Slp4-a/Granuphilin-a Interacts with Syntaxin-2/3 in a Munc18-2-dependent Manner*§

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Slp4-a/granuphilin-a was originally described as a protein specifically associated with insulin-containing granules in pancreatic β-cells, but it was subsequently found to be present on amylase-containing granules in parotid acinar cells. Although Slp4-a has been suggested to control insulin secretion through interaction with syntaxin-1a and/or Munc18-1, nothing is known about the binding partner(s) of Slp4-a during amylase release from parotid acinar cells, which do not endogenously express either syntaxin-1a or Munc18-1. In this study we systematically investigated the interaction between syntaxin-1–5 and Munc18-1–3 by co-immunoprecipitation assay using COS-7 cells and discovered that Slp4-a interacts with a closed conformation of syntaxin-2/3 in a Munc18-2-dependent manner, whereas Munc18-2 itself hardly interacts with Slp4-a at all. By contrast, Slp4-a was found to strongly interact with Munc18-1 regardless of the presence of syntaxin-2/3, and syntaxin-2/3 co-immunoprecipitated with Slp4-a only in the presence of Munc18-1–2. Deletion analysis showed that the syntaxin-2/3 (or Munc18-1-2)-binding site is a linker domain of Slp4-a (amino acid residues 144–354), a previously uncharacterized region located between the N-terminal Rab27A binding domain and the C2A domain. We also found that the Slp4-a-syntaxin-2 complex is actually present in rat parotid glands and that introduction of the antibody against Slp4-a linker domain into streptolysin O-permeabilized parotid acinar cells severely attenuates isoproterenol-stimulated amylase release, possibly by disrupting the interaction between Slp4-a and syntaxin-2/3 (or Munc18-2). These results suggest that Slp4-a modulates amylase release from parotid acinar cells through interaction with syntaxin-2/3 on the apical plasma membrane.

Small GTPase Rab27A is expressed in a wide variety of secretory cells (1) and has been suggested to control secretion by these cells through interaction with cell type- or tissue-specific Rab27A effectors (for review, see Refs. 2–4). To date three distinct groups of Rab27A-binding proteins have been reported in humans and mice (4). The first group includes the members of the syntaptotagmin-like protein (Slp) family (Slp–5) and rabphilin (5–12), all of which contain an N-terminal Rab27A binding domain (also called Slp homology domain (SHD)) and C-terminal tandem C2 domains that potentially bind phospholipids (5, 13, 14). The second group of Rab27A-binding proteins includes the members of the Slac2 family (Slac2-a–c) and Noc2, all of which contain an N-terminal Rab27A-binding domain but lack tandem C2 domains (6, 7, 11, 15–18). Both Slac2-a–melanophilin and Slac2-c–MyRIP contain a myosin binding domain at the middle of the molecule (10, 19–23) and an actin binding domain at the C terminus (17, 22). The last Rab27A-binding protein identified is Munc13-4, a putative priming factor for exocytosis (24, 25).

Although Rab27A has been shown to be involved in the control of hormone secretion by endocrine cells through interaction with Slp4-a (9, 26–29), rabphilin (11), Noc2 (11, 18), and/or Slac2-c (30, 31) and of secretion by certain immune cells through interaction with Munc13-4 (24, 25), very little is known about the expression and function of Rab27A effectors in exocrine tissues. We have recently found that Slp4-a and Slac2-c are localized on amylase-containing granules in rat parotid acinar cells and that the latter protein is involved in amylase release from acinar cells through interaction with Rab27B, a closely related isoform of Rab27A, and actin filaments (32). Although Slp4-a has been suggested to promote docking of dense-core vesicles with the plasma membrane of pancreatic β-cell lines through interaction with syntaxin-1a (26, 27) and/or Munc18-1 (29, 33), Slp4-a must be involved in the control of amylase release in a manner different from its involvement in hormone secretion because neither syntaxin-1a nor Munc18-1 is expressed in rat parotid glands (34, 35). Because other syntaxin isoforms (e.g. syntaxin-2–4) and other Munc18 isoforms (e.g. Munc18-2–3) are expressed in the rat parotid gland and have been suggested to control amylase release (34–37), Slp4-a may regulate amylase release through interaction with these syntaxins and/or Munc18s. However, these possibilities have never even been investigated in vitro.

