Molecular Biology of the Cell
Vol. 18, 688–696, February 2007

Exophilin4/Slp2-a Targets Glucagon Granules to the Plasma Membrane through Unique Ca^{2+}-inhibitory Phospholipid-binding Activity of the C2A Domain

Miao Yu,* Kazuo Kasai,* Kazuaki Nagashima,*† Seiji Torii,* Hiromi Yokota-Hashimoto,* Koichi Okamoto,† Toshiyuki Takeuchi,* Hiroshi Gomi,* and Tetsuro Izumi*

*Department of Molecular Medicine, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi 371-8512, Japan; and †Department of Neurology, Gunma University School of Medicine, Maebashi 371-8511, Japan

Submitted October 12, 2006; Revised November 21, 2006; Accepted November 30, 2006
Monitoring Editor: Akihiko Nakano

Rab27a and Rab27b have recently been recognized to play versatile roles in regulating the exocytosis of secretory granules and lysosome-related organelles by using multiple effector proteins. However, the precise roles of these effector proteins in particular cell types largely remain uncharacterized, except for those in pancreatic β cells and in melanocytes. Here, we showed that one of the Rab27a/b effectors, exophilin4/Slp2-a, is specifically expressed in pancreatic α cells, in contrast to another effector, granuphilin, in β cells. Like granuphilin toward insulin granules, exophilin4 promotes the targeting of glucagon granules to the plasma membrane. Although the interaction of granuphilin with syntaxin-1a is critical for the targeting activity, exophilin4 does this primarily through the affinity of its C2A domain toward the plasma membrane phospholipids phosphatidylserine and phosphatidylinositol-4,5-bisphosphate. Notably, the binding activity to phosphatidylserine is inhibited by a physiological range of the Ca^{2+} concentration attained after secretagogue stimulation, which presents a striking contrast to the Ca^{2+}-stimulatory activity of the C2A domain of synaptotagmin I. Analyses of the mutant suggested that this novel Ca^{2+}-inhibitory phospholipid-binding activity not only mediates docking but also modulates the subsequent fusion of the secretory granules.

INTRODUCTION

In multicellular organisms, professional secretory cells store bioactive substances in specialized organelles, such as synaptic vesicles, secretory granules, and lysosome-related organelles, and they release them in response to specific stimuli. Although the basic exocytic protein machinery is conserved, many variations occur in the control of exocytosis that are related to the physiological role of particular cell types (Burgoyne and Morgan, 2003). It is important both biologically and medically to understand specific aspects of the exocytic mechanism, because each of the secreted materials gives a vital command to other cells to orchestrate the whole organism.

We here focused on pancreatic α and β cells that play a pivotal role in the maintenance of blood glucose levels. Although both cells harbor secretory granules to release peptide hormones, the two types of cells respond to opposite stimuli. The α cells secrete glucagon in response to a low glucose level to prevent hypoglycemia, whereas β cells secrete insulin in response to postprandial hyperglycemia to maintain a physiological range of glucose concentration. However, little is known about possible differences in the exocytic machinery between these two cell types. We previously reported a novel protein product, granuphilin, that is specifically expressed in β cells but not in α cells (Wang *et al*., 1999). Subsequent analyses indicate that granuphilin functions as an effector of Rab27a (Yi *et al*., 2002) and mediates docking of insulin granules to the plasma membrane in β cells (Torii *et al*., 2002, 2004; Gomi *et al*., 2005). It is unknown, however, why granuphilin is not expressed in pancreatic α cells if it simply executes docking of the secretory granules; nor have we identified its counterpart and its functions in α cells. The identification of such a molecule may help elucidate fundamental differences in the regulatory mechanism for hormone secretion between α and β cells.

At the beginning of the present study, we found that Rab27a was expressed in pancreatic α cells as well as in β cells. Because Rab27a is thought to function through distinct effectors in a variety of regulated secretory pathways (Izumi *et al*., 2003; Fukuda, 2005), we explored the expression of Rab27a effector proteins and discovered that exophilin4 (also called Slp2-a) was specifically expressed in pancreatic α cells. In the present study, we characterized the function of exophilin4 in cultured α cell lines and found that exophilin4 promotes the targeting of glucagon granules to the plasma membrane through the interaction with phospholipids via the C2A domain. Surprisingly, the binding of the C2A domain to phosphatidylserine (PS) was inhibited by the Ca^{2+} concentration physiologically induced by secretagogues, which presented a marked contrast to the well-known Ca^{2+}-stimulatory PS-binding activity of the synaptotagmin I-C2A domain (Südhof and Rizo, 1996; Bai and Chapman, 2004).

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06–10–0914) on December 20, 2006.

Address correspondence to: Tetsuro Izumi (tizumi@showa.gunma-u.ac.jp).

© 2007 by The American Society for Cell Biology

Experiments using exophilin4 mutants revealed that this novel Ca^{2+}-sensitive PS-binding activity plays critical roles in the docking and possibly the fusion of glucagon granules.

MATERIALS AND METHODS

DNA Construction

The cDNA clone (FLJ20163) encoding an N-terminal part of human exophilin4 was obtained from Kazusa DNA Research Institute (Kisarazu, Japan). A C-terminal part of exophilin4 cDNA was amplified from the cDNA of the HMV-II human melanoma cell line by polymerase chain reaction (PCR) and ligated to construct full-length exophilin4 cDNA. Site-directed mutagenesis of exophilin4 was performed using the following primers: 5′-GGAATTCGTTCCAGCATTTCTCCAAGA-3′ and 5′-TACATATGGGTTTGAACGCTGTTTTTTTTACATTCGCTGC-3′ for DN1, 5′-CAGCGTTCAGACCCATATGTAAAGGCC-3′, and 5′-CCCCTAGGAAACTATTGCGCTTAAATGTATTCCGATGCC-3′ for DN2, and 5′-ACACTAGTAGTGCAGCAGACC-3′ and 5′-CACTACTAGTGTCTGCTGCTGGCC-3′ for KQ. The DN mutant was constructed by ligating the cDNA fragments containing the respective DN1 and DN2 mutations. The resulting exophilin4-$C2A^{DN}$ has substitutions of asparagine for aspartate at amino acid positions 635, 642, and 698 of human exophilin4, whereas exophilin4-$C2A^{KQ}$ carries substitutions of glutamine for lysine at amino acid positions 658, 659, 660, 665, and 666.

The cDNA fragments of exophilin4 encoding 579-910 (C2AB domain), 579-742 (C2A domain), and 745-910 (C2B domain) amino acids were amplified by PCR, by using a full-length exophilin4 cDNA as a template. Amplified cDNA fragments were subcloned into the pcDNA3-HA and pGEX4T-1 to express hemagglutinin (HA)-tagged and glutathione *S*-transferase (GST)-fused protein, as described previously (Yi *et al*., 2002). The cDNA fragments of N-terminal exophilin4 encoding 1-462 and 1-381 amino acids were similarly amplified and subcloned into the pcDNA3-HA and pGEX4T-1, respectively. The GST-fused cDNA constructs containing 315-673 (C2AB domain), 315-502 (C2A domain), and 487-673 (C2B domain) amino acids of granuphilin-a and that encoding the C2A domain of rat synaptotagmin I (139-267 amino acids) were described previously (Wang *et al*., 1999). A cDNA fragment encoding the C2B domain of rat synaptotagmin I (248-421 amino acids) containing glycine at 374 (Desai *et al*., 2000) was synthesized by reverse transcription-PCR, by using rat brain RNA as a template, and fused to GST. GST fusion proteins were expressed in *Escherichia coli* (BL21) and purified by affinity chromatography with glutathione-Sepharose 4B (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) as described previously (Wang *et al*., 1999).

