Slp4-a/Granuphilin-a Inhibits Dense-core Vesicle Exocytosis through Interaction with the GDP-bound Form of Rab27A in PC12 Cells

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

Slp4-a (synaptotagmin-like protein 4-a)/granuphilin-a is specifically localized on dense-core vesicles in PC12 cells and negatively controls dense-core vesicle exocytosis through specific interaction with Rab27A via the N-terminal Slp homology domain (SHD) (Fukuda, M., Kanno, E., Saegusa, C., Ogata, Y., and Kuroda, T. S. (2002) J. Biol. Chem. 277, 39673–39678). However, the mechanism of the inhibition by Slp4-a has never been elucidated at the molecular level and is still a matter of controversy. In this study, I discovered an unexpected biochemical property of Slp4-a, that Slp4-a, but not other Rab27 effectors reported thus far, is capable of interacting with both Rab27A(T23N), a dominant negative form that mimics the GDP-bound form, and Rab27A(Q78L), a dominant active form that mimics the GTP-bound form, whereas Slp4-b specifically recognizes the GDP-bound form of Rab3A and Rab8A and does not recognize their GDP-bound form. I show by deletion and mutation analyses that the TGDWFY sequence in SHD1 is involved in Rab27A(Q78L) binding. I further show by immunoprecipitation and cotransfection assays that Munc18-1, but not syntaxin IA, directly interacts with the C-terminal domain of Slp4-a in a Rab27A-independent manner. Expression of Slp4-a mutants that lack Rab27A(T23N) binding activity (i.e. specific binding to Rab27A(Q78L)) completely reverses the inhibitory effect of the wild-type Slp4-a on high KCl-dependent neurotensin secretion in PC12 cells. The results strongly indicate that interaction of Slp4-a with the GDP-bound form of Rab27A, not with syntaxin IA or Munc18-1, is the primary reason that Slp4-a expression inhibits dense core vesicle exocytosis in PC12 cells.

Granuphilin-a was originally identified as a protein that is abundantly expressed on insulin-containing vesicles in pancreatic β-cells (1), and overexpression studies have shown that it modulates dense-core vesicle exocytosis in pancreatic β-cells, AtT20 cells, and PC12 cells through interaction with Rab27A (or Rab27B), one of the small GTP-binding proteins (2–5). Granuphilin-a was subsequently reported to be the fourth member of the Slp (synaptotagmin-like protein) family and was therefore renamed Slp4-a (6, 7). The Slp family consists of five members (Slp1–5) in humans and mice (1, 7–9), and a Slp protein is defined as a protein having an N-terminal Rab27-binding domain (also called the Slp homology domain (SHD)) and C-terminal tandem C2 domains (called the C2A domain and the C2B domain) (7, 10) (reviewed in Ref. 11). Since patients with human type I Griscelli syndrome and the corresponding model mice, ashen (i.e. both of which have mutations in the rab27a gene) (12, 13) exhibit defects in granule exocytosis in cytotoxic T lymphocytes (14, 15), a great deal of attention has been focused on involvement of the Slp family in granule exocytosis (5, 11, 16). However, the Slp(s) that functions in the cytotoxic T lymphocytes has never been identified. The SHD has also been found in the Slac2 (Slp homologue lacking C2 domains) family without tandem C2 domains at the C terminus (Slac2-a/melanophilin, Slac2-b, and Slac2-c/MYRIP) (17–21). Slac2 regulates melanosome transport in melanocytes through interaction with Rab27A and myosin Va (18, 22–24), and Slac2-c has been suggested to regulate retinal melanosome transport through interaction with Rab27A and myosin VIIa (20, 21).

Expression of Slp4-a (or Slp4-b/granuphilin-b, an alternatively spliced isoform of Slp4-a lacking the C2B domain) has recently been shown to inhibit regulated secretion in endocrine cells (2, 4, 5). The precise mechanism of inhibition by Slp4-a, however, is still a matter of controversy (2, 4) and has never been elucidated. In this study, I show by means of neurotensin (NPY) cotransfection assay combined with deletion, mutation, and chimeric analyses that the SHD of Slp4-a is an exception and binds both the GTP- and GDP-bound forms of Rab27A, and that the Slp4-a-GD-Rab27A complex has an inhibitory effect on high KCl-dependent NPY secretion in PC12 cells. Based on these results, I propose that binding of Slp4-a to GDP-Rab27A inhibits a specific GTP/GDP exchange cycle required for dense-core vesicle exocytosis.