In this study we systematically investigated the interaction between syntaxin-1–5 and Munc18-1–3 in vitro and found that Slp4-a interacts with syntaxin-2/3 (specifically the closed conformation of syntaxin-2/3) only in the presence of Munc18-2 and not with syntaxin-2/3 alone or Munc18-2 alone. By contrast, Slp4-a interacts with Munc18-1 regardless of the presence of syntaxin-1a (29) or syntaxin-2/3. Systematic deletion analysis further showed that syntaxin-2/3 (or Munc18-1-2) binds the linker domain of Slp4-a (amino acid residues 144–354) located between the SHD and the C2A domain. We also found that Slp4-a-syntaxin-2 complex is actually present in rat parotid glands and that introduction of the recombinant linker domain of Slp4-a or the antibody against the Slp4-a linker domain (i.e. anti-Slp4-a-linker antibody) into streptolysin O (SLO)-permeabilized parotid acinar cells severely attenuates isoproterenol (I PR)-stimulated amylase release. Based on these results, we propose that Slp4-a is a novel regulator of the closed conformation of syntaxin-2/3 in amylase release from parotid acinar cells.
Munc18-2-dependent Interaction between Slp4-a and Syntaxin-2/3

EXPERIMENTAL PROCEDURES

Materials—Anti-syntaxin-2 rabbit polyclonal antibody was obtained from Synaptic Systems (Göttingen, Germany). Anti-Munc18 and anti-syntaxin-3 rabbit polyclonal antibodies were obtained from Merck Biosciences Calbiochem. Anti-T7 tag antibody-conjugated agarose and horseradish peroxidase (HRP)-conjugated anti-T7 tag mouse monoclonal antibody were from Merck Biosciences Novagen. HRP-conjugated anti-glutathione S-transferase (GST) antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-FLAG M2 affinity gel, HRP-conjugated anti-FLAG M2 mouse monoclonal antibody, and HRP-conjugated anti-HA tag mouse monoclonal antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-FLAG M2 affinity gel, maxiprep kits according to the manufacturer’s notes.

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Co-immunoprecipitation Assay in COS-7 Cells—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin at 37 °C under 5% CO2. pEF-T7, pEF-FLAG, and/or pEF-HA vectors (a total of 4 μg of plasmids) were transfected into COS-7 cells (7.5 × 105 cells, the day before transfection/10-cm dish) by using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s notes. Three days after transfection cells were harvested and homogenized, and total cell lysates were prepared as described previously (39, 41, 44). The total cell lysates (400 μl) were incubated with either anti-T7-tag antibody-conjugated agarose beads or anti-FLAG M2 affinity gel (v/v) with gentle agitation at 4 °C for 1 h, and the proteins bound to the beads were analyzed on 10% SDS-PAGE followed by immunoblotting with HRP-conjugated anti-T7-tag antibody (1/10,000 dilution), HRP-conjugated anti-FLAG M2 antibody (1/10,000 dilution), and/or HRP-conjugated anti-HA tag antibody (1/10,000 dilution) as described previously (39). The immunoreactive bands were visualized by means of enhanced chemiluminescence (Amersham Biosciences).

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at 4 °C. After washing the beads 5 times with 50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 1 mM MgCl₂, 0.2% Triton X-100, and protease inhibitors, the proteins bound to the beads were analyzed by 10% (or 7.5%) SDS-PAGE followed by immunoblotting with anti-syntaxin-2 (1/500 dilution), anti-syntaxin-3 (1/250 dilution), anti-Munc18 (1/250 dilution), anti-Rab27B (6 μg/ml), and anti-Slp4-a-SHD rabbit polyclonal antibodies (2.6 μg/ml). SDS-PAGE and the immunoblot analysis were performed as described previously (32, 39). The immunoreactive bands were visualized by means of enhanced chemiluminescence.

GST Pull-down Assay—T7-GST-Slp4-a-linker was expressed in COS-7 cells as described above, and the expressed GST fusion proteins were affinity-purified on glutathione-Sepharose beads (wet volume 20 ml) as described previously (44). The GST-Slp4-a-linker beads or beads coupled with GST alone as a control were incubated with ~100 μl of total lysates of rat parotid glands (10 mg/ml; see above). After washing the beads 3 times with 50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 1 mM MgCl₂, 0.2% Triton X-100, and protease inhibitors, proteins bound to the beads were analyzed by 10% SDS-PAGE followed by immunoblotting with anti-syntaxin-2, anti-syntaxin-3, anti-Munc18, anti-Rab27B rabbit polyclonal antibody, and HRP-conjugated anti-GST antibody (1/10,000 dilution) as described above.

Inhibition of the Slp4-a-Syntaxin-2 Interaction by Anti-Slp4-a Linker IgG in Vitro—GST-Slp4-a-linker was expressed in bacteria and affinity-purified on glutathione-Sepharose beads as described previously (44). New Zealand White rabbits were immunized with the purified GST-Slp4-a-linker proteins, and anti-Slp4-a-linker IgG was affinity-purified as described previously (44). Antibody inhibition experiments in COS-7 cells were performed essentially as described previously (32). In brief, agarose beads coupled with T7-Slp4-a were preincubated for 30 min at 4 °C with 10 μg of anti-Slp4-a-linker IgG or control IgG and then incubated for 1 h at 4 °C with COS-7 cell lysates containing FLAG-Munc18-2 and FLAG-syntaxin-2 (or FLAG-Rab27A). Proteins bound to the beads were analyzed as described above.