Cell Culture, Cloning, and Transfection

αTC1.6 cells, kindly provided by K. Hamaguchi (Oita University, Oita, Japan), were grown in high-glucose (25 mM) DMEM supplemented with 20% fetal calf, 1% L-glutamine, 1% sodium pyruvate, and 1% minimum essential medium nonessential amino acids (Invitrogen, Carlsbad, CA). Although the αTC1.6 cell line was established as a clonal cell line that specifically produces glucagon but not insulin (Hamaguchi and Leiter, 1990) from the original αTC1 cell line (Powers *et al*., 1990), immunostaining analysis revealed that each cell shows variable levels of glucagon expression. We thus performed the recloning of αTC1.6 cells by the limiting dilution method. Among the seven clones established, clone 6 (designated αTC1.6.6) that expresses a relatively high level of glucagon was used in some immunostaining analyses to examine the protein colocalization with glucagon granules. The stable αTC1.6 clones that express phogrin-enhanced green fluorescent protein (EGFP) were established as described previously using the phogrin-EGFP plasmid (Torii *et al*., 2004). Transfections were performed with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Antibodies, Immunostaining, Immunoblotting, and Immunoprecipitation

Rabbit anti-exophilin4 antibodies (αExo4N) were raised against GST-fused N-terminal human exophilin4 protein. The rabbit anti-granuphilin antibodies (αGrp-N) were described previously (Yi *et al*., 2002). Mouse anti-Rab27a and Rab3a monoclonal antibodies were purchased from BD Biosciences Transduction Laboratories (Lexington, KY). Mouse anti-Xpress monoclonal antibodies were purchased from Invitrogen. Rat anti-somatostatin and anti-HA (clone 3F10) monoclonal antibodies were purchased from Chemicon International (Temecula, CA) and Roche Diagnostics (Mannheim, Germany), respectively. Guinea pig anti-glucagon and anti-pancreatic polypeptide sera were purchased from Linco Research (St. Charles, MO). Guinea pig anti-porcine insulin serum was a gift from H. Kobayashi (Gunma University, Maebashi, Japan).

Indirect immunofluorescence analyses were performed as described previously (Wang *et al*., 1999; Torii *et al*., 2002). The mouse pancreas specimen was observed with an epifluorescence microscope (BX-50; Olympus, Tokyo, Japan) equipped with a SenSys charge-coupled device camera (Photometrics, Tucson, AZ). Cultured cells were observed with a confocal microscope LSM5 PASCAL (Carl Zeiss, Jena, Germany). Tissue extracts for immunoblotting analysis were prepared as follows. Tissue excised from C3H/He mice (0.5 g each) was homogenized using a glass-Teflon homogenizer (1000 rpm; 10 strokes) in buffer containing 10 mM HEPES, pH 7.4, 1 mM EGTA, and 2 mM phenylmethylsulfonyl fluoride. It was then lysed on ice for 1 h in the same buffer with 0.1 M NaCl and 1% Triton X-100. After microcentrifugation at 11,000 rpm for 30 min at 4°C, the supernatant was stored at −80°C until use. Pancreatic islets were isolated by pancreatic duct injection of collagenase solution as described previously (Kasai *et al*., 2005). Immunoblotting, immunoprecipitation, and in vitro binding assay with GST fusion proteins were performed as described previously (Nagashima *et al*., 2002).

Phospholipid Binding Assays

Preparation of ^{3}H-labeled liposomes and measurement of phospholipid binding were performed according to the methods described previously (Wang *et al*., 1999; Hosaka *et al*., 2004). All phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Liposomes were made from 75 weight % of phosphatidylcholine (PC) and 25 weight % of PS, or 98.5 weight % of PC, and 1.5 weight % of phosphatidylinositol-4, 5-biphosphate (PIP_2), with 20 μCi of 1,2-dipalmitoyl, L-3-phosphatidyl[N-*methyl*-^{3}H]choline (GE Healthcare). Phospholipids dissolved in chloroform were dried under a stream of nitrogen gas and resuspended in 5 ml of buffer A (50 mM HEPES, pH 7.4, and 0.1 M NaCl). The mixture was shaken for 1 min and sonicated for 15 s by using a probe sonicator. Liposomes were centrifuged at 10,000 × *g* for 10 min before use to remove aggregates. The phospholipid binding assay contained 15 μg of recombinant protein bound to 10 μl of glutathione beads. Beads were prewashed twice with the respective test solutions, and resuspended in 0.1 ml of buffer A containing ^{3}H-labeled liposomes (~0.1 μCi/16.7 μg of phospholipid) and 2 mM EGTA with or without the addition of 2.1 mM $CaCl_2$. For measuring the Ca^{2+} dependence of liposome binding, the free Ca^{2+} concentration was adjusted by changing the concentration of the $CaCl_2$ solution, and it was calculated using WEBMAX-C software (Patton *et al*., 2004). The mixture was incubated at room temperature for 10 min with vigorous shaking, and then it was briefly centrifuged at 2200 rpm in a tabletop centrifuge. The beads were washed three times with 1 ml of the incubation buffer, and the liposome binding was quantified by liquid scintillation counting.

Plasma Membrane Targeting and Hormone Secretion Assays

Recombinant adenoviruses bearing wild-type and mutant exophilin4 cDNAs were prepared as described previously (Yi *et al*., 2002). AxCALacZ, which expresses bacterial β-galactosidase (β-gal), was used as a control adenovirus. A targeting assay was performed as described previously (Torii *et al*., 2004; Izumi *et al*., 2005) with modifications. Briefly, αTC1.6/phogrin-EGFP cells (clone 6) were cultured on poly-L-lysine–coated eight-well chamber slides and infected with adenoviruses encoding β-gal, or wild-type or mutant HA-exophilin4. The cells were then indirectly immunostained with anti-HA antibodies to detect the exogenous exophilin4. Intrinsic EGFP and antibody staining signals were observed by confocal microscopy. A peripheral pattern of EGFP signals was quantified as follows. Cells that revealed a linear distribution along 76–100% of the whole plasma membrane were counted as 1, those along 51–75% as 0.75, those along 26–50% as 0.5, those <25% as 0.25, and those that showed no linear distribution as 0. The summed counts divided by the total number of cells examined were defined as the targeting activity. For each experiment, ~60 cells were assessed unambiguously.

The effect of overexpressed exophilin4 on glucagon secretion in αTC1.6 cells was examined as described previously for insulin secretion in MIN6 cells (Yi *et al*., 2002), with some modifications. Briefly, 5×10^5 cells seeded in poly-L-lysine–coated 12-well dishes on the preceding day were infected with recombinant adenoviruses 40 h before the secretion experiment. The cells were washed once with glucose-free modified Krebs-Ringer buffer (KRB; 120 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 15 mM HEPES, pH 7.4, and 0.1% bovine serum albumin) and then incubated for 30 min in the indicated buffer in the absence or presence of high K^+ (60 mM KCl and 65 mM NaCl). Secreted glucagon was measured using Glucagon, Human, EIA High Sensitivity kit (Peninsula Laboratories, San Carlos, CA).

RESULTS

Expression of Exophilin4

Polyclonal antibodies generated against the N terminus of exophilin4 recognized a single 130-kDa protein in the extracts of a mouse melanocyte cell line melan-a (Figure 1A), where exophilin4 is known to be expressed (Kuroda and Fukuda, 2004). The corresponding protein band was barely detected in the other tissues and cell lines examined, except for the pancreatic α cell line αTC1.6 (Figure 1A). Immunohistochemical analysis of mouse pancreas specimens revealed that exophilin4 is expressed only in peripheral islet

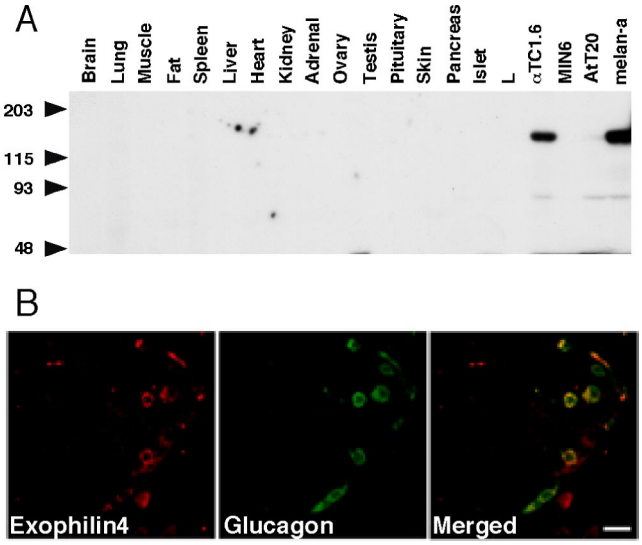

Figure 1. Tissue and cell expression of exophilin4. (A) An equal amount of protein (35 μg) from tissue and cell extracts was loaded onto a polyacrylamide gel. Immunoblotting was performed using anti-exophilin4 antibodies. Numbers to the left of the panel are molecular masses in kilodaltons. (B) The pancreata of 16-wk-old male C3H/He mice were double immunostained with anti-exophilin4 and anti-glucagon antibodies. Merged fluorescent signals are also shown. Bar, 20 μm.

