Novel Rabphilin-3-like Protein Associates with Insulin-containing Granules in Pancreatic Beta Cells*

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A novel rabphilin-3-like gene, granuphilin, has been identified in pancreatic beta cells by comparing genes expressed in pancreatic alpha and beta cell lines using mRNA differential display. The domain structure of the protein products of the granuphilin gene contains an amino-terminal zinc-finger motif and carboxyl-terminal C2-domains, similar to that of the rabphilin-3 gene. There are two isoforms: the larger isoform, granuphilin-a, has two C2-domains, whereas the smaller one, granuphilin-b, contains only the first C2-domain. Granuphilin is specifically expressed in pancreatic beta cells and the pituitary gland, but not in pancreatic alpha cells, the adrenal gland, or other major organs such as the brain. A portion of granuphilin associates with insulin-containing dense-core granules, but not with synaptic-like microvesicles in beta cells. Thus, its distribution pattern presents a striking contrast with that of rabphilin-3, which associates with small synaptic vesicles in neurons. The first C2-domain of granuphilin binds phospholipids in a Ca2+-independent manner, whereas the second one does not. These distinctive characteristics of granuphilin suggest that it is not a simple counterpart of rabphilin-3 in endocrine cells and that it has a unique role in the regulated exocytosis of dense-core granules in endocrine tissues.

A combination of genetics in yeast and biochemistry in animal cell-free systems has revealed basic molecular mechanisms of constitutive exocytosis (1, 2). For its cells to adapt to changes in environments and to communicate with other cells, a multicellular organism further differentiates a regulated exocytosis that occurs only in the presence of secretagogues (3). This pathway is highly developed in neuronal, endocrine, and exocrine cells, where neurotransmitters, hormones, and digestive enzymes are released by exocytosis in response to an intracellular Ca2+-signal generated by secretagogues. These cells must be equipped with specific machinery that permits them, for example, to sense the Ca2+ signal and then release their cargo and replenish the stored material. Biochemical studies of synaptic vesicles at the nerve terminal have identified a number of molecules possibly involved in these processes (4). A group of proteins with two C2-domains has drawn particular attention because this motif has biochemical properties that sense a Ca2+ signal and then bind membranes through interaction with phospholipids (5). Furthermore, some C2-domains interact with other proteins in a Ca2+-dependent manner (5). These C2-domain-containing molecules include synaptotagmins (5), rabphilin-3 (6), Doc2 (7), and RIM (8), although their precise functions remain elusive.

Both neuron and endocrine cells contain at least two types of secretory vesicles (9). One type of vesicle, the small synaptic vesicle (SSV) in neurons and the synaptic-like microvesicle (SLMV) in endocrine cells, releases classical neurotransmitters. These are relatively small and electron-lucent and cycle between the plasmalemma and the early endosome compartment. The other type of vesicle, the large dense-core vesicle (LDCV) or the secretory granule in neuroendocrine cells, mainly secretes polypeptides, although some of these in specific subpopulations secrete monoamines. These LDCVs directly form from the trans-Golgi network and have an electron-dense core. The physiologic and pharmacologic properties of the membranes of the two types of vesicles differ. For example, LDCV exocytosis is triggered by the lower Ca2+ concentrations reached away from the sites of Ca2+ entry (3-30 μM calcium for half-maximal stimulation for release), whereas SSV exocytosis is triggered by high Ca2+ concentrations close to the entry site (200 μM) (10). The synaptic events occur as brief as 60 μs after stimulation for SSVs, whereas the latency of LDCVs in neuroendocrine cells is 100 times longer (10). Furthermore, in motor nerve terminals, the exocytosis of acetylcholine-containing SSVs is sensitive to α-latrotoxin, the toxin of black widow spider venom, which does not affect the exocytosis of calcitonin gene-related peptide-containing LDCVs (11). Thus, the secretory pathways of SSVs/SLMVs and LDCVs/granules are different. It is also likely that their protein compositions are at least partly distinct; however, due to their low abundance, there has been less biochemical characterization of LDCVs/granules.