MATERIALS AND METHODS

Molecular Cloning of Mouse Slac2-b, Slp5, and Munc18-1 cDNAs—The chromosome locations and genome sequences of the mouse slac2-b gene (GenBank accession number AC079869; chromosome 9) and slp5 gene (XM_141661; chromosome X) were obtained by data base searching (standard BLAST search) using a human Slac2-b sequence and Slp5 cDNA sequence, respectively, as a query (9, 10). cDNAs transcribed into the opposite strand of the gene. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received for publication, December 23, 2002, and in revised form, January 21, 2003
Published, JBC Papers in Press, February 17, 2003, DOI 10.1074/jbc.M213090200

¶ To whom correspondence may be addressed: Fukuda Initiative Research Unit, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan. Tel.: 81-48-462-4994; Fax: 81-48-462-4995; E-mail: mnfukuda@brain.riken.go.jp.
encoding the open reading frame of mouse Slac2-b and Slp5 were estimated by sequence comparison with the human Slac2-b and Slp5, respectively, and were then amplified from adult mouse lung, testis, or spleen cDNA from mouse MTC panel I (Clontech) by reverse transcriptase-PCR as described previously (25). The following oligonucleotides were used for amplification of the mouse Slac2-b and Slp5 cDNAs: 5'-CCGATCCATGACGAAAGTTCCTCAGG-3' (Slac2-b N1 primer, sense), 5'-GCGGTACCGCTCTAGCCCTG-3' (Slac2-b C2 primer, antisense), 5'-GGGAGATTGCAGGAGCTTTGCCCCAG-3' (Slac2-b N2 primer, sense), 5'-AGGCCTCGAGCCAGAAAAGG-3' (Slac2-b N3 primer, sense), 5'-TCACCTATATGAGGGGGTGTT-3' (Slac2-b SHD-3 primer, sense), 5'-GTCGAGGAGCAAGGATATCT-3' (Slac2-b SHD1 C1 primer, sense), 5'-CGTTCCACTTTCCTGTGAT-3' (Slac2-b SHD1 C2 primer, sense), 5'-AGCTCTCCTCTTGCAATT-3' (Slac2-b C3 primer, antisense), 5'-TCACTGTTCTGACCTCTTTAT-3' (Slac2-b stop primer, antisense), 5'-GGCCAGCTACATAGACTCAGGGATT-3' (Slp5 Met primer, sense), 5'-TACGCCATGATCCTCGCCCATC-3' (Slp5 ΔC2AB primer, antisense), 5'-GGATCCAGAAGTCCAGGGTCTGAAG-3' (Slp5 stop primer, antisense), 5'-CCCGCGGTGAAAGCTGGGGA-3' (Slp5 ΔHD2 primer, antisense).

The purified PCR products were subcloned into the pGEM-T Easy vector (Promega; Madison, WI), and both strands were completely sequenced as described previously (25). Full-length mouse Slac2-b or Slp5 cDNA was constructed on the pGEM-T Easy vector by using appropriate restriction enzyme sites. I identified one alternative splicing site (amino acid residues 387–408) in the mouse Slp5, and this splicing event was tissue-specific (data not shown). The addition of the T7 tag (or FLAG tag) to the N terminus of Slac2-b and Slp5 and construction of expression vectors (pEF-T7-Slac2-b, pEF-T7-Slp5, and pEF-FLAG-Munc18-1) were performed as described previously (25–28).

Construction of Deletion Mutants of the Slp4-a SHD—pEF-T7-GST (glutathione S-transferase)-Slp4-SHD1, pEF-T7-Slp4-ΔSHD1, and pEF-T7-Slp4-ΔSHD2 were essentially constructed by PCR using the following oligonucleotides with appropriate restriction enzyme sites (underlined) and/or stop codons (in boldface type) as described previously (25–27): 5'-GGCAGTACGACAGGAGCTTCATTGAGAGC-3' (Slp4-SHD1-5' primer, antisense), 5'-GGATCCACGAGCAGAAAGAAGA-3' (Slp4-SHD1-3' primer, sense), 5'-GCCACTAGTCATGCCCCAGCACCACCT-3' (Slp4-ΔSHD1 primer, sense), 5'-GCCATAGTCATGCCCCAGCACCACCT-3' (Slp4-ΔSHD2 primer, sense), and 5'-GCCACTAGTCATGCCCCAGCACCACCT-3' (Slp4-ΔSHD3 primer, sense). Other expression constructs (pEF-T7-Slp5s, pEF-T7-Slac2s, pEF-T7-Rabphilin, pEF-FLAG-Rabs, pEF-FLAG-sytIα, pEF-FLAG-snap-25 (synaptosom-associated protein of 25 kDa), and pEF-FLAG-VAMP-2 (vesicle-associated membrane protein-2) were prepared as described previously (9, 10, 21, 29).

