The Slp Homology Domain of Synaptotagmin-like Proteins 1–4 and Slac2 Functions as a Novel Rab27A Binding Domain*

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rab27A, which encodes a small GTP-binding protein, was recently identified as a gene in which mutations caused human hemophagocytic syndrome (Griscelli syndrome) and ashen mice, which exhibit defects in melanosome transport as well as in regulated granule exocytosis in cytotoxic T lymphocytes. However, little is known about the molecular mechanism of Rab27A-dependent membrane trafficking or the specific effector molecules of Rab27A. In this study, we discovered that the Slp (synaptotagmin-like protein) homology domain (SHD) of Slp1–3 and Slac2-a/b specifically and directly binds the GTP-bound form of Rab27A both in vitro and in intact cells but not of the other Rabs tested (Rab1, Rab2, Rab3A, Rab4, Rab5A, Rab6A, Rab7, Rab8, Rab9, Rab10, Rab11A, Rab17, Rab18, Rab20, Rab22, Rab23, Rab25, Rab28, and Rab37). Immunocytochemical analysis revealed that Slp2 (or Slp1) colocalized with Rab27A in the melanosomes of melanoma cells. Slp2 and Rab27A were distributed to the periphery of the cells (especially at the dendritic tips) in the wild-type melanoma cells, whereas they accumulated in the perinuclear region in the melanosome transport-defective cells (S91/Cloudman). These results strongly indicated that the SHD of Slp1–3 and Slac2 functions as an in vivo Rab27A binding domain.

The C2 domain is a protein module of ~130 amino acids that is often found in various signaling molecules and proteins involved in vesicular trafficking in all eukaryotes (reviewed in Refs. 1 and 2). The prototypical C2 domains (e.g. protein kinase C C2 domain) are known to bind both several Ca2+-ions and phospholipid membranes and to regulate various Ca2+-dependent signaling processes (2). Recently, however, Ca2+-independent type C2 domains, which fail to bind Ca2+-ions as a result of amino acid variations in residues critical for Ca2+ binding, have been reported, and some of them function as a protein interaction site (3–5) or a nuclear localization signal (6, 7).

Among the various C2 domain-containing proteins, the C-terminal-type (C-type) tandem C2 proteins (known as the C2A and C2B domains), in which the tandem C2 domains are located at the C terminus, are often found among proteins involved in vesicular trafficking (8). To date, four different types of C-type tandem C2 proteins (i.e. the synaptotagmin (Syt) family (3–5, 9), rabphilin-3 (10), the Doc2 family (11–14), and the Syt-like protein (Slp) family (8, 15)) have been reported, and they are distinguished from each other by their unique N-terminal domains (8, 15).

The members of the Slp family are coded by at least four distinct genes in mouse and human and have several alternatively splicing isoforms (15): Slp1/JFC1 (8, 16), Slp2–a–d, Slp3–a/b (8, 15), and Slp4/granulphilin-a (17). The Slp family contains two conserved domains at the N terminus, referred to as Slp homology domain 1 (SHD1) and Slp homology domain 2 (SHD2) (15) (see Fig. 1, A and C). The SHD1 and SHD2 of Slp3-a and Slp4 are separated by a sequence containing two zinc-finger motifs, whereas Slp1 and Slp2 lack such zinc-finger motifs, and their SHD1 and SHD2 are linked together. The SHD has also been found in other proteins, including Slac2-a (Slp homologue lacking C2 domains-a) (15), suggesting a more general role of the SHD in cellular signaling. However, no research on the function of the SHD domains has ever been attempted. The only available information is that the SHD shows weak homology with Rab3 binding domains (RBD) of rabphilin-3 (18), RIM (19), and Noc2 (20) (see also Fig. 1A).