To analyze the molecular machinery of regulated exocytosis of LDCVs/granules, we studied pancreatic beta cells, which secrete insulin in response to blood glucose levels. Because beta cells are one of the most physiologically characterized endocrine cells and possess both insulin-containing dense-core granules and γ-aminobutyric acid-containing SLMVs (12), they are
a good model for dissecting both types of secretory vesicles. The limited number and anatomical position of beta cells, however, have hampered their biochemical analysis. Therefore, we attempted molecular characterization by analyzing the genes expressed in the cells. The expression levels of beta cell genes were then compared with those in closely related pancreatic alpha cells by mRNA differential display (13), which resulted in the identification of several novel genes that are preferentially expressed in beta cells.2 In this study, one of these genes, granuphilin, was characterized. This gene encodes a protein whose domain structure is similar to that of rabphilin-3. Rabphilin-3 is specifically expressed in neurons, contains two C2-domains, and is thought to be involved in the regulated secretion of SSVs (6, 14). Granuphilin, however, has a number of distinct characteristics compared with rabphilin-3: it is specifically expressed in pancreatic beta cells and in the pituitary, but not in other major organs such as the brain. Furthermore, it is associated with insulin-containing dense-core granules, but not with the SLVs in beta cells. In addition to a difference in the distribution, other distinctive properties of granuphilin suggest that it is not a simple counterpart of rabphilin-3 in endocrine cells and that it has a unique role in the regulated exocytosis of dense-core granules in endocrine tissues.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Tissue Preparation**—Pancreatic beta cell lines (βTC9 and MIN6) and an alpha cell line (αTC1.6) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum under 5% CO2 atmosphere at 37 °C as described previously (15). Tissues were immediately excised from 8–12-week-old C57BL/6J mice killed by cervical dislocation, frozen in a liquid nitrogen bath, and stored at −80 °C. Pancreatic islets were isolated as described previously (16).

**RNA Preparation and Northern Blotting**—Total RNA from cell lines (80% confluent culture at a density of 1–2 × 106 cells/10-cm plate) and tissues was prepared as described previously (17). For Northern blotting, 20 μg of total RNA was separated on a 1% agarose and 6.7% formaldehyde gel and transferred onto Hybond-N membranes (Amerham Pharmacia Biotech, Buckinghamshire, United Kingdom). Radioactive probes were generated with [α-32P]dCTP (1000–3000 Ci/mmol; Amerham Pharmacia Biotech) using the random priming DNA labeling system (Amerham Pharmacia Biotech), followed by centrifugation through Sephadex G-50 spin columns to remove unincorporated dNTPs.

**Molecular Cloning of Granuphilin cDNAs**—Expression levels of genes in βHC9 cells were compared with those in αTC1.6 cells by mRNA differential display (13). One of the cDNA fragments preferentially expressed in βHC9 cells as a probe (DBJ/GenBank N17221 accession number AB005756), granuphilin cDNAs were cloned from a βHC9/MIN6 cell cDNA library (15). Sequencing was performed using an ABI PRISM cycle sequencing kit (Perkin-Elmer).

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**—Total RNA (1 μg) was reverse-transcribed using oligo(dT)17 primer as described previously (15). The 5′- and 3′-primers used for PCR were derived from sequences flanking the granuphilin-a-specific region so that the sizes of PCR products from the two isoforms are discriminated. The 5′-primer, 5′-ACTTTCTCTTGGAGAGGGCGGA-3′, was combined with either of the 3′-primers, 5′-CATCATTCTTGTGATAACAC-3′ or 5′-CCTCGGTGCATGCTGCGAT-3′. The expected sizes of PCR products with the former 3′-primer are 936 base pairs (bp) for granuphilin-a and 518 bp for granuphilin-b, and those with the latter 3′-primer are 588 bp for granuphilin-a and 170 bp for granuphilin-b. PCR was carried out in 20 μl of 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 200 μM each dNTP, 1.5 units of Taq DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany), 12.5 pmol of the 5′- and 3′-primers, and the RT mixture described above. Following incubation for 5 min at 94 °C for denaturation, the samples were incubated at 94 °C for 90 s, 56 °C for 30 s, and 72 °C for 1 min for 27 cycles. The products were resolved on 2% agarose gels and visualized with ethidium bromide.