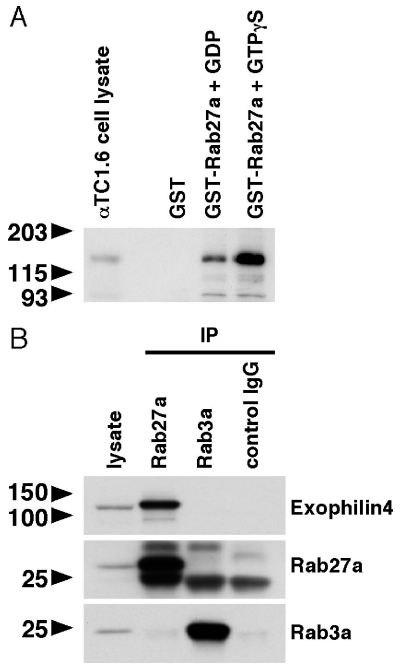

Figure 2. Complex formation between exophilin4 and Rab27a. (A) Aliquots of glutathione beads containing either GST alone or GST-fused Rab27a preloaded with GDP or GTPγS (1 μg of protein) were incubated with αTC1.6 cell extracts (1 mg of protein) and then washed three times. Proteins that bound to the GST fusion proteins and an aliquot of the original cell lysates (20 μg of protein) were analyzed by immunoblotting with anti-exophilin4 antibodies. (B) Extracts of αTC1.6 cells (1 mg of protein) were incubated with anti-Rab27a, anti-Rab3a antibodies, or control mouse IgG and then with protein G-Sepharose beads. After washing the beads, immunoprecipitates (IP) and an aliquot of the original cell lysates (20 μg of protein) were analyzed by immunoblotting using anti-exophilin4 and anti-Rabs antibodies. Numbers to the left of each panel are molecular masses in kilodaltons.

cells but not in exocrine cells (Figure 1B). Double immunostaining analysis using antibodies against pancreatic hormones indicated that it is specifically expressed in glucagon-producing α cells and pancreatic polypeptide-positive cells, but not in insulin-secreting β cells or somatostatin-secreting δ cells (Figure 1B and Table 1). Consistent with a previous finding (Wang *et al*., 1999), granuphilin was detected only in β cells (Table 1). In contrast, Rab27a was expressed in all four types of endocrine cells but was not detected in exocrine cells (Table 1). Thus, although Rab27a is expressed in all endocrine cells in the pancreas, their effectors are differentially expressed.

Exophilin4 has a putative Rab27a binding sequence without a zinc-finger motif at the N terminus (Izumi *et al*., 2003; Fukuda, 2005). To confirm the interaction with Rab27a, we first incubated extracts of αTC1.6 cells with bacterially expressed GST-fused Rab27a protein that had been immobilized on glutathione-Sepharose beads and preloaded with either GDP or guanosine 5′-*O*-(3-thio)triphosphate (GTPγS). Bound exophilin4 was then detected by immunoblotting. Exophilin4 preferentially bound to GTPγS-loaded GST-Rab27a, although a weak interaction with GDP-bound Rab27a was detected (Figure 2A). To investigate whether the interaction occurs under physiological conditions, the presence of exophilin4 was directly examined in Rab27a immunoprecipitates from αTC1.6 cells. Exophilin4 was coimmunoprecipitated with endogenous Rab27a but not with Rab3a, although Rab3a was also expressed in αTC1.6 cells (Yi *et al*., 2002) and immunoprecipitated under this condition (Figure 2B). These findings are consistent with a previous finding that exophilin4 interacts with Rab27a in the B16-F1 mouse melanoma cell line (Kuroda and Fukuda, 2004).

Table 1. Summary of the distributions of exophilin4, granuphilin, and Rab27a in the pancreas

Cell type	Exophilin4	Granuphilin	Rab27a
β (insulin)	−	+	+
α (glucagon)	+	−	+
δ (somatostatin)	−	−	+
Pancreatic polypeptide	+	−	+
Exocrine	−	−	−

Intracellular Localization of Exophilin4

Because Rab27a and its effector granuphilin are localized on insulin granules in pancreatic β cells (Yi *et al*., 2002), we considered that Rab27a and exophilin4 would locate on glucagon granules in α cells. To confirm this, we performed an immunostaining analysis. Our anti-exophilin4 antibody, however, did not yield immunostaining signals strong enough to determine the intracellular localization of endogenous exophilin4 in αTC1.6 cells. Therefore, we expressed HA-tagged exophilin4 and examined its localization by anti-HA antibodies. HA-exophilin4 was concentrated just beneath the plasma membrane and colocalized with peripheral glucagon granules (Figure 3A, a–c). Glucagon signals tended to accumulate in a more peripheral region of the transfected cells than they did in neighboring untransfected cells. The effect was specific because similarly expressed Rab27a was colocalized with glucagon in a more interior cell