Site-directed Mutagenesis and Construction of a Chimera between Slp3-a and Slp4-a—Mutant Slp4-a plasmids carrying a TGGWY-to-AGAAAY substitution at amino acid positions 115–120 (named Slp4-a(A4)) or a C102A/C105A substitution were obtained by two-step PCR techniques using the following oligonucleotides with an artificial A/H1 site (underlined) as described previously (30): 5'-CTTAAGGCTCTATCTCTTCCCTGC-3' (Slp4-a(A4) 3' primer, antisense), 5'-CTTAAGGCTCTATCTCTTCCCTGC-3' (Slp4-a(A4) 5' primer, sense), 5'-CTTAAGGCTCTATCTCTTCCCTGC-3' (Slp4-a(C102A/C105A) 3' primer, antisense), 5'-CTTAAGGCTCTATCTCTTCCCTGC-3' (Slp4-a(C102A/C105A) 5' primer, sense), and 5'-CTTAAGGCTCTATCTCTTCCCTGC-3' (Slp4-a(C102A/C105A) 5' primer, sense). Other expression constructs (pEF-T7-Slp5s, pEF-T7-Slac2s, pEF-T7-Rabphilin, pEF-FLAG-Rabs, pEF-FLAG-sytIα, pEF-FLAG-snap-25 (synaptosos-associated protein of 25 kDa), and pEF-FLAG-VAMP-2 (vesicle-associated membrane protein-2) were prepared as described previously (9, 10, 21, 29).

Site-directed Mutagenesis and Construction of a Chimera between Slp3-a and Slp4-a—Mutant Slp4-a plasmids carrying a TGGWY-to-AGAAAY substitution at amino acid positions 115–120 (named Slp4-a(A4)) or a C102A/C105A substitution were obtained by two-step PCR techniques using the following oligonucleotides with an artificial A/H1 site (underlined) as described previously (30): 5'-CTTAAGGCTCTATCTCTTCCCTGC-3' (Slp4-a(A4) 3' primer, antisense), 5'-CTTAAGGCTCTATCTCTTCCCTGC-3' (Slp4-a(A4) 5' primer, sense), 5'-CTTAAGGCTCTATCTCTTCCCTGC-3' (Slp4-a(C102A/C105A) 3' primer, antisense), 5'-CTTAAGGCTCTATCTCTTCCCTGC-3' (Slp4-a(C102A/C105A) 5' primer, sense), and 5'-CTTAAGGCTCTATCTCTTCCCTGC-3' (Slp4-a(C102A/C105A) 5' primer, sense). Other expression constructs (pEF-T7-Slp5s, pEF-T7-Slac2s, pEF-T7-Rabphilin, pEF-FLAG-Rabs, pEF-FLAG-sytIα, pEF-FLAG-snap-25 (synaptosos-associated protein of 25 kDa), and pEF-FLAG-VAMP-2 (vesicle-associated membrane protein-2) were prepared as described previously (9, 10, 21, 29).