Preparation of Parotid Acinar Cells and Measurement of Amylase Release from SLO-permeabilized Parotid Acinar Cells—The animal protocol obeyed Guidelines of The Nippon Dental University for the Care and Use of Laboratory Animals. Preparation of rat parotid acinar cells and permeabilization of the cells with SLO in the presence or absence of GST-Slp4-a-linker, GST alone, anti-Slp4-a-linker IgG, or control rabbit IgG were essentially performed as described previously (32). The cell suspension was stimulated with 1 μl IPR for 20 min. The reaction was stopped by the addition of 900 μl of incubation medium, and the reaction medium was immediately collected by passage through a glass filter paper. To measure the total amylase activity, the acinar cells were homogenized in 0.1% Triton X-100. The amylase activity was then measured according to the method described by Bernfeld (45).

RESULTS

Slp4-a Interacts with Syntaxin-2/3 in a Munc18-2-dependent Manner—In our previous studies we showed that Slp4-a directly interacts with Munc18-1 in PC12 cells (29) and that Slp4-a is also expressed in exocrine parotid acinar cells (32), which do not endogenously express either Munc18-1 or syntaxin-1a (34, 35). These results have suggested that Slp4-a interacts with other Munc18 isoforms or other syntaxin isoforms in parotid acinar cells and regulates amylase release (4). Because rat parotid glands express two Munc18 isoforms (i.e. Munc18-2 and Munc18-3) and at least four syntaxin isoforms (i.e. syntaxin-2–5) (34–37), we first cloned their cDNAs and systematically tested for an interaction between T7-tagged Munc18s and FLAG-tagged syntaxins by co-immunoprecipitation assay using COS-7 cells (Fig. 1). Consistent with previous reports by other groups (46–48), Munc18-1 and Munc18-2 interacted with syntaxin-1a, -2, and -3 but not with syntaxin-4 or -5 (lanes 1 and 2 in Fig. 1), whereas Munc18-3 strongly interacted with syntaxin-4, weakly with syntaxin-1a and -2, and not at all with syntaxin-3 or -5 (lane 3 in Fig. 1). We therefore decided to focus on the Munc18-2-syntaxin-3/2 complex and Munc18-3-syntaxin-4 complex for subsequent analysis of the function of Slp4-a in parotid glands.

In the first set of experiments we investigated the interaction between Slp4-a and Munc18-2-syntaxin-2/3 complex and its individual components by co-immunoprecipitation assay using COS-7 cells, in which only a small amount of syntaxin-3 and none of the other proteins (i.e. Slp4-a, Munc18-1/2, and syntaxin-2) is endogenously expressed (supplemental Fig. 1A). To our surprise Slp4-a interacted with syntaxin-2 and syntaxin-3 only in the presence of Munc18-2 (closed arrowheads, lane 2 in the middle panels of Fig. 2, C and D, respectively), whereas Munc18-2 itself marginally interacted with Slp4-a (open arrowheads, lane 2 in the middle panels of Fig. 2, C and D). By contrast, neither Munc18-2 nor syntaxin-2/3 alone interacted with Slp4-a (lanes 1 and 3 in the middle panels of Fig. 2, C and D). The Munc18-2-dependent interaction between Slp4-a and syntaxin-2/3 was also observed when purified samples or tagless recombinant proteins were used (Fig. 2F and Supplemental Fig. 1, B and C). These results suggest that Slp4-a first recognizes the Munc18-2-syntaxin-2/3 complex rather than its individual components and that Slp4-a then may interact with syntaxin-2 as a result of replacement of Munc18-2 with Slp4-a.

Despite the high sequence similarity between Munc18-1 and Munc18-2, the mode of interaction between Slp4-a and Munc18-2 was quite different from the mode of interaction between Slp4-a and
Munc18-1. Slp4-a directly interacted with Munc18-1 regardless of the presence of syntaxin-2/3, whereas syntaxin-2/3 alone did not interact with Slp4-a (open arrowheads, lanes 1–3) (or beads alone; lane 4) were incubated with COS-7 cell lysates containingFLAG-Munc18s, FLA-GMunc18s plus FLAG-syntaxins, or FLAG-syntaxins alone as described under “Experimental Procedures.” Proteins bound to the beads were analyzed by 10% SDS-PAGE followed by immunoblotting with HRP-conjugated anti-FLAG M2 antibody (Blot: anti-FLAG; IP: anti-T7; middle panels) and HRP-conjugated anti-T7-tag antibody (Blot: anti-T7; IP: anti-T7; bottom panels). Input means 1/80 volume of the reaction mixtures (top panels). Open and closed arrowheads indicate the positions of Munc18s and syntaxins, respectively. Note that Slp4-a interacted with syntaxin-2/3 only in the presence of Munc18-1/2 and that only Munc18-1, and not Munc18-2/3, was capable of interacting with Slp4-a irrespective of the presence of syntaxin-2/3. 

Mapping of the Munc18-1/2 Interaction Site and Syntaxin-2/3 Interaction Site in Slp4-a—We previously showed that the N-terminal SHD of Slp4-a is necessary and sufficient for Rab27A binding and that Munc18-1 directly interacts with the large C-terminal part of Slp4-a (i.e., ASHD, amino acid residues 144–673) (29). In the next set of experiments we attempted to determine the minimal Munc18-1/2 interaction site in the Slp4-a molecule. We initially thought that Munc18-1 and Munc18-2 might interact with different domains of Slp4-a and that Munc18-2 and syntaxin-2/3 might interact with the C2B domain of Slp4-a, a putative phospholipid and/or protein interaction site (4, 49), because Slp4-b, an alternative splicing isoform that lacks the C2B domain (50), exhibited only weak Munc18-2-dependent syntaxin-2/3 binding activity (closed arrowheads, lane 2 in the middle panels of Fig. 3, C and D), whereas it interacted normally with Munc18-1, the same as Slp4-a (Fig. 2, A and B, and Fig. 3, A and B).

To define the Munc18-1/2 and/or syntaxin-2/3-binding site in the Slp4-a molecule, we prepared a series of Slp4-a deletion mutants (Fig. 4) and tested their binding activity with Munc18-1 alone, Munc18-1/2, Munc18-2, and syntaxin-2/3. Co-immunoprecipitated Munc18-1 and syntaxin-2/3 proteins were easily detected even by Amido Black staining of the blots, and the amount of Munc18-1 (or the Munc18-1/syntaxin-2/3 complex) protein was less than half that of the Slp4-a protein immunoprecipitated (Supplemental Fig. 1B). We also investigated the interaction between Slp4-a and Munc18-3/syntaxin-4 complex, but neither the complex nor its components interacted with Slp4-a (Fig. 2E).

**FIGURE 2.** Slp4-a interacts with syntaxin-2 and syntaxin-3 in a Munc18-2-dependent manner. **A and B,** interaction between T7-Slp4-a and FLAG-syntaxin-2/3 in the presence of FLAG-Munc18-1. **C and D,** interaction between T7-Slp4-a and FLAG-syntaxin-2/3 in the presence of FLAG-Munc18-2. **E,** interaction between T7-Slp4-a and FLAG-syntaxin-4 in the presence of FLAG-Munc18-3. Aagarose beads coupled with T7-Slp4-a (lanes 1–3) or beads alone (lane 4) were incubated with COS-7 cell lysates containing FLAG-Munc18s, FLAG-Munc18s plus FLAG-syntaxins, or FLAG-syntaxins alone as described under “Experimental Procedures.” Proteins bound to the beads were analyzed by 10% SDS-PAGE followed by immunoblotting with HRP-conjugated anti-FLAG M2 antibody (Blot: anti-FLAG; IP: anti-T7; middle panels) and HRP-conjugated anti-T7-tag antibody (Blot: anti-T7; IP: anti-T7; bottom panels). Input means 1/80 volume of the reaction mixtures (top panels). Open and closed arrowheads indicate the positions of Munc18s and syntaxins, respectively. Note that Slp4-a interacted with syntaxin-2/3 only in the presence of Munc18-1/2 and that only Munc18-1, and not Munc18-2/3, was capable of interacting with Slp4-a irrespective of the presence of syntaxin-2/3. **F,** direct interaction between T7-Slp4-a and FLAG-syntaxin-2 in the presence of FLAG-Munc18-2. T7-Slp4-a beads were incubated with purified FLAG-syntaxin-2 and FLAG-Munc18-2 as described under “Experimental Procedures.” Proteins bound to the beads were detected as described above.
interaction between T7-Slp4-b and FLAG-syntaxin-2/3 in the presence of FLAG-Munc18-1, C and D, interaction between T7-Slp4-b and FLAG-syntaxin-2/3 in the presence of FLAG-Munc18-2, 4–5), suggesting that the N-terminal SHD adjacent to the linker domain of Slp4-a (amino acid residues 144–354), a previously uncharacterized region between the SHD and the C2A domain (open arrowhead, lanes 1–3 in the middle panel of Fig. 4B) and that the C2 domains (putative protein interaction sites) were unnecessary for Munc18-1 binding. Syntaxin-3 also interacted with the linker domain of Slp4-a in a Munc18-1-dependent manner, and the amounts of Munc18-1 and syntaxin-3 proteins bound to the Slp4-a beads were almost identical based on estimates of the intensity of the immunoreactive bands on x-ray film (lanes 7–9 in the middle panel of Fig. 4B). It should be noted that the linker domain of Slp4-a alone interacted with Munc18-1 and syntaxin-3 much more strongly than did the full-length protein (compare lanes 7 and 9 in the middle panel of Fig. 4B).