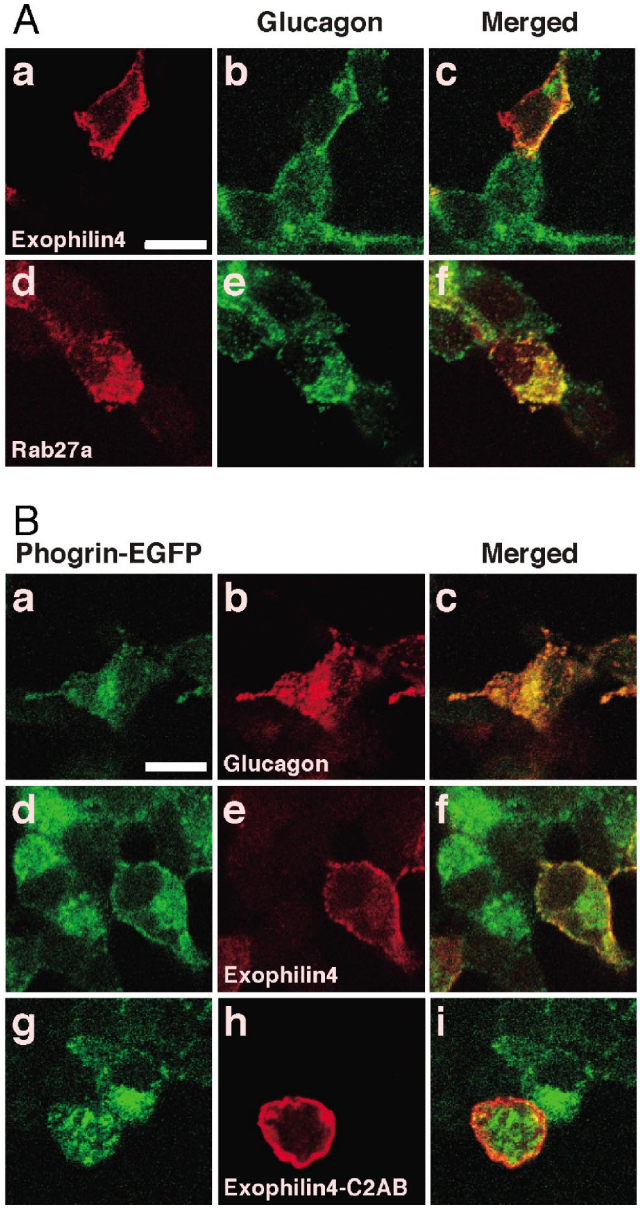

Figure 3. Intracellular distribution of exophilin4, Rab27a, and glucagon granules. (A) αTC1.6.6 cells were transfected with a plasmid encoding either HA-tagged exophilin4 (a–c) or Xpress-tagged Rab27a (d–f). They were then double-immunostained with anti-glucagon and either anti-HA (a–c) or anti-Xpress antibodies (d–f), and observed with a confocal microscope. Merged fluorescent signals are shown in c and f. (B) αTC1.6/phogrin-EGFP cells (a–c) or those transiently transfected with an expression plasmid encoding HA-tagged full-length (d–f) or C-terminal exophilin4 (579-910 amino acids; g–i) were fixed and stained with anti-glucagon (a–c) or anti-HA antibodies (d–i). Intrinsic EGFP fluorescence signals (a, d, and g), immunostaining signals (b, e, and h), and merged signals (c, f, and i) are shown. Bars, 10 μm.

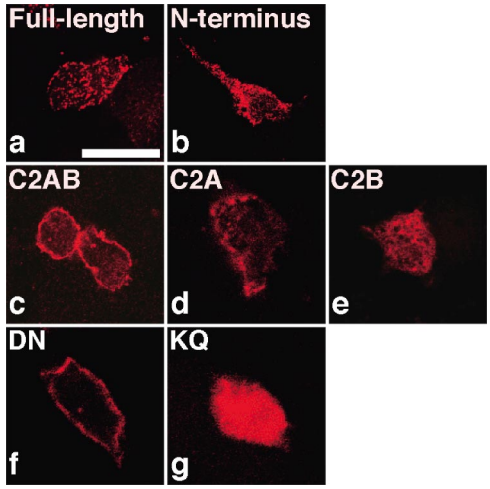

Figure 4. Subcellular localization of N-terminal and C-terminal portions of exophilin4. αTC1.6 cells were transfected with an expression plasmid encoding the full-length (a), N terminus (b), C2AB domain (c), C2A domain (d), or C2B domain (e) of exophilin4 with the HA-tag. The HA-tagged C2A domain harboring the DN (f) or KQ mutation (g) was similarly expressed in αTC1.6 cells. The cells were fixed and stained with anti-HA antibodies and observed with a confocal microscope. Bar, 10 μm.

area and did not induce the peripheral redistribution of the granules (Figure 3A, d–f). These findings suggest that exophilin4 is localized on glucagon granules in the cell periphery and may target interior granules close to the plasma membrane.

To examine the peripheral targeting activity more directly, we established an αTC1.6 cell line that stably expressed phogrin-EGFP. We previously used this fusion protein as a fluorescent marker of secretory granules and showed that overexpression of granuphilin promotes the plasma membrane targeting of insulin granules in a β cell line (Torii *et al.*, 2004). Immunostaining analysis indicated that phogrin-EGFP was colocalized with glucagon on the secretory granules, although it was also colocalized with it around the perinuclear Golgi/endosomal region (Figure 3B, a–c). We thus can follow the motion of glucagon granules by tracking the EGFP fluorescence in these cells. When HA-tagged exophilin4 was transiently overexpressed, EGFP signals were clearly redistributed at the peripheral region exactly where exogenous exophilin4 was colocalized (Figure 3B, d–f). In contrast, neighboring untransfected cells showed only punctate EGFP signals mainly distributed in the interior cytoplasm. The peripheral redistribution of EGFP signals was not seen by the expression of the C-terminal exophilin4 lacking the Rab binding region (Figure 3B, g–i). These observations support the notion that exophilin4 induces translocation of glucagon granules toward the plasma membrane, as granuphilin does for insulin granules (Torii *et al.*, 2004).

We previously showed that granuphilin mediates the docking of insulin granules to the plasma membrane, in which the interaction of its N-terminal region with the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein syntaxin-1a is critical (Torii *et al.*, 2002, 2004; Gomi *et al.*, 2005). Thus, in the present study, we explored whether exophilin4 also interacts with the plasma membrane SNARE proteins syntaxin-1a and/or SNAP-25 by using coimmunoprecipitation experiments. However, we failed to detect such interactions, although the possibility remains that our anti-exophilin4 antibodies raised against the N-terminal region might have sterically hindered them. To identify the region most important for the plasma membrane-targeting activity, we expressed a portion of exophilin4 protein with the HA tag in αTC1.6 cells and determined the subcellular localization by an immunostaining analysis (Figure 4, a–e). We found that full-length exophilin4 is dis-

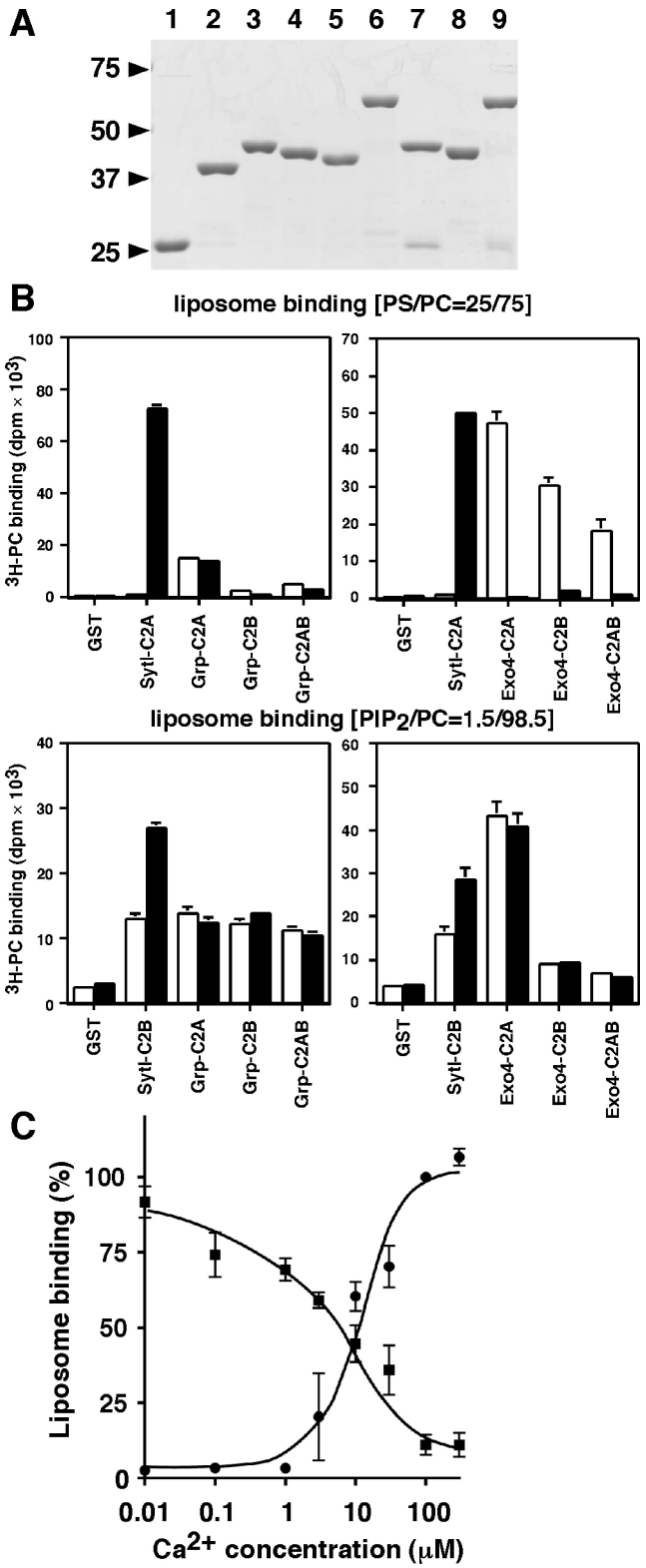

Figure 5. Liposome binding to the C2 domains of synaptotagmin I, granuphilin, and exophilin4. (A) Recombinant proteins were produced in *E. coli* and purified with glutathione-Sepharose 4B. Equivalent amounts (2 μg) of each GST-fused protein were electrophoresed on a 10% polyacrylamide gel and stained with Coomassie blue: GST protein (lane 1); synaptotagmin I-C2A (lane 2) and synaptotagmin-C2B (lane 3); exophilin4-C2A (lane 4), exophilin4-C2B (lane 5), and exophilin4-C2AB (lane 6); and granuphilin-C2A (lane 7), granuphilin-C2B (lane 8), and granuphilin-C2AB (lane 9). Numbers to the left of the panel are molecular masses in kilodaltons. (B) GST-fused recombinant proteins bound to glutathione-Sepharose 4B were incubated with ^{3}H-labeled liposomes in the absence (open bars) or presence (black bars) of 100 μM Ca^{2+}. ^{3}H-labeled liposomes were composed of PC mixed with either PS (top) or PIP_2 (bottom). Phospholipid binding was measured by scintillation counting of the beads after extensive washing and are represented as the means ± SEM (n = 7). (C) Binding of ^{3}H-labeled liposomes containing PS to synaptotagmin I-C2A (circles) or exophilin4-C2A (squares) was examined in buffers containing various free Ca^{2+} concentrations. The data are normalized to the binding of synaptotagmin I-C2A at 100 μM Ca^{2+} or that of exophilin4-C2A without the addition of the $CaCl_2$ solution, respectively, and are represented as means ± SEM (n = 3).

tributed in both peripheral and interior regions, whereas the Rab27a-interacting N-terminal region showed only an intracellular punctate pattern. In contrast, the C-terminal region containing the C2AB domains or the single C2A domain was concentrated in the periphery, although the single C2B domain was localized diffusively in the cytoplasm. These findings suggest a role of the C2 domains, especially the C2A domain, for the targeting activity.

Ca^{2+} and Phospholipid Binding of Exophilin4 C2 Domains

Although some C2 domains reportedly interact with other proteins, their unequivocal function is the ability to bind phospholipids either in a Ca^{2+}-dependent or -independent manner (Nalefski and Falke, 1996). To examine the Ca^{2+}- and phospholipid-binding activity, GST-fused C2 domains of exophilin4 were produced in *E. coli* and purified (Figure 5A). For comparison, the C2 domains of synaptotagmin I and granuphilin were produced. As established previously (Davletov and Südhof, 1993), the first C2 domain of synaptotagmin I (GST-SytI-C2A) showed a high binding activity to the ^{3}H-labeled liposomes containing PS, a negatively charged, plasma membrane phospholipid, only in the presence of 100 μM Ca^{2+} (Figure 5B, top left). Also as reported previously (Wang *et al.*, 1999), the first C2 domain of granuphilin (GST-Grp-C2A) had a weak, Ca^{2+}-independent binding activity to the PS liposome, whereas the second C2 domain (GST-Grp-C2B) had no binding activity to it. By contrast, the C2 domains of exophilin4 (GST-Exo4-C2A, -C2B, and -C2AB), especially the C2A domain, exhibited binding activities comparable to GST-SytI-C2A, but surprisingly, only in the absence of Ca^{2+} (Figure 5B, top right). Thus, the PS-binding activity of GST-Exo4-C2A had a Ca^{2+} sensitivity that was completely opposite to that of GST-SytI-C2A. We then measured the Ca^{2+} sensitivity of these two proteins in detail in buffers containing various free Ca^{2+} concentrations (Figure 5C). Consistent with a previous report (Davletov and Südhof, 1993), Ca^{2+} cooperatively stimulated the PS-liposome binding of GST-SytI-C2A (EC_{50} = 11.6 μM). In contrast, Ca^{2+} inhibited the binding of GST-Exo4-C2A in a dose-dependent manner (EC_{50} = 6.2 μM). Because regulated exocytosis generally occurs with a Ca^{2+} dependence in the 2–30 μM range (Burgoyne and Morgan, 2003), exophilin4 may physiologically dissociate from PS on the plasma membrane, as synaptotagmin I associates with it, in response to a secretory stimulus.

We next examined the binding activity of these C2 domains toward PIP_2, a highly charged phospholipid that is implicated in the fusion of secretory vesicles with the plasma membrane in response to a secretagogue (Martin, 2001). The

second C2 domain of synaptotagmin I (GST-SytI-C2B) exhibited a significant binding activity to the PIP_2 liposome, which was modestly stimulated by Ca^{2+} (Figure 5B, bottom), as reported previously (Bai *et al.*, 2004). GST-Exo4-C2A had a higher PIP_2-binding activity than GST-SytI-C2B both in the presence and absence of Ca^{2+}. By contrast, the C2B domain of exophilin4 and both C2 domains of granuphilin showed relatively weak, Ca^{2+}-independent binding activities to the PIP_2 liposome. GST-Exo4-C2AB exhibited weaker binding activities toward both PS and PIP_2 than GST-Exo4-C2A or -C2B, suggesting inefficient folding of the larger C2AB protein in bacteria and/or interference in lipid binding by the intramolecular interaction between the C2A and C2B domains.

Ca^{2+} and Phospholipid Binding of Exohpilin4 Mutants

To examine the functional role of the Ca^{2+}- and phospholipid-binding activity, we introduced mutations into the C2A domain of exophilin4. One of the mutations, designated KQ, changes a cluster of five lysine residues to glutamine residues. The corresponding mutant in mouse exophilin4 was previously shown to lose the PS-binding activity (Kuroda and Fukuda, 2004). Another novel mutant, designated DN, carries three substitutions of asparagine for aspartate. The corresponding aspartate residues in the C2A domain of synaptotagmin I are crucial for the Ca^{2+} binding (Südhof and Rizo, 1996; Bai and Chapman, 2004), although only three of the five aspartates in synaptotagmin I are conserved in exophilin4. These mutant proteins were bacterially produced (Figure 6A) and used for the liposome binding assay as described above. GST-Exo4-$C2A^{KQ}$ lacked the binding activity to PS regardless of the presence of Ca^{2+} (Figure 6B). By contrast, GST-Exo4-$C2A^{DN}$ exhibited a high PS-binding activity even in the presence of Ca^{2+} and largely lost the Ca^{2+} sensitivity, although a small Ca^{2+}-dependent decrease was still observed. We then examined the binding activity to the PIP_2 liposome (Figure 6C). GST-Exo4-$C2A^{KQ}$ again lost the binding activity to PIP_2, whereas GST-Exo4-$C2A^{DN}$ showed a Ca^{2+}-independent PIP_2-binding activity similar to the wild type. These findings indicate that the series of positively charged lysine residues in the C2A domain are essential for binding to both PS and PIP_2 and that the three aspartates whose homologous residues are involved in the Ca^{2+} binding of the synaptotagmin I-C2A domain are critical for the Ca^{2+}-inhibitory PS-binding activity of exophilin4.