**Antibodies, Immunostaining, and Immunoblotting**—Rabbit antibodies against the carboxyl-terminal granuphilin-a peptide, CTLQLRS-SMVQKQGLGV, were produced as described previously (15). Rabbit antibodies against vesicle-associated membrane protein (VAMP)-2 (18) and synaptotagmin III (19) were gifts from Dr. M. Takahashi (Mitsubishi Kasei Institute of Life Sciences). Mouse monoclonal antibodies against synaptophysin (SY 58) and guinea pig anti-glucagon serum were purchased from Progen Biotechnik (Heidelberg, Germany) and Linco Research (St. Charles, MO), respectively. Guinea pig anti-porcine insulin serum was a gift from Drs. K. Wakabayashi and H. Kobayashi (Institute for Molecular and Cellular Regulation, Gunma University). Immunostaining on the mouse pancreas specimen was performed as described previously (15).

**Immunoblotting**—Proteins were solubilized in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 5 mM EDTA, and the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride and 10 μg/mL each aprogin, pepstatin A, and leupeptin. The supernatant of the whole-cell extract was prepared by sedimenting the insoluble material by centrifugation at 15,000 rpm for 15 min. After SDS-polyacrylamide gel electrophoresis, proteins were transferred onto an Immobilon-P membrane (Millipore Corp., Bedford, MA). Immunoblotting was performed using enhanced chemiluminescent Western blotting detection reagents (Amersham Pharmacia Biotech).

**Equilibrium Sucrose Density Gradient—SLMs and dense-core granules were separated according to the method described previously (12), with modifications. MIN6 cells (5 × 107 cells/10-cm plate) were resuspended in 1.5 ml of homogenization buffer (0.3 M sucrose, 20 mM MOPS, pH 7.0) and then centrifuged at 30,000 rpm for 15 h at 4 °C in an RPS40/4 rotor (Hitachi Koki, Katsuta, Japan). Fractions (870 µl) were collected from the bottom of the gradient. The sucrose molarity of each fraction was determined by measuring its refractive index. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis, followed by immunoblotting. Immunoreactive insulin was extracted with acidic ethanol (sample/ethanol/concentrated HCl = 28:5:70:1.5) overnight at 4 °C. After sonication and centrifugation, the insulin was measured using anti-insulin antibodies as described previously (16).

**Immunoprecipitation of Secretory Vesicles**—This was performed according to the method described previously (19). Briefly, the post-nuclear supernatant was prepared from five confluent 10-cm plates of MIN6 cells as described above with the addition of resuspension in 3 ml of 14 mM Hepes, 140 mM KCl, 10 mM sucrose, 10 mM HEPES, pH 7.2, 1.5 mM MgCl2, 1% bovine serum albumin, and the protease inhibitors described above. A portion of the supernatant (0.6 ml) was incubated with the indicated antibodies at 4 °C for 2 h. The antibodies were then bound to protein G-Sepharose 4FF (Amersham Pharmacia Biotech) at 4 °C for 1 h with gentle rotation. The immunoprecipitates were washed four times with the homogenization buffer. Tricine/SDS-polyacrylamide gel electrophoresis (17) was used to detect small peptides such as insulin and proinsulin, and proteins were transferred onto an Immobilon-P membrane (Millipore Corp.). Immunoblotting was then performed using anti-insulin antibodies.

**Production of Glutathione S-Transferase (GST)-fused Recombinant Proteins**—Two cDNA fragments of granuphilin-a encoding amino acids 315–502 and 487–673 that contained the first and second C2-domains, respectively, were amplified by PCR using a full-length granuphilin-a cDNA as a template. Amplified cDNA fragments were subcloned into pGEX4T-1 (Amersham Pharmacia Biotech). A cDNA fragment encoding the first C2-domain of rat synaptotagmin I (amino acids 139–267), synthesized by RT-PCR using rat brain RNA as a template, was ligated into the ligation-independent cloning vector pGEX4T-1, followed by affinity chromatography with glutathione-Sepharose 4B (Amersham Pharmacia Biotech).

**Phospholipid Binding Assay**—Preparation of ‘H-labeled liposomes and measurement of phospholipid binding were performed according to the methods described previously (21). All phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Liposomes were made from 20 μg/mL of phosphatidylincholine, 0.42 mg/mL of brain phosphatidylethanolamine, 20 μg/mL of 1,2-di-palmitoyl-sn-2-phosphatidyl-(N)-methyl-L-homocine (Amersham Pharmacia Biotech). When indicated, phosphatidylinerine was replaced by either phosphatidylinositol or phosphatidylethanolamine. Phospholipids dissolved in chloroform were mixed and dried under a stream of nitrogen gas. After resuspension in 5 ml of buffer A (50 mM HEPES, pH 7.4, and 0.1 mM NaCl) by vigorous shaking for 1 min, phospholipid suspensions were sonicated for 15 s and centrifuged at 15,000 rpm for 15 min. After SDS-polyacrylamide gel electrophoresis, proteins were transferred onto an Immobilon-P membrane (Millipore Corp., Bedford, MA). Immunoblotting was performed using enhanced chemiluminescent Western blotting detection reagents (Amersham Pharmacia Biotech).

