Slp4-a/Granuphilin-a Regulates Dense-core Vesicle Exocytosis in PC12 Cells*

Received for publication, May 30, 2002, and in revised form, July 22, 2002
Published, JBC Papers in Press, August 9, 2002, DOI 10.1074/jbc.M205349200

Mitsunori Fukuda‡, Eiko Kanno, Chika Saegusa, Yukie Ogata, and Taruho S. Kuroda

From the Fukuda Initiative Research Unit, RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

The synaptotagmin-like protein (Slp) family was originally identified as proteins that share homology to tandem C2 domains of the synaptotagmin (Syt) family (1), and it was subsequently defined as proteins that contain a unique N-terminal Slp homology domain (SHD) (2) and C-terminal tandem C2 domains (known as the C2A domain and C2B domain), which are putative Ca\(^{2+}\)-binding motifs (2). To date, five members of the Slp family (Slp1/Jfc1, Slp2-a, Slp3-a, Slp4-a/granuphilin-a, and Slp5) have been described in the mouse and human (1–5), and several alternative splicing isoforms have been identified in Slp2, Slp3, and Slp4 (2, 4).

The SHD consists of two conserved domains, referred to as SHD1 and SHD2 (2). The SHD1 and SHD2 of Slp3-a, Slp4-a, and Slp5 are separated by a sequence containing two zinc-finger motifs, whereas Slp1 and Slp2-a lack such zinc-finger motifs, and their SHD1 and SHD2 are linked (2). The SHD has also been found in other proteins, including Slac2-a (Slp homologue lacking C2 domains-a)/melanophilin (2, 6, 7), Slac2-b/KIAA0624 (8, 9), and Slac2-c/MyRIP (10).2 We recently discovered that the SHD of the Slp family and Slac2 functions as an effector domain for specific Rab (7, 9), a small GTP-binding protein believed to be essential for membrane trafficking in eukaryotic cells (reviewed in Refs. 11–13). All SHDs directly interact with the GTP-bound form of Rab27A (and possibly Rab27B), both in vitro and in intact cells (5, 7, 9, 10, 14–16), but the Slp4-a SHD is exceptional because it can also interact with Rab8 and Rab3A (9, 16), whereas the others only recognize Rab27 isoforms among the Rabs we have tested (more than 20 Rabs) (5, 7, 9).

Since mutations in the rab27A gene cause defects in granule exocytosis in cytotoxic T lymphocytes and melanosome transport in human Griscelli syndrome and ashen mice (17–22), we hypothesized previously that the Slp family and Slac2 are involved in such membrane trafficking (9). Consistent with this, Slac2-a has recently been identified as a “missing link” between Rab27A in the melanosome and myosin Va, an actin-based motor, indicating that formation of a tripartite protein complex (Rab27A/Slac2-a/myosin Va) is crucial for melanosome transport (7, 16, 23–25). In contrast, however, functional involvement of the Slp family in Rab27-dependent membrane trafficking (especially in regulated granule exocytosis) remained to be clarified.

In this study, we demonstrated that the Slp4-a-Rab27A complex is formed on dense-core vesicles in vivo and controls regulated secretion (high KCl-dependent neuropeptide Y (NPY) secretion) by using PC12 cells in which Slp4-a, Rab3A, Rab8, and Rab27A were endogenously expressed. Based on our findings, we discuss the function of the Slp family as effectors for Rab27A in regulated dense-core vesicle exocytosis.

EXPERIMENTAL PROCEDURES

Antibody Production—cDNA encoding the C2B domain of mouse Slp4-a/granuphilin-a (amino acids 489–673) (4, 9) was amplified by the conventional PCR and subcloned into the pcDNA3 vector (named pGEX-Slp4-a/C2B) (Amersham Biosciences), as described previously (26). GST (glutathione S-transferase) fusion proteins were expressed and purified on glutathione-Sepharose beads by the standard method (27). New Zealand White rabbits were immunized with purified GST-Slp4-a/C2B, and anti-Slp4-a antibody was affinity-purified by exposure to antigen-bound Affi-Gel 10 beads (Bio-Rad), as described previously (28). The specificity of the antibody was checked by immunoblotting.