Site-directed Mutagenesis and Construction of a Chimera between Slp3-a and Slp4-a—Mutant Slp4-a plasmids carrying a TGGWY-to-AGAAAY substitution at amino acid positions 115–120 (named Slp4-a(A4)) or a C102A/C105A substitution were obtained by two-step PCR techniques using the following oligonucleotides with an artificial A/H1 site (underlined) as described previously (30): 5'-CTTAAGGCTCTATCTCTTCCCTGC-3' (Slp4-a(A4) 3' primer, antisense), 5'-CTTAAGGCTCTATCTCTTCCCTGC-3' (Slp4-a(A4) 5' primer, sense), 5'-CTTAAGGCTCTATCTCTTCCCTGC-3' (Slp4-a(C102A/C105A) 3' primer, antisense), 5'-CTTAAGGCTCTATCTCTTCCCTGC-3' (Slp4-a(C102A/C105A) 5' primer, sense), and 5'-CTTAAGGCTCTATCTCTTCCCTGC-3' (Slp4-a(C102A/C105A) 5' primer, sense). Other expression constructs (pEF-T7-Slp5s, pEF-T7-Slac2s, pEF-T7-Rabphilin, pEF-FLAG-Rabs, pEF-FLAG-sytIα, pEF-FLAG-snap-25 (synaptosos-associated protein of 25 kDa), and pEF-FLAG-VAMP-2 (vesicle-associated membrane protein-2) were prepared as described previously (9, 10, 21, 29).
with 50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 0.2% Triton X-100, and Slp4-a-C2B IgG (5) or control rabbit IgG (20). After homogenization of PC12 cells, the supernatant was incubated with either anti-Slp4-a (Slp4-a antibody, 1:1000 dilution; Transduction Laboratories), or anti-Slp4-a antibody (1:25 dilution; Transduction Laboratories) for 1 h at 4°C. Released NPY-T7-GST was recovered by incubation with glutathione-Sepharose beads and analyzed by immunoblotting with horseradish peroxidase (HRP)-conjugated anti-T7 tag antibody (Novagen, Madison, WI). The intensity of the immunoreactive bands on x-ray film was quantified by Lane Analyzer (version 3.0) (ATTO Corp., Tokyo, Japan) and normalized by total expressed NPY-T7-GST.

**Miscellaneous Procedures**—T7-tagged Slp4-a mutants, HA-tagged Rab27A, and/or various FLAG-tagged proteins (Rab3A, Rab5A, Rab27A, syntaxin IA, SNAP-25, VAMP-2, or Munc18-1) were coexpressed in COS-7 cells (32), and associations between these proteins were evaluated by immunoprecipitation as described previously (18, 25). The amounts of pEF-T7-Slp, pEF-T7-Slac2, or pEF-BOS, a vector control by using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer’s notes (31). Since the expression levels of recombinant T7-tagged proteins varied among the Slp (or Slac2) family, the amounts of pEF-T7-Slp (or Slac2) plasmids used for transfection were varied so that similar amounts of T7-tagged proteins would be expressed in total cell lysates of PC12 cells. Three days after transfection, cells were stimulated with high KCl buffer (56 mM KCl, 95 mM NaCl, 2.2 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, and 15 mM HEPES-KOH, pH 7.4) for 10 min at 37°C. Released NPY-T7-GST was recovered by incubation with glutathione-Sepharose beads and analyzed by immunoblotting with horseradish peroxidase (HRP)-conjugated anti-T7 tag antibody (Novagen, Madison, WI). The intensity of the immunoreactive bands on x-ray film was quantified by Lane Analyzer (version 3.0) (ATTO Corp., Tokyo, Japan) and normalized by total expressed NPY-T7-GST.

NPY Release Assay—NPY-T7-GST secretion assay in PC12 cells was essentially performed as described previously (5, 31). Briefly, PC12 cells (6-cm dish) were cotransfected with pShooter-NPY-T7-GST and pEF-T7-Slp, pEF-T7-Slac2, or pEF-BOS, a vector control by using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer’s notes (31). Since the expression levels of recombinant T7-tagged proteins varied among the Slp (or Slac2) family, the amounts of pEF-T7-Slp (or Slac2) plasmids used for transfection were varied so that similar amounts of T7-tagged proteins would be expressed in total cell lysates of PC12 cells. Three days after transfection, cells were stimulated with high KCl buffer (56 mM KCl, 95 mM NaCl, 2.2 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, and 15 mM HEPES-KOH, pH 7.4) for 10 min at 37°C. Released NPY-T7-GST was recovered by incubation with glutathione-Sepharose beads and analyzed by immunoblotting with horseradish peroxidase (HRP)-conjugated anti-T7 tag antibody (Novagen, Madison, WI). The intensity of the immunoreactive bands on x-ray film was quantified by Lane Analyzer (version 3.0) (ATTO Corp., Tokyo, Japan) and normalized by total expressed NPY-T7-GST.