**Final Modification—**
10,000 \times g for 10 min to remove aggregates. The supernatant was transferred to a new tube; 5 ml of buffer A was added; and then it was stored at 4 °C for as long as 1 month.

The phospholipid binding assay contained 15 \mu g of recombinant protein bound to 10 \mu l of glutathione beads. Beads were prewashed twice with the respective test solutions and resuspended in 0.1 ml of buffer A containing 1\textsuperscript{H}-labeled liposomes (~0.1 \mu Ci/16.7 \mu g of phospholipid) and 2 \mu g of ETGA with or without the addition of 2.1 \mu M CaCl\textsubscript{2}. The mixture was incubated at room temperature for 10 min with vigorous shaking and then briefly centrifuged at 2200 rpm in a tabletop centrifuge. The beads were washed three times with 1 ml of the incubation buffer without liposomes. After eluting the proteins with 250 \mu l of buffer containing 1% SDS and 10 mM EDTA, liposome binding was quantified by liquid scintillation counting.

**RESULTS**

Molecular Cloning of a Novel Rabphilin-3-like Gene—Although the pancreatic alpha and beta cells are developmentally very close, they have an opposite role in maintaining glucose homeostasis: the alpha cells secrete glucagon in response to a low glucose level, and the beta cells increase insulin secretion in response to a high glucose level. Therefore, there must exist fundamental differences in their glucose recognition and metabolism and in their regulation of peptide hormone secretion. A comparison of the genes expressed in the two cells may reveal the molecules involved in these processes. It is difficult to purify considerable amounts of these cells from islets, and so we instead used cultured alpha and beta cell lines (\alpha TC1.6 and \beta HC9) derived from transgenic mice. Using mRNA differential display (13), we identified several novel genes that are specifically or preferentially expressed in \beta HC9 cells.\textsuperscript{2}

Molecular cloning of the full-length cDNAs revealed one novel gene, granuphilin, which encodes a protein whose domain structure is similar to that of rabphilin-3 \textsuperscript{(6)}. Two isoforms were found among the multiple independent clones in the \beta HC9/\textit{MIN6} cDNA library. One (granuphilin-a) encodes a protein of 673 amino acids that has a zinc-finger motif at the N terminus and two C\textsubscript{2}-domains at the C terminus (Fig. 1). The other (granuphilin-b) lacks 418 nucleotides (from positions 1641 to 2058) of granuphilin-a, which causes a frameshift at residue 486, located between the two C\textsubscript{2}-domains of granuphilin-a, and generates a stop codon at residue 503. Thus, granuphilin-b is a protein of 502 amino acids and has only the first C\textsubscript{2}-domain of granuphilin-a and a different C-terminal sequence, GSVMAKWWTGWIRLVKK (Fig. 1). The calculated molecular mass and isoelectric point for granuphilin-a are 76,000 Da and 9.2, and those for granuphilin-b are 57,000 Da and 9.2, respectively. Despite the similarity of the domain structure, the overall identity of the amino acid sequences of granuphilin-a and rabphilin-3 only amounts to 22%.