* This work was supported in part by grants from the Science and Technology Agency to Japan (to M. F.) and Grant 13780624 from the Ministry of Education, Science, and Culture of Japan (to M. F.).1 To whom correspondence should be addressed. Tel.: 81-48-462-4994; Fax: 81-48-462-4995; E-mail: mnfukuda@brain.riken.go.jp.

2 The abbreviations used are: Slp(a), synaptotagmin-like protein(s); GST, glutathione S-transferase; HRP, horseradish peroxidase; NPY, neuropeptide Y; SHD, Slp homology domain; Slac2, Slp homologue lacking C2 domains; Syt, synaptotagmin; GTP\(\alpha\), guanosine 5’-O-(3-thiotriphosphate).

The Journal of Biological Chemistry © 2002 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 277, No. 42, Issue of October 18, pp. 39673–39678, 2002
Printed in U.S.A.
with recombinant T7-tagged Slp1, Slp2a, Slp3a, Slp4-a, Slp5, rabphilin, and Syt I expressed in COS-7 cells (29, 30) (see Fig. 1B). Anti-Rab3A rabbit polyclonal antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Rab3A, anti-Rab8, and anti-Rab27A mouse monoclonal antibodies were from Transduction Laboratories (Lexington, KY). Horseradish peroxidase (HRP)-conjugated anti-FLAG tag and anti-synaptophysin mouse monoclonal antibodies were from Sigma. HRP-conjugated anti-T7 tag antibody was from Novagen (Madison, WI).

Construction of Expression Vectors and Transfections—pEF-T7-Slp4-a-2SHD (amino acids 144–673), pEF-T7-GST-Slp4-a, and pEF-T7-GST vectors were constructed by PCR essentially as described previously (7). pEF-FLAG-Rab27A mutants (E14A, I18A, V21A, or D32A) were similarly constructed by PCR using the following mutated oligonucleotides with appropriate restriction enzyme sites (underlined below): 5’-CGAGTCATCTGGGAGTAATTACCTCTCTCTCTTCGCGATGGCAAGG-3’ (E14A primer), 5’-GGATCCATGTCGGAGATACTAGACCTCTCTTTTCTGTCCGAGATGGAAAGGGATTTGGCCCTC-3’ (I18A primer), 5’-ATCTCTGCGGAGAACGACCTCTTG-3’ (V21A primer), and 5’-GGAGTCTACCTCTCTCTTCGCGAATCCTTTTCTCGACTGGTGC-3’ (D32A primer). pEF-FLAG-Rab27A/T23N and -Rab27A/Q78L were prepared as described elsewhere. Other expression vectors (pEF-T7-Slp1, -Slp2a, -Slp3a, -Slp5, -rabphilin, -Syt I, 1⁄500 dilution), as described previously (31). The blots shown in this study are representative of at least two or three independent experiments.

Immunoprecipitation and Immunoblotting—PC12 cells (two confluent 10-cm dishes) were homogenized in a buffer containing 1 ml of 50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 0.5 mM GTP-γS, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10 μM pepstatin A) in a glass Teflon Potter homogenizer with 10 strokes at 900–1000 rpm, and proteins were solubilized with 1% Triton X-100 at 4 °C for 1 h. After removal of insoluble materials by centrifugation at 15,000 rpm for 10 min, the supernatant was incubated with either anti-Slp4-a-C28 IgG or control rabbit IgG (10 μg/ml) for 1 h at 4 °C and then incubated with protein A-Sepharose beads (Amersham Biosciences) for 1 h at 4 °C. After washing the beads five times with 50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 0.2% Triton X-100, and protease inhibitors, the immunoprecipitates were subjected to 10% SDS-PAGE followed by immunoblotting with anti-Rab3A, anti-Rab8, anti-Rab27A (1⁄200 dilution), or anti-Slp4-a antibody (1⁄250 dilution) mouse monoclonal antibodies, or anti-Slp4-a (1 μg/ml dilution), as described previously (31). A 4-μg amount of each plasmid was transfected into COS-7 cells (7.5 × 105 cells, the day before transfection/10-cm dish) or PC12 cells (3 × 105 cells, the day before transfection/6-cm dish coated with collagen type I) by using LipofectAMINE Plus or LipofectAMINE 2000 reagent (Invitrogen), respectively, according to the manufacturer's notes (28, 32, 33).

RESULTS

Expression of Rab3A, Rab8, Rab27A, and Slp4-a in PC12 Cells—Our previous in vitro GST pull-down assay showed that the SHD of Slp4-a preferentially binds Rab8 and Rab27A and weakly interacts with Rab3A, whereas other SHD's specifically bind Rab27A (9). To attempt to determine which Rab's are in vivo binding partners of Slp4-a, we used specific antibodies to search for cell line(s) in which these three Rabs and Slp4-a are endogenously expressed. The specificity of each antibody used in this study (anti-Rab3A, anti-Rab8, anti-Rab27A, and anti-Slp4-a antibodies) was confirmed by immunoblotting with recombinant FLAG-Rabs and recombinant T7-Slps, rabphilin,
Slp4-a Functions as a Rab27A Effector in PC12 Cells

and Syt I (Fig. 1A, lanes 1–3 and Fig. 1B, lanes 1–7). The anti-Slp4-a antibody only recognized T7-Slp4-a, not the other Slps, including Slp5, a close homologue of Slp4-a (5). PC12 cells were found to express all four proteins (Fig. 1A, lanes 1–3, and 1B, lane 8). Quantitative analysis indicated that the ratio of the amounts of the three Rabs in descending order was Rab8: Rab3A:Rab27A = 1:0.4:< 0.1 (Fig. 1A). The level of expression of Slp4-a was lower than in the pancreas (i.e., pancreatic β-cells; data not shown). To investigate whether Slp4-a-Rab complex is actually formed in vivo, an immunoprecipitation experiment was performed using anti-Slp4-a-specific antibody (Fig. 2A, lane 3). Consistent with our previous in vitro binding experiments (9), Rab8 and Rab27A were found to co-immunoprecipitate with Slp4-a, whereas hardly any Rab3A was detected in the anti-Slp4-a IgG immunoprecipitates (Fig. 2A, lane 3). In contrast, none of the Rabs were detected in the control IgG immunoprecipitates (Fig. 2A, lane 2). Since the amounts of bound Rab8 and Rab27A were significantly reduced in the absence of GTP-γS, Slp4-a should recognize the GTP-bound form of Rab8 and Rab27A even under the in vivo conditions (data not shown). Similar results were obtained when exogenously expressed GST-Slp4-a was used (Fig. 2B, lane 2): GST-Slp4-a bound both Rab8 and Rab27A but only a trace amount of Rab3A. Although the relative amount of Rab27A was less than 10% of that of Rab8 in PC12 cells, Slp4-a efficiently co-immunoprecipitated with Rab27A, indicating that Slp4-a prefers Rab27A to Rab8 or Rab3A as an in vivo binding partner.

Slp4-a-Rab8 and Slp4-a-Rab27A Complexes on Dense-core Vesicles in PC12 Cells—PC12 cells contain two types of secretory vesicles, i.e., dense-core vesicles and synaptic-like microvesicles, and they are often utilized for analysis of regulated secretion. Since Slp4-a/granuphilin-a was first identified as a protein that specifically associated with insulin-containing dense-core vesicles (4), we next investigated whether Slp4-a and three Rabs are localized on the dense-core vesicles of PC12 cells. As shown in Fig. 3, Rab3A (Fig. 3A, green), Rab8 (Fig. 3D, green), Rab27A (Fig. 3G, green), and Slp4-a (Fig. 3, B, E, and H, red) were found to be predominantly localized in the distal portion of neurites, where dense-core vesicles were enriched (Fig. 3, C, F, and I, yellow). Dotted signals for Rab8 were found throughout the cell body in addition to the neurites (Fig. 3D), whereas the others were almost exclusively expressed in the distal portion of the neurites. Similar expression patterns were observed when Slp4-a or Rabs were exogenously expressed in PC12 cells (data not shown).

A subcellular fractionation study was performed to further confirm the presence of Slp4-a and the three Rabs on dense-core vesicles (Fig. 4). As expected, Rab3A, Rab27A, and Slp4-a were co-distributed with Syt IX (dense-core vesicle marker (DCV); fractions 5–8) but not with syntrophin (synaptic-like microvesicle marker (SLMV); fractions 2–4) (29, 35, 36). In contrast, Rab8 was also detected in lighter fractions in addition to the dense-core vesicle-enriched fractions, consistent with the immunocytochemical analysis (Fig. 3D). We therefore concluded that the Slp4-a-Rab8 and Slp4-a-Rab27A complexes are indeed formed on dense-core vesicles in intact PC12 cells.

Opposite Effects of Slp4-a and Rab27A on Regulated Secretion in PC12 Cells—Finally, we used an NPY-T7-GST secretion assay to investigate whether the Slp4-a-Rab8 and/or Slp4-a-Rab27A complexes control regulated exocytosis in PC12 cells (see ref. 34 for details). When Rab27A was transiently coexpressed with NPY in PC12 cells, high KCl-dependent NPY secretion was significantly promoted (Fig. 5A, closed bar), but the Rab27A expression had no effect on low KCl-dependent NPY secretion (data not shown). The effect of Rab27A should be GTP-dependent because expression of Rab27AT23N), a dominant negative form that mimics the GDP-bound state, did not promote NPY secretion, consistent with the previous report (14) (data not shown). In contrast, expression of Rab8 had no significant effect on high KCl-dependent NPY secretion (Fig. 5A, open bar). The slight effect of Rab8 on regulated secretion was not due to its expression levels in PC12 cells because

Fig. 2. In vivo formation of Slp4-a-Rab complex in PC12 cells. A, in vivo formation of Slp4-a-Rab8 and Slp4-a-Rab27A in PC12 cells. Anti-Slp4-a IgG, but not control IgG, immunoprecipitated Rab8 and Rab27A, but no Slp4-a-Rab3A complex was detected under our experimental conditions (lane 3). The asterisk indicates the light chain of IgG. B, exogenously expressed T7-GST-Slp4-a, but not T7-GST alone, preferentially interacts with Rab8 and Rab27A but essentially not with Rab3A (lane 2). In both panels, input means ½ volume of the reaction mixture used for immunoprecipitation. The positions of the molecular weight markers (× 10⁻²) are shown on the left.
SLP4-A Functions as a Rab27A Effector in PC12 Cells

Fig. 3. Colocalization of Rab3A, Rab8, and Rab27A with Slp4-a in the distal portion of the neurites of PC12 cells. PC12 cells were fixed, permeabilized, and stained with anti-Rab3A (A, green), anti-Rab8 (D, green), anti-Rab27A (G, green), and anti-Slp4-a (B, E, and H, red) antibodies. Note that Slp4-a and three Rabs were colocalized in the distal portion of the neurites, where dense-core vesicles are enriched (C, F, and I, yellow) in merged images of Slp4-a and the Rabs, suggesting that Slp4-a and the three Rabs are present on the dense-core vesicles. Scale bar indicates 50 μm.

Fig. 4. Rab3A, Rab8, Rab27A, and Slp4-a are localized on dense-core vesicles in PC12 cells. PC12 cells were fractionated on a 0.6–1.8 m sucrose gradient. The fractions were analyzed by immunoblotting with anti-Slp4-a (top panel), anti-Rab3A (second panel), anti-Rab8 (third panel), anti-Rab27A (fourth panel), anti-Syt IX (fifth panel); a marker for dense-core vesicles (DCV), and anti-synaptophysin antibody (bottom panel); a marker for synaptic-like microvesicles (SLMV). Note that Rab3A, Rab8, and Slp4-a were co-distributed with the dense-core vesicle-enriched fractions (fractions 5–8), the same as Syt IX, not with the synaptic-like microvesicle-enriched fractions (fractions 2–4).

similar amounts of FLAG-Rab8 and -Rab27A were detected in PC12 cells by immunoblotting (Fig. 5A, inset). Similarly, expression of Rab3A did not promote NPY secretion, as described previously (14, 37–39) (data not shown).

When Slp4-a was expressed in PC12 cells, high KCl-dependent NPY secretion was significantly reduced (Fig. 5B, open bar), but it had no effect on low KCl-dependent secretion (data not shown). Interestingly, expression of Slp3-a, a Rab27A-specific Slp exhibiting Ca2+-dependent phospholipid binding activity (1, 40), in PC12 cells strongly promoted high KCl-dependent NPY secretion (Fig. 5B, closed bar). To further determine whether the effect of Slp4-a (or Slp3-a) expression in high KCl-dependent NPY secretion is indeed Rab27A-dependent, an Slp4-a mutant incapable of Rab27A binding (∆SHD) was expressed in PC12 cells (Fig. 6A, lane 18). As expected, expression of Slp4-a-∆SHD completely reversed the inhibitory effect (Fig. 6B, closed bar). A similar result was obtained for Slp3-b (2), an alternatively splicing isoform that lacks an N-terminal SHD (i.e. no significant effect on high KCl-dependent NPY secretion; Fig. 6B, closed bar). We therefore concluded that the observed effect of Slp4-a (or Slp3-a) expression in NPY secretion depends on the presence of the SHD (i.e. Rab27A-binding site).

Since deletion of the SHD of Slp4-a also abrogated Rab3A and Rab8 binding (Fig. 6A, lanes 16 and 17), we could not completely rule out the possibility that the Slp4-a-Rab3A or Slp4-a-Rab8 complex, but not Slp4-a-Rab27A complex, has an inhibitory effect on high KCl-dependent NPY secretion. When Ala-based site-directed mutagenesis was performed to resolve this issue, a single amino acid substitution in the SHD1 was found to alter the Rab binding specificity of Slp4-a (Fig. 6A). Slp4-a(V21A) and Slp4-a(118A) mutants specifically recognized Rab27A but not Rab3A and Rab8 (Fig. 6B, lanes 4–9), whereas the Slp4-a(V21A) mutant recognized both Rab8 and Rab27A but not Rab3A (lanes 10–12). In contrast, a D32A mutation had almost no effect on the Rab binding specificity of Slp4-a (lanes 13–15). If Slp4-a-Rab3A or Slp4-a-Rab8 complex plays a major role in inhibition of high KCl-dependent NPY secretion, expression of a 118A or V21A mutant should reverse the inhibitory effect. However, when these mutants of Slp4-a were expressed in PC12 cells, no difference in high KCl-dependent NPY secretion was detected between the wild-type and mutant Slp4-a (Fig. 6B, open bars). These results strongly indicated that Slp4-a modulates dense-core vesicle exocytosis via binding to Rab27A in PC12 cells.

DISCUSSION

The SLP family (Slp1–5) was defined as proteins with an N-terminal SHD and C-terminal tandem C2 domains (1, 2). Previously, we showed that the SHD of the Slp family binds the GDP-bound form of Rab27A (5, 9), but involvement of the Slp family in Rab27A-dependent membrane trafficking (i.e. melanosome transport in melanocytes and granule exocytosis in cytotocxic T lymphocytes observed in type I human Griscelli syndrome and ashen mice (17–22)) has never been elucidated.
Slp4-a Functions as a Rab27A Effector in PC12 Cells

A

Fig. 6. Effect of expression of Slp4-a mutants and Slp3-b on regulated secretion in PC12 cells. A, pEF-T7-Slp4-a one-point mutants (or pEF-T7-Slp4-a-ΔSHD) and pEF-FLAG-Rabs (Rab3A, Rab8, or Rab27A) were coexpressed into COS-7 cells. Co-immunoprecipitated (IP) FLAG-Rabs and immunoprecipitated T7-Slp4-a proteins are shown in the middle panel (Blot, anti-FLAG; IP, anti-T7) and bottom panel (Blot, anti-T7; IP, anti-T7), respectively. The top panel indicates the total FLAG-Rabs expressed (% of volume of the reaction mixture; input) that were used for immunoprecipitation. Note that the E14A, I18A, and V21A mutations altered the Rab binding specificity of Slp4-a (lanes 4–12), whereas deletion of the SHD completely abolished Rab binding activity (lanes 16–18). B, effect of expression of Slp4-a mutants and Slp3-b on regulated secretion in PC12 cells (**, p < 0.01, Student’s unpaired t test). The NPY-T7-GST secretion assay was performed as described previously (34). The results are expressed as percentages of NPY-T7-GST secretion in control (Cont) samples (shaded bars) without expression of recombinant proteins. Bars indicate the means ± S.E. of three determinations. The results shown are representative of three independent experiments. The inset shows recombinant proteins visualized with anti-T7 tag antibody. Note that expression of Slp4-a-ΔSHD or Slp3-b had no effect on high KCl-dependent NPY secretion (closed bar), whereas expression of Slp4-a(I18A) (Rab27A-specific) and Slp4-a(V21A) (Rab8- and Rab27A-specific) inhibited NPY secretion, the same as the wild-type (WT) protein (open bars).

In this study, we demonstrated for the first time that Rab27A and its effector molecules (Slp4-a and Slp3-a) control dense-core vesicle exocytosis in PC12 cells.

Although Slp4-a has been found to interact with Rab3A, Rab8, and Rab27A in vitro and in overexpression studies (9, 16), endogenous Slp4-a in PC12 cells preferentially interacted with Rab27A on dense-core vesicles (Figs. 2–4). The Slp4-a–Rab8 complex was also observed on dense-core vesicles in PC12 cells, but the majority of Rab8 (especially in the cell body; Fig. 3F) was free of Slp4-a, and significant amounts of Slp4-a–Rab3A were not detected. In addition, we discovered that expression of Rab27A, but not of Rab8, promoted high KCl-dependent NPY secretion in PC12 cells (Fig. 5A), whereas expression of Slp4-a and Slp3-a had opposite effects on NPY secretion (Fig. 5B). These effects should be Rab27A-dependent because Slp mutants incapable of Rab27A binding had no effect on NPY secretion (Fig. 6B). The opposite effects of Slp4-a and Slp3-a may be attributable to the different biochemical properties of their C2 domains (i.e. the Ca2+-independent phospholipid binding activity of Slp4-a (4) versus the Ca2+-dependent phospholipid binding activity of Slp3-a (40)) because Ca2+-dependent type Syt I and Ca2+-independent type Syt IV were found to have a positive effect and inhibitory effect, respectively, on transmitter secretion (41–43). While this manuscript was being reviewed, an inhibitory effect of Slp4-a on dense-core vesicle exocytosis has also been reported in pancreatic β-cell lines (44), strongly supporting our finding that the Slp4-a–Rab27A complex controls dense-core vesicle exocytosis.

Although the function of Slp4-a–Rab8 in PC12 cells is unknown, we speculate that Slp4-a–Rab8 may have a function different from regulated secretion, in which Slp4-a–Rab27A plays a major role. One possible function of Slp4-a–Rab8 may be transport of dense-core vesicles (from the trans-Golgi network to the cell periphery) because Rab8 has been shown to regulate polarized membrane traffic (from the trans-Golgi network to the basolateral plasma membrane) in epithelial cells (45). The function of Slp4-a–Rab8 complex is now under investigation in our laboratory.

In summary, this study is the first to demonstrate involvement of the Slp family and Rab27A in dense-core vesicle exocytosis in PC12 cells. It will be interesting to determine which Slps function in granule exocytosis in cytotoxic T lymphocytes as well as platelets, where granule exocytosis is known to be defective in Rab27A mutant animals (17, 18, 20, 21).

REFERENCES
1. Fukuda, M., and Mikoshiba, K. (2001) Biochem. Biophys. Res. Commun. 281, 1226–1233
2. Fukuda, M., Saegusa, C., and Mikoshiba, K. (2001) Biochem. Biophys. Res. Commun. 283, 513–519
3. McAdara Berkowitz, J. K., Catz, S. D., Johnson, J. L., Ruedi, J. M., Thon, V., and Babior, B. M. (2001) J. Biol. Chem. 276, 18855–18862