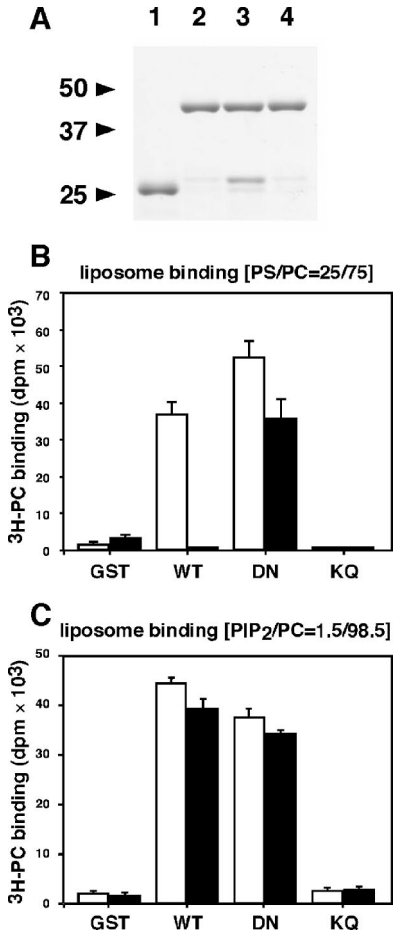

Figure 6. Liposome binding to the wild-type and mutant C2A domains of exophilin4. (A) Equivalent amounts (2 μg) of GST protein (lane 1), wild type (lane 2), DN mutant (lane 3), and KQ mutant, GST-fused exophilin4-C2A proteins (lane 4) were electrophoresed and stained by Coomassie blue. Numbers to the left of the panel are molecular masses in kilodaltons. (B and C) GST-fused recombinant proteins were incubated with ^{3}H-labeled liposomes containing either PS (B) or PIP_2 (C) in the absence (open bars) or presence (black bars) of 100 μM Ca^{2+}. Data are shown as means ± SEM (B, n = 7; C, n = 6).

The Granule-targeting Activity of Exophilin4 Mutants

We next examined whether the drastic changes in the affinity to phospholipids affect the subcellular localization of these mutants. The HA-tagged, mutant C2A domain was expressed in αTC1.6 cells and subjected to the immunostaining analysis. As shown before (Figure 4, d), the wild-type C2A domain was located in both interior and peripheral cell areas. The DN mutant exhibited a more prominent peripheral distribution (Figure 4, f). By contrast, the KQ mutant was diffusively localized in the cytoplasm (Figure 4, g), as was found in melan-a cells (Kuroda and Fukuda, 2004). Thus, the plasma membrane localization of the C2A proteins is highly correlated with the binding activity to phospholipids, especially to PS.

We then expressed full-length exophilin4 protein that contained either the wild-type or mutant C2A domain in αTC1.6/phogrin-EGFP cells to examine the subcellular localization and the granule-targeting activity. Exogenous human exophilin4 was expressed at levels that were ~3 times higher than those of endogenous mouse exophilin4 by recombinant adenoviruses (Figure 7A). The localization of the full-length exophilin4 proteins was highly correlated with that of each C2A domain (Figure 7B, left column). Thus, the wild type was located in both interior and peripheral regions, whereas the DN mutant was strongly concentrated in the periphery. By contrast, the KQ mutant was exclusively located in the interior. In contrast to the single C2A domains; however, the full-length proteins were punctately localized in the cytoplasm probably via the N-terminal interaction with Rab27a on glucagon granules. These findings suggest that the C2A domain has a predominant role in determining the plasma membrane localization of the whole exophilin4 protein. The EGFP fluorescence reflecting the granule distribution was concentrated exactly where the exogenous exophilin4 was accumulated (Figure 7B, middle and right columns). Cells infected with the adenovirus expressing wild-type exophilin4 displayed a more peripheral redistribution of EGFP-labeled granules compared with those expressing control β-gal protein. The DN mutant showed a significant enhancement in the granule-targeting activity. Quantitative measurement revealed an ~1.7-fold increase compared with the wild type (Figure 7C). By contrast, the KQ mutant com-

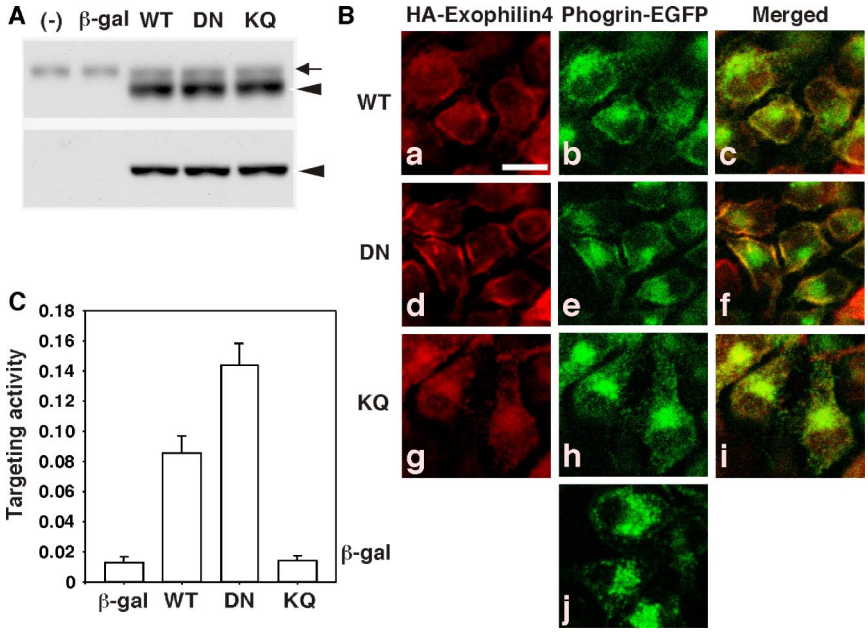

Figure 7. Granule-targeting activity of wild-type and mutant exophilin4. (A) αTC1.6/phogrin-EGFP cells were infected with adenovirus bearing β-gal, HA-tagged exophilin4^{WT}, exophilin4^{DN}, or exophilin4^{KQ} cDNA. The expression levels of endogenous (arrow) and exogenous exophilin4 (arrowhead) were determined by immunoblotting with anti-exophilin4 antibodies (top). Exogenous human exophilin4 migrated slightly faster than endogenous mouse exophilin4. The expression levels of exogenous exophilin4 (arrowhead) were also examined by immunoblotting with anti-HA antibodies (bottom). (B) αTC1.6/phogrin-EGFP cells were infected with recombinant adenovirus encoding HA-tagged exophilin4^{WT} (a–c), exophilin4^{DN} (d–f), or exophilin4^{KQ} (g–i), as well as control adenovirus encoding β-gal (j), under the same conditions as for A. The cells were fixed and stained with anti-HA and Cy3-labeled anti-rat IgG antibodies. Cy3 fluorescence (a, d, and g), intrinsic EGFP fluorescence (b, e, h, and j), and merged fluorescence (c, f, and i) are shown. Bar, 10 μm. (C) The targeting activities of wild-type and mutant exophilin4 examined as in B were quantified as described in *Materials and Methods*. Data are shown as means ± SEM (n = 6).

pletely lost the activity to induce the peripheral redistribution (Figure 7, B and C). These findings indicate that the binding activity of the C2A domain to phospholipids, especially to PS, primarily determined the targeting activity of exophilin4.

Exophilin4 Modulates the Regulated Exocytosis of Glucagon Granules

Finally, we investigated the effect of wild-type and mutant exophilin4 on the exocytosis of glucagon granules. Infection with recombinant adenovirus expressing β-gal affected neither basal nor high K^+-stimulated glucagon release (Figure 8A). Although expression of wild-type exophilin4 did not influence basal glucagon secretion, it significantly inhibited high K^+-induced secretion by ~30%. The inhibitory effect was specific to the exocytosis of glucagon granules because exogenous exophilin4 similarly expressed in the β-cell line MIN6 did not affect insulin secretion in either a basal or a high K^+-stimulated state. Expression of the KQ and DN mutants produced distinct effects on evoked glucagon secretion in αTC1.6 cells, although neither mutant affected basal secretion (Figure 8B). The DN mutant inhibited evoked secretion as the wild type and had a tendency to decrease it further. By contrast, the KQ mutant did not reduce evoked secretion and instead tended to increase it compared with noninfected cells or those expressing control β-gal protein. Therefore, the effect of wild-type and mutant exophilin4 on fusion reactions was inversely correlated with the targeting activity of each C2A domain.

DISCUSSION

The present study indicated that the Rab27a effector exophilin4 is specifically expressed in pancreatic α cells and promotes the targeting of fluorescently labeled glucagon granules to the plasma membrane. Using a similar method, we previously showed that another Rab27a effector granuphilin promotes the targeting of insulin granules to the plasma membrane in MIN6/phogrin-EGFP cells (Torii *et al.*, 2004). We then demonstrated by electron microscopy that granuphilin-deficient mouse pancreatic β cells exhibit severe impairment in the docking of insulin granules to the plasma membrane (Gomi *et al.*, 2005). The targeting activity of wild-type and mutant granuphilins overexpressed in cultured β cells (Torii *et al.*, 2004) is highly correlated with their activity to restore docked granules in granuphilin-deficient pancreatic β cells (Gomi *et al.*, 2005). Therefore, it is highly likely that the targeting activity of exophilin4 found here in αTC1.6/phogrin-EGFP cells reflects its activity to dock glucagon granules to the plasma membrane in vivo. Consistent with this idea, knockdown of exophilin4 by small-interfering RNAs has been shown to markedly reduce the number of melanosomes in the cell periphery of cultured melanocytes (Kuroda and Fukuda, 2004). In this case, however, the knockdown has at the same time induced a more rounded shape of melanocytes, which makes it difficult to judge whether the change in melanosome distribution is a primary effect or a secondary phenomenon resulting from the rounding of the cell shape.

The mechanism for exophilin4 to mediate the docking of secretory granules seems to be distinct from that of granuphilin. We previously showed that granuphilin interacts with one of the plasma membrane SNARE proteins, syntaxin-1a, through its N-terminal domain (Torii *et al.*, 2002). The L43A granuphilin mutant that possesses the intact C2 domains but largely loses the interaction with syntaxin-1a in cells (Torii *et al.*, 2002) neither promotes the targeting of fluorescently labeled granules in MIN6 cells (Torii *et al.*, 2004) nor restores docked granules in granuphilin-null β cells (Gomi *et al.*, 2005). Furthermore, granuphilin-b, another isoform expressed in β cells, which lacks the C2B domain but shows stronger interaction with syntaxin-1a (Torii *et al.*, 2002), has a higher targeting activity than canonical granuphilin-a in MIN6/phogrin-EGFP cells (Torii *et al.*, 2004). These findings suggest that the interaction of granuphilin with syntaxin-1a is essential for the docking activity, although the C2 domains with a modest binding activity to the plasma-membrane phospholipids (Wang *et al.*, 1999; the present study) likely plays another role to appose granules to the plasma membrane. However, we

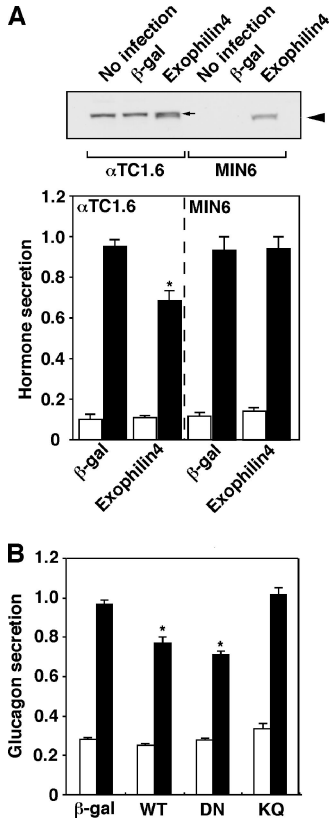

Figure 8. Effect of overexpression of wild-type and mutant exophilin4 on glucagon and insulin secretion. (A) αTC1.6 and MIN6 cells were infected with adenoviruses bearing either β-gal or wild-type human exophilin4 cDNA. The expression levels of endogenous and exogenous exophilin4 were determined by immunoblotting with anti-exophilin4 antibodies (top). Exogenous human exophilin4 (arrowhead) migrates slightly faster than endogenous mouse exophilin4 (small arrow). Note that MIN6 cells do not express exophilin4 endogenously. The infected αTC1.6 cells were incubated for 30 min in either modified KRB (16.7 mM glucose; open bars) or the buffer modified to include high K^+ (60 mM KCl and 1.38 mM glucose; solid bars). The infected MIN6 cells were incubated for 30 min in either modified KRB (including 1.38 mM glucose; open bars) or the buffer modified to include high K^+ (60 mM KCl and 16.7 mM glucose; solid bars). Secreted glucagon or insulin in the media was measured (bottom). Note that exogenous expression of exophilin 4 does not affect evoked insulin secretion in MIN6 cells, in contrast to the inhibition of glucagon secretion in αTC1.6 cells. (B) αTC1.6 cells infected by adenovirus as in Figure 7A and the effect of expression of wild-type and mutant exophilin4 on glucagon secretion was examined as in A. Values are normalized to the release of glucagon or insulin from uninfected cells stimulated by high K^+ and are given as means ± SEM. *$p < 0.001$ versus high K^+-stimulated cells infected with the same titer of the virus bearing β-gal cDNA (A and B, n = 6).

failed to detect such protein interactions for exophilin4. Instead, the C2 domains, particularly the C2A domain, of exophilin4 showed markedly higher phospholipid-binding activities than those of granuphilin. The phospholipid-binding activity of the C2A domain should play a predominant role because its KQ mutation that entirely destroys the affinity to PS and PIP_2 impaired both the plasma membrane localization and the granule-targeting activity of the whole protein. This finding is consistent with a previous report that showed that the homologous mutant in mouse exophilin4 lacks both the plasma membrane localization and the activity to induce peripheral distribution of melanosomes (Kuroda and Fukuda, 2004). Therefore, even if other protein interactions might be hidden, the phospholipid binding activity of the C2A domain is crucial in the docking function of exophilin4. Recently, rabphilin, another Rab3/Rab27 effector, has been reported to interact with the plasma-membrane SNARE protein, SNAP-25, via its C2B domain (Tsuboi and Fukuda, 2005). Thus, different Rab27a effectors, granuphilin, exophilin4, and rabphilin, seem to use distinct strategies to mediate the docking process.

We found a robust binding activity of the C2A domain of exophilin4 to PIP_2 as well as to PS, although the affinity to PIP_2 was not previously detected using a dot blot assay on nitrocellulose membrane (Kuroda and Fukuda, 2004). This discrepancy is likely due to differences in the binding assays used because we found that the dot blot assay produces highly variable results depending on the buffer components (e.g., detergent and blocking reagents) and sometimes exhibits data discrepant from those obtained by the more physiological liposome assay adopted here (Yu and Izumi, unpublished observation). We cannot discriminate between PS and PIP_2 as a target of the C2A domain of exophilin4 because the docking-incompetent mutant KQ loses the binding activity to both lipids. However, the mutant DN, that has a higher PS- but a comparable PIP_2-binding activity compared with the wild type, showed a stronger targeting activity, indicating a primary role of PS. Alternatively, the C2B domain of exophilin4 that has a considerable PS-binding activity but only a weak PIP_2-binding activity was not be able to associate with the plasma membrane when it was singly expressed in αTC1.6 cells, suggesting that the PIP_2-binding activity is also important. PS has a single net negative charge and is present evenly throughout the cytoplasmic leaflet of the plasma membrane (McLaughlin and Murray, 2005). In contrast, PIP_2, although a minor component of the plasma membrane lipids, is highly charged and sequestered adjacent to any protein with a cluster of four or more basic residues located at the plasma membrane (McLaughlin and Murray, 2005). Therefore, we suggest that the C2A domain of exophilin4 first attaches to PS and then recruits PIP_2 to form a microdomain through its basic cluster to stabilize the plasma membrane association.