Rabphilin-3 is specifically localized on the synaptic vesicles at the nerve terminals and is associated with Rab3A through the N-terminal fragment containing the zinc-finger motif (14, 22). Although the amino acid conservation in the N-terminal regions of granuphilin and rabphilin-3 is only 31%, the distribution of cysteine residues in granuphilin (Fig. 1, boxed), C\textsubscript{X}\textsubscript{C}\textsubscript{X}\textsubscript{12–13}C\textsubscript{X}\textsubscript{X}C\textsubscript{X}\textsubscript{X}C\textsubscript{X}\textsubscript{10–18}C\textsubscript{X}C, is similar to that in rabphilin-3 (6), RIM (8), and FGD1 protein (23), all of which bind small G proteins. Crystal structural analysis of the Rab3A–rabphilin-3 complex has revealed that a deep pocket in Rab3A called the Rab complementarity-determining region, directly interacts with an SGAFF motif in rabphilin-3 (24). Although the SGAFWF motif is conserved among other members of the rabphilin-3 family, including RIM (8) and Noe\textsubscript{2} (25), the corresponding sequence of granuphilin is distinct, TGD-WFY (Fig. 1, boxed). Like rabphilin-3, there is a single stretch of glutamate residues between the zinc-finger motif and the first C\textsubscript{2}-domain, although its functional role remains unknown (Fig. 1, \textit{dashed underlining}). The primary sequences within the C\textsubscript{2}-domains of granuphilin and rabphilin-3 also vary considerably (31% conservation). Particularly, the five aspartate residues participating in Ca\textsuperscript{2+} binding in the C\textsubscript{2A}-domain of synaptotagmin I (5) are only partially conserved in the C\textsubscript{2}-domains of granuphilin (Fig. 1, \textit{asterisks}).

Preferential Expression of Granuphilin in Pancreatic Beta Cells—Tissue expression of granuphilin was examined. Northern blot analysis revealed that it was not expressed in major tissues such as the brain, kidney, liver, heart, muscle, and spleen (Fig. 2A). As expected from our finding using mRNA differential display (data not shown), it was specifically expressed in \beta HC9 cells, but not in \alpha TC1.6 cells. The double bands of signals in \beta HC9 cells suggest that both granuphilin-a and -b are transcribed. This interpretation was further supported by RT-PCR analysis. The PCR products consisted of two discrete bands, whose sizes correspond to those expected for the two isoforms, in the pancreatic islets, \beta HC9 cells, and \textit{MIN6} cells (Fig. 2B, \textit{lanes 2–4 and 7–9}). In contrast, they were not found in either \alpha TC1.6 cells or brain (Fig. 2B, \textit{lanes 1, 5, 6, and 10}). These results indicate that both isoforms are expressed in pancreatic beta cells.

A polyclonal antibody was produced against the C-terminal peptide of granuphilin-a. In this study, the larger isoform, granuphilin-a, was further characterized using this antibody. Immunoblot analysis showed that the antibody recognized a single 78-kDa protein (the value of which is consistent with the calculated molecular mass of granuphilin-a) in the extracts of \beta HC9 cells (Fig. 2C) and \textit{MIN6} cells (data not shown, but see Fig. 4A). Consistent with the finding from the Northern blot analysis (Fig. 2A), there were no immunoreactive proteins in either \alpha TC1.6 cells or other major organs (Fig. 2C). Among the endocrine tissues examined, granuphilin-a was most abundantly expressed in pancreatic islets. It was also expressed in the pituitary, but was not significantly expressed in the testis, ovary, and adrenal gland. This expression pattern is in striking contrast to that of rabphilin-3, which is specifically expressed in the brain, but is not expressed in endocrine tissues, except for the adrenal gland (6, 26, 27).

Northern blot analysis in cultured cell lines suggests that granuphilin is expressed in pancreatic beta cells, but not in alpha cells. Transformed cell lines, however, may acquire properties different from those of the original progenitors, as in the case of the insulin receptor-related receptor, which is expressed in pancreatic alpha cells, but not in \alpha TC1.6 cells (15). Therefore, we performed immunohistochemical analysis directly on the mouse pancreas specimen. Granuphilin-a was highly expressed in the insulin-positive beta cells (Fig. 3, \textit{a}, \textit{c}, and \textit{e}). In contrast, granuphilin-a immunoreactivity was not detected in either alpha or exocrine cells (Fig. 3, \textit{b}, \textit{d}, and \textit{f}).

Association of Granuphilin-a with Insulin-containing Dense-core Granules—Next, the subcellular localization of granuphilin-a was biochemically addressed. Because of the difficulty in obtaining large amounts of materials from pancreatic islets and because of the heterogeneous cell components in the islets, biochemical studies were performed using the beta cell lines \beta HC9 and \textit{MIN6}. Both cell lines were established from transgenic mice expressing simian virus 40 large T antigen under the control of the rat insulin promoter, and they preserve some distinctive characteristics of the normal progenitors, such as glucose-inducible insulin secretion (28, 29). \textit{MIN6} cells were mainly used for biochemical analysis in this study because they contain a larger number of insulin granules and have a higher insulin content than do \beta HC9 cells (30). \textit{MIN6} cells were homogenized and then fractionated on continuous sucrose gradients. Similar procedures have permitted the separation of the two types of secretory vesicles present in beta cells: insulin-
containing dense-core granules and γ-aminobutyric acid-containing SLMVs (12). Insulin immunoreactivity peaked at fraction 10 (Fig. 4A, upper panel), whereas synaptophysin was enriched at fractions 6 and 7 (lower panel). Synaptophysin, an integral membrane protein of SSVs in neurons, is absent in the insulin granules, but present in the SLMVs in beta cells (12, 19, 31). Thus, the two types of secretory vesicles were clearly separated. Granuphilin-a immunoreactivity showed two peaks, fractions 2–4 and fractions 9 and 10 (Fig. 4A, middle panel). These results suggest that a portion of granuphilin-a associates with the insulin granules. The two separate fractions of granuphilin-a may reflect its different functional states.

To obtain further evidence for the association of granuphilin-a with dense-core granules, intact secretory vesicles were incubated with anti-granuphilin-a antibodies. The immunoprecipitates were then examined for the presence of insulin immunoreactivity (Fig. 4A, lower panel). The results suggest that granuphilin-a is associated with dense-core granules, providing further evidence for its role in insulin secretion.

**Fig. 1. Amino acid sequence of granuphilin.** The amino acid sequence of mouse (m) granuphilin is aligned with that of mouse rabphilin-3 (DDBJ/GenBank™/EBI accession number D29965) (26). Dashes indicate individual amino acid gaps to generate optimal alignment. Two isoforms, granuphilin-a and -b, are found. Granuphilin-b lacks 418 bp of the internal sequence of granuphilin-a. This causes a frameshift at residue 486, which generates a 17-amino acid different sequence, followed by a stop codon. Thus, granuphilin-b contains only the first C2-domain. Conserved cysteine residues in the zinc-finger motifs are boxed. An SGAWFF structural element of rabphilin-3 (24) and the corresponding sequence of granuphilin are boxed. The C2-domains are indicated by dashed underlining, following which the sequence data are available from DDBJ/GenBank™/EBI under accession numbers AB025258 (granuphilin-a) and AB025259 (granuphilin-b).
munnoreactivity. Granuphilin-a immunoprecipitates contained both insulin and proinsulin (Fig. 4B, lane 3). The association is specific because neither control IgG nor anti-synaptotagmin III and anti-VAMP-2 precipitates possessed insulin and proinsulin (Fig. 4B, lanes 2 and 4). Synaptotagmin III is localized in the insulin granules (19), whereas VAMP-2 exists in both granules and SLMVs in beta cells (31). These results indicate an association of granuphilin-a with insulin-containing dense-core granules in beta cells.

**Ca**<sup>2+</sup> and Phospholipid Binding of Granuphilin—As described above, the primary sequences of the C<sub>2</sub>-domains of granuphilin varied from those of the C<sub>2</sub>A-domain of synaptotagmin I (Fig. 1), including the amino acids corresponding to the five aspartate residues that participate in Ca<sup>2+</sup> binding (5). In addition, the granuphilin-b isoform lacks the second C<sub>2</sub>-domain. To determine the biochemical property of each C<sub>2</sub>-domain of granuphilin, recombinant proteins fused to GST were produced in *E. coli* (Fig. 5A). A recombinant protein consisting of the first C<sub>2</sub>-domain of rat synaptotagmin I (GST-SytC<sub>2</sub>A) was similarly produced as a positive control. GST-SytC<sub>2</sub>A showed Ca<sup>2+</sup>-dependent binding of the <sup>3</sup>H-labeled liposomes (Fig. 5B). Binding was detected only in liposomes containing negatively charged phospholipids such as phosphatidylethanolamine and phosphatidylserine, but not in those containing phosphatidylethanolamine, as described previously (21). A protein derived from the first C<sub>2</sub>-domain of granuphilin (GST-GrpC<sub>2</sub>A) bound the <sup>3</sup>H-labeled liposomes, but in a Ca<sup>2+</sup>-independent manner (Fig. 5B). The binding is specific because it also required negatively charged phospholipids. Surprisingly, a recombinant protein from the second C<sub>2</sub>-domain of granuphilin (GST-GrpC<sub>2</sub>B) did not have significant affinity for the phospholipid (Fig. 5B). Although it could be that the protein does not have a proper conformation, a similar construction used for each C<sub>2</sub>-domain suggests that the C<sub>2</sub>B-domain of granuphilin does not bind phospholipids and has a distinct role other than membrane binding.

**DISCUSSION**

In this study, we have identified and characterized a novel rabphilin-3-like gene, granuphilin. Granuphilin and rabphilin-3 share several sequence characteristics: an N-terminal zinc-finger domain, C-terminal C<sub>2</sub>-domains, and a glutamate stretch between them. Granuphilin, however, has several properties distinct from those of rabphilin-3; the tissue distribution pattern and subcellular localization are particularly different. Rabphilin-3 is specifically expressed in brain and adrenal
gland tissues and is associated with SSVs at nerve terminals. On the other hand, granuphilin is selectively expressed in pancreatic beta cells and pituitary tissue. It is not significantly expressed in the brain, pancreatic exocrine cells, or other endocrine tissues such as pancreatic alpha cells, adrenal gland, testis, and ovary. It is associated with dense-core granules, but not SLMVs, in pancreatic beta cells. So far, no concomitant expression of granuphilin and rabphilin-3 has been found in tissues, suggesting that each protein is separately differentiated based on the properties of its associated secretory vesicles. To our knowledge, granuphilin is the first protein strictly associated with the membrane of dense-core granules in endocrine cells. Synaptotagmin III is expressed in pancreatic islets at low levels and associates with insulin-containing granules in MIN6 cells, but is abundantly expressed in brain tissue (19, 32). Noc2, which has a zinc-finger motif but no C2-domain, is expressed only in endocrine tissues, but is not associated with insulin-containing granules in MIN6 cells (25).

The distribution pattern of granuphilin-a suggests that its function is restricted to those dense-core granules that contain a polypeptide hormone in endocrine cells. Although it is well known that SSVs and LDCVs differ in the membrane protein constituents of their neurons (9), little is known about the difference of dense-core granules in endocrine cells. Because it is relatively easy to obtain the materials, most biochemical studies of endocrine cells have been performed on adrenal chromaffin cells or on their derivative PC12 cell lines, whose granules contain monoamines. These granules, however, may have characteristics more closely resembling SSVs. For example, in chromaffin and PC12 cells, synaptophysin exists in both SLMVs and granules (33, 34), whereas in pancreatic beta cells, it is not present in the insulin granules, but is restricted to the SLMVs (12, 19, 31). Rabphilin-3 associates with SSVs in neurons (14) and with granules in chromaffin cells (35), but is not expressed in beta cells (27). Granuphilin may have a role for the regulated exocytosis of a specific type of dense-core granules in endocrine tissues. The reason for the differential ex-
pression of granuphilin between pancreatic alpha and beta cells is currently unknown. It is possible that it reflects the different regulatory mechanism for hormone secretion in response to glucose. It will also be important to determine which cells in the pituitary, another multicellular endocrine tissue, express granuphilin.

Despite the similarity of the domain structure, the primary amino acid sequences of granuphilin and rabphilin-3 vary considerably. The five aspartate residues participating in Ca\(^{2+}\) binding in the C\(_2\)A-domain of synaptotagmin I are mostly conserved in synaptotagmins, rabphilin-3, and Doc2 (5), but not in either RIM (8) or granuphilin. This sequence feature is consistent with the Ca\(^{2+}\) independence of the phospholipid binding in the first C\(_2\)-domain of granuphilin-a. A protein consisting of the second C\(_2\)-domain had no affinity for phospholipids, suggesting that this second C\(_2\)-domain may have a functional role distinct from membrane binding, possibly relevant to differential roles between the two isoforms. Further characterization of granuphilin-b, which lacks the second C\(_2\)-domain, will require antibodies specific to this isoform.

Rabphilin-3 is bound to Rab3A/C through the N-terminal region (14, 22). All known members of Rab3 (A, B, C, and D) are expressed in rat pancreatic islets and beta cell lines (27). Because rabphilin-3 is not expressed in these cells (27), granuphilin is expressed in rat pancreatic islets and beta cell lines (27). Be-

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