**Functional Analysis of Slp4-a in Dense-core Vesicle Exocytosis**

**Fig. 2. GTP-dependent binding of Slps and Slac2s to Rab27A.** A, Rab27A(T23N), a dominant negative form that mimics the GDP-bound form, interacts with Slp4-a but not other Slps, Slac2s, and rabphilin (asterisk). Note that, with the exception of Slp4-a, all of the proteins bound Rab27A in a GTP-dependent manner (i.e. Rab27A(Q78L)) (lane 1). B, GTP-dependent interaction of Slp4-a and rabphilin with Rab3A and Rab8A. T7-Slps (T7-Slps or T7-Rabphilin) and FLAG-Rab were coexpressed in COS-7 cells, and their associations were determined by co-immunoprecipitation assay as described previously (10, 18). Co-immunoprecipitated FLAG-Rab and immunoprecipitated T7-tagged proteins were visualized with HRP-conjugated anti-FLAG tag antibody (Blot: anti-FLAG) and HRP-conjugated anti-T7 tag antibody (Blot: anti-T7), respectively. Input, one-eighthieth volume of the reaction mixture used for immunoprecipitation (top panels). The positions of the molecular mass markers (×10⁻³) are shown on the left.
this paper are representative of at least two or three independent experiments.

RESULTS

The Slp Homology Domain of Slp4-a Interacts with Both GDP- and GTP-bound Forms of Rab27A—The SHD basically consists of two α-helical regions (named SHD1 and SHD2), separated by two zinc finger motifs (Fig. 1A), and SHD1 alone (but not SHD2 and the zinc finger motifs) functions as an autonomous specific GTP-dependent Rab27A/B-binding site in Slac2-a (33). The only exception is the Slp4-a SHD, because it can also interact with Rab3A and Rab8A (4, 5, 10, 22). Why and how the Slp4-a SHD recognizes Rab3A and Rab8A in addition to Rab27A, however, had never been elucidated, although this information is important to understanding the physiological function of the Rab3A-Slp4-a and the Rab8A-Slp4-a complex. Deletion and mutation analyses clearly showed that three subdomains of the Slp4-a SHD (SHD1, SHD2, and zinc finger motifs) contribute differently to the recognition of Rab3A, Rab8A, and Rab27A (Fig. 1, A and B). The SHD1 of Slp4-a (i.e. GST-SHD1) functions as a specific Rab3A-binding sitesite, the same as the Slac2-a SHD (33), whereas the SHD1 + zinc finger motifs (i.e. ΔSHD2) function as a Rab3A-binding site, and the whole SHD is required for recognition of Rab8A. Since deletion of the SHD1 of Slp4-a (i.e. ΔSHD1) completely abrogated binding activity toward all three Rabs (Fig. 1B), the zinc finger motifs and the SHD2 of Slp4-a themselves are not an autonomous Rab binding domain, the same as the Slac2-a SHD (33).

Next, I investigated the GTP/GDP dependence of the Rab binding activity of the Slp4-a deletion mutants. To my surprise, the entire SHD of Slp4-a bound both the dominant active form (Q78L; mimics GTP-bound state) and the dominant negative form of Rab27A (T23N; mimics GDP-bound form), despite the fact that the SHD1 alone (or the ΔSHD2) recognizes only the dominant active form (Fig. 1C, compare lanes 5 and 6). By contrast, however, the Slp4-a SHD specifically recognized the GTP-bound form of both Rab3A and Rab8A (i.e. dominant active form) but not their GDP-bound forms (i.e. dominant negative forms), the same as rabphilin (Figs. 1C (lanes 1–4) and 2B).