The most notable finding in the present study was the Ca^{2+}-inhibitory PS-binding activity of the exophilin4-C2A domain, which was completely the reverse of that of the synaptotagmin I-C2A domain. Because the Ca^{2+} sensitivity was in a range of the concentration induced by secretagogue stimulation, this novel property of exophilin4 probably has physiological relevance. Although only three aspartate residues are conserved in the C2A domain of exophilin4 among the five critical aspartates for Ca^{2+} binding of synaptotagmin I (Südhof and Rizo, 1996; Bai and Chapman, 2004), the replacement of these residues largely disrupts the inhibition by Ca^{2+} and results in a constitutive PS-binding activity. Furthermore, expression of this DN mutant heavily accumulated granules close to the plasma membrane and impeded stimulus-induced exocytosis. In contrast, the mutant KQ, which loses both the PS-binding and the granule-targeting activities, did not affect evoked exocytosis. These findings are consistent with our previous proposal that stable docking is not necessarily essential but inhibitory for fusion and that undocked vesicles are releasable with a functionally meaningful time course in regulated exocytosis (Gomi *et al.*, 2005). It seems that the docking machinery must be displaced from exocytic sites so that the opposing membrane can fuse. Although the exact function of the Ca^{2+}-inhibitory PS-binding activity of the exophilin4-C2A domain remains

unknown, it may play a role in the disassembly of the preformed docking machinery. The Ca^{2+} increase induced by external secretagogues, however, may not be sufficient because we failed to detect morphological detachment of exophilin4 from the plasma membrane when cells were stimulated with Ca^{2+} ionophores, at least on the level of light microscopy (Kasai and Izumi, unpublished observation). This failure may simply reflect the fact that the PIP_2 binding of exophilin4, which would happen subsequent to the PS binding as described above, is no longer Ca^{2+} sensitive. The alternative and not incompatible possibility is that the Ca^{2+}-inhibitory PS-binding activity prevents stable docking during secretory stimulation. Further investigation is required to explore the coupling and uncoupling mechanisms between docking and fusion processes.

Finally, the present study discloses that professional secretory cells use distinct machinery and molecular interaction for vesicle docking, even between developmentally close cells such as pancreatic α and β cells. This suggests that the docking machinery is differentially configured to meet the biological demands of particular cell types. The first glimpse of distinct exocytotic machinery between pancreatic α and β cells should lead to further understanding of the differential mechanism of secretion of the two vital hormones, glucagon and insulin, in both physiological and pathological states.

ACKNOWLEDGMENTS

We thank Dr. S. Zhao for assistance with the immunostaining analyses at the beginning of this project and Dr. M. Hosaka for advice on the liposome assay. This work was supported by grants in aid for scientific research and a grant of the 21st Century COE program from the Ministry of Education, Culture, Sports, Science and Technology of Japan. It was also supported in part by grants from Astellas Foundation for Research on Metabolic Disorders and Novo Nordisk Insulin Study Award (to T.I.).

REFERENCES

Bai, J., and Chapman, E. R. (2004). The C2 domains of synaptotagmin–partners in exocytosis. Trends Biochem. Sci. *29*, 143–151.

Bai, J., Tucker, W. C., and Chapman, E. R. (2004). PIP_2 increases the speed of response of synaptotagmin and steers its membrane-penetration activity toward the plasma membrane. Nat. Struct. Mol. Biol. *11*, 36–44.

Burgoyne, R. D., and Morgan, A. (2003). Secretory granule exocytosis. Physiol. Rev. *83*, 581–632.

Davletov, B. A., and Südhof, T. C. (1993). A single C_2 domain from synaptotagmin I is sufficient for high affinity Ca^{2+}/phospholipid binding. J. Biol. Chem. *268*, 26386–26390.

Desai, R. C., Vyas, B., Earles, C. A., Littleton, J. T., Kowalchyck, J. A., Martin, T.F.J., and Chapman, E. R. (2000). The C2B domain of synaptotagmin is a Ca^{2+}-sensing module essential for exocytosis. J. Cell Biol. *150*, 1125–1135.

Fukuda, M. (2005). Versatile role of Rab27 in membrane trafficking: focus on the Rab27 effector families. J. Biochem. *137*, 9–16.

Gomi, H., Mizutani, S., Kasai, K., Itohara, S., and Izumi, T. (2005). Granuphilin molecularly docks insulin granules to the fusion machinery. J. Cell Biol. *171*, 99–109.

Hamaguchi, K., and Leiter, E. H. (1990). Comparison of cytokine effects on mouse pancreatic α-cell and β-cell lines: viability, secretory function, and MHC antigen expression. Diabetes *39*, 415–425.

Hosaka, M., Suda, M., Sakai, Y., Izumi, T., Watanabe, T., and Takeuchi, T. (2004). Secretogranin III binds to cholesterol in the secretory granule membrane as an adapter for chromogranin A. J. Biol. Chem. *279*, 3627–3634.

Izumi, T., Gomi, H., Kasai, K., Mizutani, S., and Torii, S. (2003). The roles of Rab27 and its effectors in the regulated secretory pathways. Cell Struct. Funct. *28*, 465–474.

Izumi, T., Gomi, H., and Torii, S. (2005). Functional analysis of Rab27a effector granuphilin in insulin exocytosis. Methods Enzymol. *403*, 216–229.

Kasai, K., Ohara-Imaizumi, M., Takahashi, N., Mizutani, S., Zhao, S., Kikuta, T., Kasai, H., Nagamatsu, S., Gomi, H., and Izumi, T. (2005). Rab27a mediates the tight docking of insulin granules onto the plasma membrane during glucose stimulation. J. Clin. Investig. *115*, 388–396.

Kuroda, T. S., and Fukuda, M. (2004). Rab27A-binding protein Slp2-a is required for peripheral melanosome distribution and elongated cell shape in melanocytes. Nat. Cell Biol. *6*, 1195–1203.

Martin, T.F.J. (2001). $PI(4,5)P_2$ regulation of surface membrane traffic. Curr. Opin. Cell Biol. *13*, 493–499.

McLaughlin, S., and Murray, D. (2005). Plasma membrane phosphoinositide organization by protein electrostatics. Nature *438*, 605–611.

Nagashima, K., Torii, S., Yi, Z., Igarashi, M., Okamoto, K., Takeuchi, T., and Izumi, T. (2002). Melanophilin directly links Rab27a and myosin Va through its distinct coiled-coil regions. FEBS Lett. *517*, 233–238.

Nalefski, E. A., and Falke, J. J. (1996). The C2 domain calcium-binding motif: structural and functional diversity. Protein Sci. *5*, 2375–2390.

Patton, C., Thompson, S., and Epel, D. (2004). Some precautions in using chelators to buffer metals in biological solutions. Cell Calcium *35*, 427–431.

Powers, A. C., Efrat, S., Mojsov, S., Spector, D., Habener, J. F., and Hanahan, D. (1990). Proglucagon processing similar to normal islets in pancreatic α-like cell line derived from transgenic mouse tumor. Diabetes *39*, 406–414.

Südhof, T. C., and Rizo, J. (1996). Synaptotagmins: C_2-domain proteins that regulate membrane traffic. Neuron *17*, 379–388.

Torii, S., Takeuchi, T., Nagamatsu, S., and Izumi, T. (2004). Rab27 effector granuphilin promotes the plasma membrane targeting of insulin granules via interaction with syntaxin 1a. J. Biol. Chem. *279*, 22532–22538.

Torii, S., Zhao, S., Yi, Z., Takeuchi, T., and Izumi, T. (2002). Granuphilin modulates the exocytosis of secretory granules through interaction with syntaxin la. Mol. Cell. Biol. *22*, 5518–5526.

Tsuboi, T., and Fukuda, M. (2005). The C2B domain of rabphilin directly interacts with SNAP-25 and regulates the docking step of dense core vesicle exocytosis in PC12 cells. J. Biol. Chem. *280*, 39253–39259.

Wang, J., Takeuchi, T., Yokota, H., and Izumi, T. (1999). Novel rabphilin3-like protein associates with insulin-containing granules in pancreatic beta cells. J. Biol. Chem. *274*, 28542–28548.

Yi, Z., Yokota, H., Torii, S., Aoki, T., Hosaka, M., Zhao, S., Takata, K., Takeuchi, T., and Izumi, T. (2002). The Rab27a/granuphilin complex regulates the exocytosis of insulin-containing dense-core granules. Mol. Cell. Biol. *22*, 1858–1867.