In this study, we first discovered that the SHD of Slp1, Slp3-a, and Slac2 is a novel binding domain for the GTP-bound form of Rab27A (21–23), one of the small GTP-binding proteins that are believed to be essential components of the membrane-trafficking mechanism of eukaryotic cells (reviewed in Ref. 24). Mutation in the rab27A gene causes defects in melanosome transport as well as defects in granule exocytosis in cytotoxic T lymphocytes in human hemophagocytic syndrome (Griscelli syndrome) and ashen mice (22, 25, 26). Immunocytochemical analysis of melanoma cells showed that Slp2 (or Slp1) and Rab27A colocalized in the melanosome of the wild-type and the melanosome transport-defective melanoma cells. Based on our findings, we discuss the possible role of the SHD of Slps and Slac2s in Rab27A-dependent membrane trafficking.

EXPERIMENTAL PROCEDURES

Molecular Cloning of the Mouse Rab Family and Slp4 cDNAs—cDNA encoding a full open reading frame of the mouse Rab family (Rab1, one S-transferase; HRP, horseradish peroxidase; PVDF, polyvinylidene difluoride; RBD, Rab3 binding domain; SHD, Slp homology domain; Slac2, Slp homologue lacking C2 domains; Slp(s), synaptotagmin-like protein(s); Syt, synaptotagmin(s); TRP-1, tyrosinase-related protein-1; GTP-yS, guanosine 5’-O-(thiotriphosphate).
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Rab2, Rab3A, Rab4A, Rab5A, Rab6A, Rab7, Rab8, Rab9, Rab10, Rab11A, Rab17, Rab18, Rab20, Rab22, Rab23, Rab25, Rab27A, Rab28, and Rab37) was amplified from the Marathon-Ready adult brain cDNA (CLONTECH) by reverse transcriptase-PCR as described previously (27) using the following pairs of oligonucleotides with restriction enzyme sites underlined (sense) and 5′-TGAAGCAGC-CTCCACCTG-3′ (Rab1-Met primer; sense) and 5′-TGAAGCAGC-CTCCACCTG-3′ (Rab1-stop primer; antisense; accession number X70804); 5′-TCAACAGGACCTCCCTCCCTG-3′ (Rab2-Met primer; sense) and 5′-TCAACAGGACCTCCCTCCCTG-3′ (Rab2-stop primer; antisense; accession number NM_009001); 5′-ATCCATGGCCACGACGACTG-3′ (Rab3A-Met primer; sense) and 5′-ATCCATGGCCACGACGACTG-3′ (Rab3A-stop primer; antisense; accession number D86563); 5′-ATCCATGGCCACGACGACTG-3′ (Rab4A-met primer; sense) and 5′-ATCCATGGCCACGACGACTG-3′ (Rab4A-stop primer; antisense; accession number AB041575); 5′-ATCCATGGCCACGACGACTG-3′ (Rab5A-Met primer; sense) and 5′-ATCCATGGCCACGACGACTG-3′ (Rab5A-stop primer; antisense; accession number NM_008999); 5′-ATCCATGGCCACGACGACTG-3′ (Rab7-Met primer; sense) and 5′-ATCCATGGCCACGACGACTG-3′ (Rab7-stop primer; antisense; accession number NM_016678); 5′-ATCCATGGCCACGACGACTG-3′ (Rab9-Met primer; sense) and 5′-ATCCATGGCCACGACGACTG-3′ (Rab9-stop primer; antisense; accession number NM_017397); 5′-ATCCATGGCCACGACGACTG-3′ (Rab10-Met primer; sense) and 5′-ATCCATGGCCACGACGACTG-3′ (Rab10-stop primer; antisense; accession number NM_016678); 5′-ATCCATGGCCACGACGACTG-3′ (Rab11A-Met primer; sense) and 5′-ATCCATGGCCACGACGACTG-3′ (Rab11A-stop primer; antisense; accession number AB041575); 5′-ATCCATGGCCACGACGACTG-3′ (Rab27A-Met primer; sense) and 5′-ATCCATGGCCACGACGACTG-3′ (Rab27A-stop primer; antisense; accession number AB151111); and 5′-ATCCATGGCCACGACGACTG-3′ (Rab37 Met primer; sense) and 5′-ATCCATGGCCACGACGACTG-3′ (Rab37 stop primer; antisense; accession number NM_016678). Reactions were carried out in the presence of the expression vectors were performed as described previously (27–30).