The interaction between SHD and Rab27A(T23N) was found to be a unique event in Slp4-a (asterisk in Fig. 2A), because, except for Slp4-a, all of the Rab27-binding proteins reported thus far specifically recognize Rab27A(Q78L) and do not recognize Rab27A(T23N) (Fig. 2A, compare lanes 1 and 2). Based on the results of deletion analysis of the Slp4-a SHD, I hypothesized that the SHD1 alone can function as a GTP-Rab27A-binding site, the same as the Slac2-a SHD1 (i.e. RBD27, Rab-binding domain specific for Rab27 isoforms) (33), that the SHD2 is involved in an auxiliary GDP-Rab27A binding, because the SHD2 alone does not recognize Rab27A at all, and that the zinc finger motifs are not necessary for Rab27A recognition. To verify this hypothesis, I performed Ala-based site-directed mutagenesis (Fig. 3A). When one of the conserved amino acids among the SHD1 of the Slp and Slac2 families (Glu-14, Ile-18, Val-21, or Asp-32 of Slp4-a) (5, 33) was replaced by Ala, the mutant Slp4-a SHD1 (T7-GST-Slp4-SHD1) completely abolished Rab27A binding activity (Fig. 3B, lanes 2–5), indicating that the Slp4-a SHD1 is critical for GTP-Rab27A binding activity of each Slp4-a mutant is indicated (− or +). ND, not determined. B, effect of the single amino acid substitution in the SHD1 of Slp4-a on Rab27A binding activity (see the arrowheads in A). C, effect of the mutations in the zinc finger motifs (C102A/C105A) or the TGDWFY motif in the SHD2 of Slp4-a on Rab27A binding. Note that the Slp4-a-SHD(A4) and Slp4-a(ΔSHD) carrying the SHD of Slp3-a specifically bound the GTP-bound form of Rab27A(Q78L) and did not bind the GDP-bound form of Rab27A(T23N) (lanes 1, 2, 5, and 6). T7-Slp4-a mutants and FLAG-Rab27A were coexpressed in COS-7 cells, and their associations were determined by co-immunoprecipitation assay as described previously (10, 18). Co-immunoprecipitated FLAG-Rab and immunoprecipitated T7-Slp4-a mutants were visualized with HRP-conjugated anti-FLAG tag antibody (Blot: anti-FLAG, middle panels) and HRP-conjugated anti-T7 tag antibody (Blot: anti-T7, bottom panels), respectively. Input, one-eighthieth volume of the reaction mixture used for immunoprecipitation (top panels). The positions of the molecular mass markers (×10–7) are shown on the left.
expression of the mouse Slp or Slac2 on high KCl-dependent NPY secretion in PC12 cells. Note that only Slp4-a expression significantly inhibited high KCl-dependent NPY secretion (cross-hatched bar in A). B, effect of expression of Slp4-a mutants on high KCl-dependent NPY secretion in PC12 cells. Note that the Slp4-a mutants lacking the Rab27A(T23N) binding activity had no effect on high KCl-dependent NPY secretion in PC12 cells (hatched bars in B). The NPY-T7-GST secretion assay was performed as described previously (31). The results are expressed as percentages of NPY-T7-GST secretion in control samples (control, closed bar) without expression of recombinant proteins. Bars, means ± S.E. of three determinations. The results shown are representative of at least three independent experiments. **, p < 0.01; *, p < 0.05, Student’s unpaired t test. Insets in A and B show expressed recombinant proteins visualized with HRP-conjugated anti-T7 tag antibody. Similar amounts of the wild-type and mutant Slp4-a were detected in the total cell lysates by immunoblotting (data not shown), indicating that the lack of effects of the Slp4-a mutants is not attributable to lower protein expression levels.

Binding, the same as the Slac2-a SHD1 (33). When the TGD-WFY sequence in the SHD2 was mutated to AGAAAY, the mutant Slp4-a(A4) specifically bound Rab27A(Q78L) but did not bind Rab27A(T23N) (Fig. 3C, lanes 1 and 2). By contrast, mutation of the zinc finger motifs (C102A/C105A) of Slp4-a had no effect on interaction with either Rab27A(T23N) or Rab27A(Q78L) (Fig. 3C, lanes 3 and 4) but has been shown to reduce the Rab3A binding activity (4). These results indicate that the SHD1 and the SHD2 of Slp4-a are involved in recognition of GTP-Rab27A and GDP-Rab27A, respectively.

Slp4-a, but Not Other Slps and Slac2s, Inhibits Dense-core Vesicle Exocytosis in PC12 Cells—Since appropriate GTP/GDP exchange (or GTPase activity) of Rab proteins is generally believed to be essential for expression of their function and Rab effectors are also generally believed to bind the GTP-bound, activated form of Rab proteins, I hypothesized that binding of Slp4-a to the GDP-bound form of Rab27A is responsible for the inhibition of dense-core vesicle exocytosis in endocrine cells (2, 4, 5). Consistent with this hypothesis, expression of other Slps and Slac2s that specifically interact with the GTP-bound form of Rab27A did not inhibit high KCl-dependent NPY secretion in PC12 cells (Fig. 4A, shaded bars); only the Slp4-a expression significantly inhibited high KCl-dependent NPY secretion (Fig. 4A, cross-hatched bar). Expression of Slp3-a and Slp5, Ca2+-dependent type Slp (5, 6, 9, 16), significantly promoted high KCl-dependent NPY secretion, and promotion by Slp3-a was always greater than by Slp5 expression. Expression of Slp1 and Slp2-a slightly stimulated high KCl-dependent NPY secretion, but their effect was not statistically significant under our experimental conditions. Expression of Slac2-a, Slac2-b, and Slac2-c, on the other hand, had no effect at all. Since, with the exception of Slac2-b, the expression levels of the recombinant proteins did not differ much (Fig. 4A, insets), the effects observed in Fig. 4A were unlikely to be attributable to differences in protein expression levels. Expression of Slac2-b protein was difficult to detect in total cell lysates (Fig. 4A, inset, lane 8), and as a result the effect of Slac2-b expression on NPY secretion may have been underestimated. Since similar expression levels of NPY were observed both in Slp4-a (or Slp3-a)-transfected cells and in nontransfected cells and the Slp4-a (or Slp3-a) expression had no significant effect on low KCl-dependent NPY secretion (Refs. 4 and 5 and data not shown), both Slp4-a and Slp3-a are likely to be involved in a late step of dense-core vesicle exocytosis (i.e. postdocking steps) rather than dense-core vesicle biogenesis, transport step, or docking step. Consistent with this, Rab27A has been shown to be required for a late step in granule exocytosis in cytotoxic T-lymphocytes (14, 15).

To further verify my hypothesis, I expressed loss-of-function type Slp4-a mutants that interact only with the GTP-bound form of Rab27A and not the GDP-bound form of Rab27A, in PC12 cells (Fig. 3C, lanes 1, 2, 5, and 6), and I tested their effect on NPY secretion. As expected, expression of Slp4-a(A4) or Slp4-a(A3-SHD), a chimera between Slp3-a and Slp4-a, had no significant effect on NPY secretion (Fig. 4B, hatched bars).
cells, where neuronal SNARE-related proteins are not endogenously expressed (29). As shown in Fig. 6C (lanes 4 and 8), Munc18-1 interacted with Slp4-a irrespective of the presence of Rab27A, but syntaxin IA did not interact with Slp4-a even in the presence of Rab27A under my experimental conditions (lanes 1 and 5). SNAP-25 and VAMP-2 also did not interact with Slp4-a, consistent with the immunoprecipitation experiments described above. Similar results were obtained for Slp3-a, but the interaction of Slp3-a with Munc18-1 was relatively weak. I also mapped the Munc18-1 binding site to the C-terminal domain of Slp4-a, but not the N-terminal SHD (Fig. 6D, lanes 3 and 6 in the third panel), consistent with the fact that the Slp4-a-Munc18-1 interaction is Rab27A-independent. The interaction between Slp4-a and Munc18-1 should be direct, because purified components formed a complex even in vitro (Fig. 6E).

DISCUSSION

Since the effectors for Rab small GTP-binding proteins are generally believed to bind the GDP-bound, activated form of Rab and not the GDP-bound, inactivated form, my finding that Slp4-a interacts with the dominant negative form of Rab27A(T23N) (mimics the GDP-bound form) was unexpected and surprising, with other Rab27 effectors (Slp1, Slp2-a, Slp3-a, Slp5, Slac2-a, Slac2-b, Slac2-c, and rabphilin) only recognizing the dominant active form of Rab27A(Q78L) (Figs. 1 and 2). It should be noted that binding of Slp4-a to GDP-Rab is specific to Rab27A but not to Rab3A and Rab8A, indicating that Slp4-a recognizes Rab3A, Rab8A, and Rab27A in a different fashion. Consistent with this notion, the SHD1, zinc finger motifs, and SHD2 of Slp4-a contribute differently to Rab3A, Rab8A, and Rab27A recognition. The SHD1 of Slp4-a functions as a specific GTP-Rab27A binding site, whereas the SHD2 (i.e. TGDWFY motif) is involved in GDP-Rab27A recognition, although the SHD2 alone is not an autonomous Rab27A-binding site. Recognition of Rab3A by Slp4-a requires the SHD1 and zinc finger motifs, whereas recognition of Rab8A by Slp4-a requires the whole SHD. Despite sharing a similar (T/S)(G/L)WF(Y/F) motif in the SHD2 of other Slps and Slac2s (7, 10), they are unable to interact with Rab27A(T23N). Why only Slp4-a recognizes Rab27A(T23N) is currently unknown. The nonconserved sequences around the TGDWFY motif of the Slp4-a SHD2 may also be involved in Rab27A(T23N) recognition, and three-dimensional structural analysis of the Slp4-a-Rab27a complex will be necessary to answer this question.