In contrast to the interaction between the Slp4-a linker and the Munc18-1-syntaxin-3 complex, Munc18-2 alone did not interact with the Slp4-a linker, but it did interact with the Slp4-a linker in the presence of syntaxin-3 (open arrowhead, lanes 3 and 9, respectively, in the middle panel of Fig. 4C), although a much smaller amount of Munc18-2 than of syntaxin-3 bound to the Slp4-a linker beads. It should be noted that neither Munc18-2 nor syntaxin-3 interacted with Slp4-a-ΔSHD despite containing the linker domain (lane 8 in the middle panel of Fig. 4C), suggesting that the N-terminal SHD adjacent to the linker domain may affect the interaction between Slp4-a and syntaxin-3. We, therefore, investigated the effect of the Rab27A binding to the SHD of Slp4-a on the interaction between syntaxin-3 and Slp4-a; however, Rab27A binding to the SHD had no effect on the interaction between Slp4-a and Munc18-1/syntaxin-3, or between Slp4-a and Munc18-2/syntaxin-3 (supplemental Fig. 2). These results indicated that the Slp4-a SHD (or the C2B domain) is required for stable interaction with syntaxin-3 in the presence of Munc18-2 and that Rab27A binding to the SHD is unnecessary for the syntaxin-3 binding to the Slp4-a linker domain. Similar experiments were performed using FLAG-syntaxin-2, and syntaxin-2 was also found to interact with the linker domain of Slp4-a in a Munc18-1/2-dependent manner (data not shown).

**Slp4-a Interacts with the Closed Conformation of Syntaxin-2/3—Syntaxins are thought to occur in two functional conformational states; an open conformation and a closed conformation (51, 52). The open conformation of syntaxin-1a contributes to the formation of a neuronal SNARE complex (i.e. fundamental fusion machinery of synaptic vesicle exocytosis (53)) by binding to VAMP-2/synaptobrevin-2 and SNAP-25 (synaptosome-associated protein of 25 kDa), whereas the closed conformation of syntaxin-1a specifically interacts with Munc18-1. L165A/E166A substitutions in syntaxin-1a are known to fix syntaxin-1a in the open conformation, and a syntaxin-1a(L165A/E166A) mutant forms four helix bundles with VAMP-2 and SNAP-25 rather than interacting with Munc18-1. It, therefore, seemed interesting to identify the conformational differences between syntaxin-2/3 that interact with Slp4-a, and we did so by introducing mutations into syntaxin-3 (i.e. syntaxin-3(L165A/E166A)) similar to the syntaxin-1a(L165A/E166A) mutant and testing its binding properties. The same as the syntaxin-1a(L165A/E166A) mutant, syntaxin-3(L165A/E166A) mutant exhibited dramatically less binding activity with Munc18-1 and Munc18-2 than the wild-type protein (top and second panels of Fig. 5B, respectively) and instead strongly interacted with SNAP-23 (third panel of Fig. 5B), a syntaxin-3-binding part.
Munc18-2-dependent Interaction between Slp4-a and Syntaxin-2/3

Functional Involvement of Slp4-a-Syntaxin-2/3 Complex in Amylase Release from SLO-permeabilized Rat Parotid Acinar Cells—In the final set of experiments we investigated whether endogenous Slp4-a interacts with syntaxin-2/3 in rat parotid glands. The results of the co-immunoprecipitation experiment indicated that a small but significant amount of syntaxin-2 actually interacted with Slp4-a in rat parotid glands (lane 3 in the top panel of Fig. 6A), whereas Munc18-2 did not (lane 3 in the second panel), consistent with the results of the in vitro binding experiments described above (Fig. 2). Due to the high backgrounds of the anti-syntaxin-3 antibody, however, we were unable to detect syntaxin-3 in the anti-Slp4-a immunoprecipitates (data not shown). We also investigated whether GST-Slp4-a-linker traps the endogenous Munc18-2 and syntaxin-2/3 in rat parotid gland lysates. Consistent with the results of the in vitro binding experiments described above (Fig. 4), GST-Slp4-a-linker efficiently trapped syntaxin-2 (top panel of Fig. 6B), moderately trapped Munc18-2 (third panel), and trapped syntaxin-3 to a lesser extent (second panel). By contrast, no Rab27B signals were detected in the GST-Slp4-a-linker beads, because GST-Slp4-a-linker lacks the SHD that specifically binds Rab27B (compare the third panel of Fig. 6A and the fourth panel of Fig. 6B).

To investigate the functional involvement of the Slp4-a-syntaxin-2/3 complex in amylase release, GST-Slp4-a linker (or GST alone as a negative control) was introduced into SLO-permeabilized rat parotid acinar cells, and amylase release from these cells was measured as described previously (32). As shown in Fig. 6C, GST-Slp4-a-linker, but not GST alone or heat-denatured GST-Slp4-a-linker (up to 5 µM), strongly inhibited amylase release in a dose-dependent manner.