Slac2-b/KIAA0624 was a kind gift from the Kazusa DNA Research Institute (Chiba, Japan) (31). pEF-T7-Slp1–, pEF-T7-Slp2–, pEF-T7-Slac2–, and pEF-T7-rabphilin-3 were also essentially produced by plasmid DNA was prepared from Cos-7 cells transiently expressing FLAG-tagged Rab proteins were blotted onto the PVDF membrane (Millipore Corp., Bedford, MA) and probed with the goat anti-FLAG tag antibody (Sigma Chemical Co.). A 20-

Antibody Production—New Zealand White rabbits were immunized with purified GST (glutathione S-transferase)-Slp1-C2B and GST-Slp2-C2B (8), and anti-Slp1 and anti-Slp2 antibodies were affinity-purified by affinity to antigen-bound Affi-Gel 10 beads (Bio-Rad, Hercules, CA) as described previously (32). Specificity of these antibodies was checked by immunoblotting using recombinant T7-tagged Slp1–4 expressed in COS-7 cells (32, 33).

Cell Culture, Transfections, Immunoprecipitation, and Immunocytochemistry—Transfection of pEF-T7-Slps (or T7-Slac2) and/or pEF-FLAG-Rabs into COS-7 cells (5 × 10^5 cells, the day before transfection/10-cm dish) was performed as described previously (34). Proteins were solubilized in a buffer containing 1% Triton X-100, 250 mM NaCl, 1 mM MgCl2, 50 mM HEPES-KOH, pH 7.2, 0.1% phenylmethylsulfonly fluoride, 10 μM leupeptin, and 10 μM pepstatin A at 4 °C for 1 h. T7-Slps (or T7-Slac2) were immunoprecipitated by anti-T7 tag antibody-conjugated agarose (Novagen, Madison, WI) as described previously (27). SDS-PAGE and immunoblotting analyses were also performed as described previously (27, 30).