Since I also found that the Slp4-a mutants that specifically interacted with GTP-Rab27A had no effect on dense-core vesicle exocytosis in PC12 cells (Fig. 4B) and since expression of Rab27A(T23N) or Rab27A(N133I) (mimics GDP-bound form) in melanocytes has been shown to inhibit melanosome transport from the perinuclear region to cell periphery (i.e. melanosomes accumulate in the perinuclear region) (36, 37), the most straightforward explanation of the inhibitory effect of the wild-

...
type Slp4-a expression on dense-core vesicle exocytosis is that Slp4-a interacts with GDP-bound form of Rab27A. Trapping of the GDP-bound form of Rab27A by Slp4-a may reduce the availability of the GTP-bound form of Rab27A that participates in enhancement of dense-core vesicle exocytosis, although I cannot completely rule out the possibility that interaction of Slp4-a with Munc18-1 (or syntaxin IA) also exerts an inhibitory effect on dense-core vesicle exocytosis (see below).

Very recently, Slp4-a has been shown to interact with a closed conformation of syntaxin IA in vitro, and this interaction has been suggested to inhibit insulin secretion in pancreatic β cells (2). Although small amounts of syntaxin I and Munc18-1 were co-purified with Slp4-a even in PC12 cells, the cotransfection assay in COS-7 cells in the present study clearly showed that syntaxin IA does not directly interact with Slp4-a even in the presence of Rab27A, whereas Munc18-1 interacts with the C-terminal domain of Slp4-a independent of Rab27A (Fig. 6). The most likely explanation for the apparent discrepancy is that syntaxin IA indirectly binds Slp4-a through interaction with Munc18-1, because Munc18-1 is known to directly bind syntaxin IA (38–40) and the Munc18-1-syntaxin IA interaction was prominent even in the cotransfection assay in COS-7 cells (data not shown). Alternatively, the co-immunoprecipitated syntaxin detected with anti-HPC-1 (anti-syntaxin I) may correspond to other syntaxin isoforms (e.g. syntaxin IB), because the anti-HPC-1 antibody also recognizes other syntaxin isoforms. Since I have already shown that the C-terminal portion of Slp4-a (i.e. Slp4-a-ASHD) that interacts with Munc18-1 failed to inhibit NPY secretion in PC12 cells (5), I concluded that the SHD of Slp4-a is the primary reason for the inhibitory effect on dense-core vesicle exocytosis.

In summary, I have discovered that Slp4-a is an exception and interacts with both the GTP- and GDP-bound forms of Rab27A and that the SHD1 and SHD2 of Slp4-a contribute to GTP- and GDP-Rab27A recognition in a different manner. I also found a strong correlation between the GDP-Rab27A binding activity of Slp4-a and the inhibitory effect of Slp4-a on dense-core vesicle exocytosis in PC12 cells. Based on these findings, I propose that expression of Slp4-a inhibits dense-core vesicle exocytosis (possibly postdocking steps) through interaction with the GDP-bound form of Rab27A. Since Slp4-a interacts only with the GTP-bound form of Rab8A and Rab8A, it is possible that Rab8A/Slp4-a and Rab8A/Slp4-a complexes positively control dense-core vesicle exocytosis, although the proportions of these complexes are much smaller than that of Rab27A/Slp4-a complex in PC12 cells (5). Further work is necessary to elucidate this issue.

REFERENCES


38. M. Fukuda, unpublished data.
Slp4-a/Granuphilin-a Inhibits Dense-core Vesicle Exocytosis through Interaction with the GDP-bound Form of Rab27A in PC12 Cells

Mitsunori Fukuda

doi: 10.1074/jbc.M213090200 originally published online February 17, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M213090200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2003/03/24/M213090200.DC1.html

This article cites 40 references, 26 of which can be accessed free at
http://www.jbc.org/content/278/17/15390.full.html#ref-list-1