FIGURE 4. Mapping of the sites in the Slp4-a molecule responsible for Munc18-1, Munc18-2, and syntaxin-3 binding. A, schematic representation of deletion mutants of Slp4-a. Slp4-a consists of an N-terminal SHD (hatched box) containing two zinc finger motifs (Zn²⁺) and tandem C2 domains (named the C2A domain and C2B domain; shaded boxes). The Munc18-1/2 and syntaxin-3 binding activity of each mutant (−, −−, ++, or +++) is indicated after its name. The amino acid positions are indicated on both sides. 8 and 12 interaction between T7-Slp4-a deletion mutants and FLAG-Munc18-1/2 in the presence and absence of FLAG-syntaxin-3. Agarose beads coupled with T7-Slp4-a mutants (Slp4-a, antibody (as described under “Experimental Procedures.” Proteins bound to the beads were analyzed by 12.5% SDS-PAGE followed by immunoblotting with HRP-conjugated anti-FLAG M2 antibody). The positions of the molecular mass markers (× 10⁻⁴) are indicated on the left.

B and C, co-immunoprecipitation analysis employing Munc18-1 or Munc18-2 (B) or FLAG-syntaxin-3 (C) as an immunoprecipitation control. The positions of the molecular mass markers (× 10⁻⁴) are indicated on the left.
To further investigate the direct involvement of the Slp4-a linker domain in amylase release, we produced an antibody against the linker domain of Slp4-a (named anti-Slp4-a-linker antibody). As shown in Fig.

**FIGURE 5.** Slp4-a preferentially interacts with the closed conformation of syntaxin-3 in a Munc18-2-dependent manner. A, interaction between T7-Slp4-a and the wild-type FLAG-syntaxin-3 and the FLAG-syntaxin-3(L165A/E166A) mutant in the presence and absence of FLAG-Munc18-2. A binding assay was performed as described in the legend to Fig. 2. Note that syntaxin-3(L165A/E166A) which mimics the open conformation of syntaxin-3 (see B), failed to interact with Slp4-a even in the presence of Munc18-2 (lane 4 in the middle panel). Input means 1/80 volume of the reaction mixtures (top panel). B, distinct Munc18-1/2 and SNAP-23 binding activity of the wild-type and FLAG-syntaxin-3(L165A/E166A) mutant. The binding assay was performed as described in the legend to Fig. 1. Note that the syntaxin-3(L165A/E166A) mutant showed increased SNAP-23 binding activity but reduced Munc18-1/2 binding activity (lane 4). Input means 1/80 volume of the reaction mixtures (lanes 1 and 2). C, Munc18-2-dependent interaction between T7-Slp4-a and syntaxin-3 in the presence and absence of FLAG-SNAP-23 and FLAG-VAMP-3/8. Agarose beads coupled with T7-Slp4-a were incubated with COS-7 cell lysates containing the FLAG-tagged proteins indicated as described under “Experimental Procedures.” Proteins bound to the beads were analyzed by 12.5% SDS-PAGE followed by immunoblotting with HRP-conjugated anti-FLAG M2 antibody (Blot: anti-FLAG, IP: anti-T7; middle panel) and HRP-conjugated anti-T7 tag antibody (Blot: anti-T7; IP: anti-T7; bottom panel). Input means 1/80 volume of the reaction mixtures (top panel).

**FIGURE 6.** GST-Slp4-a linker traps endogenous syntaxin-2/3 in rat parotid acinar cells and strongly attenuates amylase release from SLO-permeabilized parotid acinar cells. A, anti-Slp4-a antibody co-immunoprecipitated syntaxin-2 (top panel) and Rab27B (third panel) but not Munc18-2 (second panel). Immunoprecipitation and immunoblot analyses were performed as described under “Experimental Procedures.” B, GST-Slp4-a linker, but not GST alone, trapped syntaxin-2 (top), syntaxin-3 (second), and Munc18-2 (third) but not Rab27B (fourth panel). GST pull-down assay and immunoblotting were performed as described under “Experimental Procedures.” The positions of the molecular mass markers (×10−3) are shown on the left. C, inhibition of amylase release by recombinant Slp4-a linker from SLO-permeabilized parotid acinar cells. GST-Slp4-a-linker, but not GST alone or heat-denatured GST-Slp4-a-linker (i.e. incubation at 95 °C for 5 min), inhibited amylase release in a dose-dependent manner. IPR-stimulated amylase release from SLO-permeabilized parotid acinar cells was measured as described under “Experimental Procedures.” The amylase activity released is expressed as a percentage of the IPR-stimulated release in the absence of GST fusion proteins. Bars indicate the mean ± S.E. of 3–5 independent experiments performed in triplicate. The data were analyzed by one-way or two-way analysis of variance and Tukey’s post hoc test. *, p < 0.01 versus control value.