Recent development of the wild-type mouse (35) and Slac2b/Cloudman derived from dilute mouse (36) were obtained from American Type Culture Collection (ATCC). B16-F1 cells were cultured on glass-bottom dishes (35-mm dish, MatTek Corp., MA) in Dulbecco’s modified Eagle’s medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin at 37 °C under 5% CO2. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and stained with anti-Slp1 rabbit antibody (6.4 μg/ml), anti-Slp2 rabbit antibody (14 μg/ml), anti-Rab27 mouse monoclonal antibody (1/50 dilution, Transduction Laboratories, Lexington, KY), and anti-TRP-1 (tyrosine-related protein-1) goat antibody (1/50 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) visualized by a second antibody (1/5000 dilution, anti-rabbit Alexa 488, anti-mouse Alexa 568, or anti-goat Alexa 633 antibodies, Molecular Probes, Eugene, OR) as described previously (32, 33). The cells were then observed with a confocal fluorescence microscope (Fluoview, Olympus, Tokyo, Japan). COS-7 cells transiently expressing FLAG-tagged Rab proteins were homogenized in 50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 0.1 mM phenylmethylsulfonly fluoride, 10 μM leupeptin, and 10 μM pepstatin A, and the proteins were solubilized with 1% Triton X-100 at 4 °C for 1 h. After centrifugation at 17,360 g for 10 min at 4 °C, each of the cell lysates was appropriately diluted beforehand so that the same amount of Rab proteins would be contained in the reaction mixture (see Fig. 2A). The amount of Rab proteins in the reaction mixtures was confirmed by immunoblotting with horseradish peroxidase (HRP)-conjugated anti-FLAG tag antibody (Sigma Chemical Co.). A 20-μl volume of glutathione Sepharose 4B beads (Amersham Biosciences, Inc.) were washed several times with PBS and incubated with Rab proteins (or GST alone) was incubated with 1 ml of COS-7 cell lysate containing Rab proteins for 2 h at 4 °C. After washing the beads five times with 1 ml of the buffer (50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 0.2% Triton X-100, and protease inhibitors), proteins trapped with the beads were analyzed by 12.5% SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA) and probed with the goat anti-FLAG tag antibody (Sigma Chemical Co.).
FIG. 1. Comparison of the Rab effector domains of the Slp family, Slac2, rabphilin-3, RIM, and Noc2. A, schematic representation of proteins containing the Slp homology domain (SHD) and proteins containing the Rab3 binding domain (RBD) (black boxes): mouse Slp1/ JFC1 (8, 16), mouse Slp2-a, mouse Slp2-a (15), mouse Slp4/granuphilin-a (17), mouse Slac2-a/melanophilin (15, 44), human Slac2-b/KIAA0624 (31), mouse rabphilin-3 (10), rat RIM1 (19), and rat Noc2 (20). The SHDs of Slp1-a, Slp4, and Slac2-a are separated by a sequence containing two zinc-finger motifs (indicated as Zn$^{2+}$), the same as the Rab3 effector domains of rabphilin-3, RIM1, and Noc2. By contrast, the SHDs of Slp1, Slp2-a, and Slac2-b lack zinc-finger motifs. The two C2 domains and the PDZ domain are represented by the shaded boxes and the hatched box, respectively. Amino acid numbers are given on both sides. B, phylogenetic tree of the SHD and the RBD. This tree was drawn with the ClustalW program (available at clustalw.genome.ad.jp). Note that the SHD of the Slp family and Slac2 form a distinct branch of the Rab3 effector domains of RIM1, RIM2, rabphilin-3, and Noc2. C, sequence alignment of the SHDs of the Slp family, Slac2, RIM, rabphilin-3, and Noc2. Among the splicing isoforms of RIM1, RIM1A57–106 (19) shows the highest sequence homology with other SHDs. Residues in the sequences that are conserved and similar are shown against a black background and against a shaded background, respectively. The solid lines indicate two SHDs. The number signs (#) indicate the sequence corresponding to the Rab3 binding site (SGAWFF) of rabphilin-3 (35). The conserved Cys residues corresponding to two zinc-finger motifs are indicated by asterisks. Amino acid numbers are indicated at the right of each line.

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RESULTS AND DISCUSSION

Identification of the SHD as a Novel Rab27A Binding Domain—In our previous study, we found a novel protein motif, the Slp homology domain (SHD) in the N-terminal domain of Slp1–4 and Slac2-a (15). A data base search revealed the existence of an additional SHD-containing protein lacking two C2 domains, and we therefore named it Slac2-b (Fig. 1A). The SHD consists of SHD1 and SHD2, which are separated by two zinc-finger motifs in some molecules (asterisks in Fig. 1A), and homology search analysis indicated that the SHD of Slp1–4 and Slac2 shows striking homology to the Rab3 binding domain (RBD) of RIM1, RIM2, rabphilin-3, and Noc2 (18–20) (Fig. 1, B and C). It should be noted that all the SHD2 included a sequence similar to the Rab3-binding site of rabphilin-3 (SGAWFF; # in Fig. 1C) (38). However, because the SHD of the Slp family and Slac2 forms a distinct branch from the RBD of RIM1, RIM2, rabphilin-3, and Noc2 in the phylogenetic tree, we hypothesized that the SHD functions as a certain Rab (other than Rab3) binding domain.

To verify this hypothesis, we prepared twenty different Rab proteins (Rab1, Rab2, Rab3A, Rab4A, Rab5A, Rab6A, Rab7, Rab8, Rab9, Rab10, Rab11A, Rab17, Rab18, Rab20, Rab22,
Rab23, Rab25, Rab27A, Rab28, and Rab37), which are known to be present in distinct membrane structures and involved in membrane trafficking (reviewed in Ref. 24). Each of the Rab proteins with a FLAG tag was expressed in COS-7 cells (Fig. 2A), and a GST pull-down assay was performed by using the SHD of Slp1, Slp2-a, Slp3-a, Slp4, