* **61,** 337–362
21. Miki, T., Nagashima, K., Tashiro, F., Kotake, K., Yoshitomi, H., Tamamoto, A., Gonoi, T., Iwanaga, T., Miyazaki, J., and Seino, S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 10402–10406
22. Seghers, V., Nakazaki, M., DeMayo, F., Aguilar-Bryan, L., and Bryan, J. (2000) *J. Biol. Chem.* **275,** 9270–9277
23. Brown, J. C., Dryburgh, J. R., Ross, S. A., and Dupre, J. (1975) *Recent Prog. Horm. Res.* **31,** 487–532
24. Drucker, D. J. (1998) *Diabetes* **47,** 159–169
25. Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D. E., and Graybiel, A. M. (1998) *Science* **282,** 2275–2279
26. de Rooij, J., Rehmann, H., van Triest, M., Cool, R. H., Wittinghofer, A., and Bos, J. L. (2000) *J. Biol. Chem.* **275,** 20829–20836
27. Ozaki, N., Shibasaki, T., Kashima, Y., Miki, T., Takahashi, K., Ueno, H., Sunaga, Y., Yano, H., Matsuura, Y., Iwanaga, T., Takai, Y., and Seino, S. (2000) *Nat. Cel. Bio.* **2,** 805–811
28. Kashima, Y., Miki, T., Shibasaki, T., Ozaki, N., Miyazaki, M., Yano, H., and Seino, S. (2001) *J. Biol. Chem.* **276,** 46046–46053
29. Fujimoto, K., Shibasaki, T., Yokoi, N., Kashima, Y., Matsumoto, M., Sasaki, T., Tajima, N., Iwanaga, T., and Seino, S. (2002) *J. Biol. Chem.* **277,** 50497–50502
30. Isomoto, S., Kondo, C., Yamada, M., Matsumoto, S., Higashiguchi, O., Horio, Y., Matsuzawa, Y., and Kurachi, Y. (1996) *J. Biol. Chem.* **271,** 24321–24324
31. Higgins, C. F. (1992) *Annu. Rev. Cell Biol.* **8,** 67–113
32. Su, Y., Dostmann, W. R., Herberg, F. W., Durick, K., Xuong, N. H., ten Eyck, L., Taylor, S. S., and Varughese, K. I. (1995) *Science* **269,** 807–813
33. Rehmann, H., Prakash, B., Wolf, E., Rueppel, A., De Rooij, J., Bos, J. L., and Wittinghofer, A. (2003) *Nat. Struct. Biol.* **10,** 26–32
34. Harris, B. Z., and Lim, W. A. (2001) *J. Cell Sci.* **114,** 3219–3231
35. Zerangue, N., Schwappach, B., Jan, Y. N., and Jan, L. Y. (1999) *Neuron* **22,** 537–548
36. Charvin, N., L'Eveque, C., Walker, D., Berton, F., Raymond, C., Kataoka, M., Shoji-Kasai, Y., Takahashi, M., de Waard, M., and Seagar, M. J. (1997) *EMBO J.* **16,** 4591–4596
37. Sheng, Z. H., Yokoyama, C. T., and Catterall, W. A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 5405–5410
38. Wiser, O., Trus, M., Hernandez, A., Renstrom, E., Barg, S., Rorsman, P., and Atlas, D. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 248–253
39. Bokvist, K., Eliasson, L., Ammala, C., Renstrom, E., and Rorsman, P. (1995) *EMBO J.* **14,** 50–57