To further investigate the direct involvement of the Slp4-a linker domain in amylase release, we produced an antibody against the linker domain of Slp4-a (named anti-Slp4-a-linker antibody). As shown in Fig.
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**FIGURE 7. Functional involvement of Slp4-a in amylase release from parotid acinar cells.** A, effect of anti-Slp4-a-linker antibody on the Munc18-2-dependent interaction between Slp4-a and syntaxin-2 in *vitro*. B, effect of anti-Slp4-a-linker antibody on the interaction between Slp4-a and Rab27A in *vitro*. The T7-Slp4-a beads (bottom panel) were incubated with FLAG-Munc18-2 and FLAG-syntaxin-2 (or FLAG-Rab27A in B) in the presence and absence of the anti-Slp4-a-linker IgG or a control rabbit IgG as described under “Experimental Procedures,” and the FLAG-tagged proteins trapped by the beads were analyzed by immunoblotting with HRP-conjugated anti-FLAG M2 antibody (1/10,000 dilution; Blot: anti-FLAG; IP: anti-T7; middle panels). Note that the anti-Slp4-a-linker IgG, but not control IgG, inhibited the interaction between Slp4-a and syntaxin-2 (closed arrowhead, lane 2, in the middle panel of A) but had no effect on the Rab27A-binding to Slp4-a (lane 2 in the middle panel of B). Input means 1/10 volume of the reaction mixtures (top panels in A and B). C, inhibition of amylase release by anti-Slp4-a-linker antibody. The anti-Slp4-a-linker IgG inhibited amylase release in a dose-dependent manner, whereas control IgG (up to 10 μg/ml) had no significant effect on amylase release. IPR-stimulated amylase release from SLO-permeabilized parotid acinar cells was measured as described under “Experimental Procedures” (32). The amylase activity released is expressed as a percentage of the IPR-stimulated release in the absence of antibodies. Bars indicate the mean ± S.E. of five independent experiments performed in triplicate. The data were analyzed by one-way or two-way analysis of variance and Tukey’s post hoc test. *p < 0.01

7, A and B, the anti-Slp4-a-linker antibody strongly inhibited the Munc18-2-dependent Slp4-a-syntaxin-2 interaction but had no effect on the Slp4-a-Rab27A interaction *in vitro*, indicating that this antibody is a useful tool for specific disruption of the Slp4-a-syntaxin-2/3 (or Slp4-a-Munc18-2) complex *in vivo* without affecting the formation of the Slp4-a-Rab27A/B complex. When the anti-Slp4-a-linker antibody was introduced into SLO-permeabilized parotid acinar cells, IPR-stimulated amylase release was severely inhibited in a dose-dependent manner (Fig. 7C), whereas control antibody had no effect on amylase secretion. Taken together, these results strongly indicate that the linker domain of Slp4-a is involved in the IPR-stimulated amylase release from rat parotid acinar cells, possibly through interaction with syntaxin-2/3 (or Munc18-2).

**DISCUSSION**

Slp4-a and other members of the Slp family share an N-terminal SHD and C-terminal tandem C2 domains that are separated by a linker of varying length (2–4). Although the function of the SHD of Slp1–5 as a Rab27 binding domain (7–10) and the phospholipid binding activity of the tandem C2 domains of certain Slps (5, 13, 14, 50, 54) had already been well demonstrated, the function of the linker domain of Slp1–5 had never been elucidated. In the present study we demonstrated for the first time that Slp4-a interacts differently with Munc18 isoforms (Fig. 2) and that the Slp4-a linker domain interacts with the closed conformation of syntaxin-2/3 only in a Munc18-2-dependent manner in addition to interaction with Munc18-1 (Fig. 4). Based on an analogy to the Slp4-a-Munc18-1-syntaxin-2/3 complex, we speculate that Slp4-a first recognizes the Munc18-2 of the Munc18-2-syntaxin-2/3 complex and that Slp4-a-Munc18-2-syntaxin-2/3 complex may be formed transiently. Such a tripartite protein complex, however, should be unstable (or transient), probably because of steric hindrance by other domains adjacent to the linker domain of Slp4-a (e.g. C2A domain), and Munc18-2 may be rapidly released from the tripartite protein complex to form more stable Slp4-a-syntaxin-2/3 complex by replacement of Munc18-2 with Slp4-a. By contrast, the Slp4-a linker domain alone is capable of interacting with the Munc18-2-syntaxin-2/3 complex, probably because of the reduction of steric hindrance that results from truncation of the Slp4-a molecule. Slp4-a must contain a novel type of syntaxin-2/3- or Munc18-1-binding motif, because the Slp4-a linker domain was found not to exhibit any significant homology with the known syntaxin or Munc18 binding motifs in a data base search (data not shown). Further structural studies will be necessary to define the syntaxin or Munc18 binding motif in Slp4-a and to fully understand the distinct molecular recognition of Slp4-a by Munc18-1 and Munc18-2.

As far as we know Slp4-a is the only C-terminal type tandem C2 protein whose linker domain functions as a protein interaction site; none of the linker domains of other C-terminal-type tandem C2 proteins (e.g. synaptotagmins, rabphilin, and Doc2s) reported thus far have been described as a protein interaction site. Because the linker domains are not conserved among the Slp family members, the linker domain of Slps other than Slp4-a is very unlikely to act as a Munc18-1- or syntaxin-2/3-binding site. Consistent with the low sequence similarities of the linker domain of Slps (less than 20% identity at the amino acid level), Slp2-a, Slp3-a, and Slp5 (the closest isoform of Slp4-a) do not interact with either Munc18-1 or syntaxin-2/3 even *in vitro* (supplemental Fig. 3 and data not shown) (29), and we speculate that the linker domain of Slps other than Slp4-a may also function as an interaction site with a specific protein. It is interesting to note that several alternative splicing events occur in the linker domain of Slp2-a (5, 6) and Slp5 (29), although their physiological significance has never been elucidated. Further work is needed to determine whether such alternative splicing events actually affect protein interactions.

We have also demonstrated that Slp4-a interacts with syntaxin-2 (and possibly with syntaxin-3) but not with Munc18-2 in rat parotid...
glands and that the introduction of the recombinant Slp4-a linker domain or the anti-Slp4-a-linker antibody into SLO-permeabilized acinar cells strongly attenuates amylase release. Based on our findings together with the previous findings by other groups, we think that Slp4-a is likely to function in two possible ways during amylase release from parotid acinar cells. First, Slp4-a may be involved in controlling the docking of amylase-containing granules to the apical plasma membrane of the acinar cells through simultaneous interaction with Rab27B on the amylase-containing granules via the SHD and with syntaxin-2/3 on the apical plasma membrane (36) via the linker domain, as has been suggested in regard to the role of Slp4-a in insulin secretion in pancreatic β-cell lines (i.e. simultaneous interaction with Rab27A on granules and syntaxin-1a on the plasma membrane) (27), and in regard to the role of Slp2-a in melanosome transport in melanocytes (i.e. simultaneous interaction with Rab27A on melanosomes and phosphatidylinserine in the plasma membrane) (14). The mechanism by which Slp4-a dissociates from the closed conformation of syntaxin-2/3 after docking of the granules to the apical plasma membrane and the mechanism by which the closed conformation of syntaxin-2/3 is converted to the open conformation that contributes to the formation of a SNARE complex with SNAP-23 and VAMP-3/8 still remain unknown. Munc13 isoforms (55), putative priming factors for exocytosis, may be involved in this process (56), and Munc13-4 is of particular interest because Munc13-4 itself has recently been shown to function as a Rab27A-binding protein in cyto-toxic T-lymphocytes (25, 57) and platelets (24). Future study will clarify Munc13 isoform expression and localization in parotid acinar cells.

The other possibility is that Slp4-a may indirectly regulate SNARE complex formation (e.g. syntaxin-2/3-SNAP-23-VAMP-3/8), which is thought to be important for amylase release (36), or directly regulate the syntaxin-2/3-Munc18-2 complex by modulating the availability of syntaxin-2/3. These two possibilities are not mutually exclusive, and Slp4-a may function in both ways in amylase release. GST-Slp4-a-linker (or the anti-Slp4-a-linker antibody) is capable of both disrupting the interaction between Slp4-a and syntaxin-2/3 (i.e. inhibiting docking of amylase-containing granules to the apical plasma membrane) and reducing the availability of syntaxin-2/3 by trapping the closed configuration of syntaxin-2/3 (or by indirectly modulating the Munc18-dependent syntaxin-2/3 complex formation). Further analysis of the function of Slp4-a either by gene targeting or by gene silencing by means of RNA interference technology will be needed to fully understand the molecular mechanism of Slp4-a-dependent amylase release, although the latter approach is technically difficult because primary parotid acinar cells cannot be maintained in vitro for a long period, and no cell lines that normally secrete amylase are currently available.

In summary, we have demonstrated in vitro binding assays that Slp4-a interacts with the closed configuration of syntaxin-2/3 in a Munc18-2-dependent manner via the linker domain of Slp4-a and that the Slp4-a-syntaxin-2 complex is actually present in rat parotid acinar cells. Because introduction of the fragment of the Slp4-a linker, which efficiently trapped endogenous syntaxin-2, or the anti-Slp4-a-linker antibody into SLO-permeabilized parotid acinar cells strongly inhibited amylase release, we propose that Slp4-a is involved in the control of amylase release by modulating certain protein interaction(s) of syntaxin-2/3 and/or Munc18-2 in parotid acinar cells, whereas Slp4-a is involved in the control of hormone secretion by certain endocrine cells through interaction with syntaxin-1a and/or Munc18-1 (27, 29, 33).

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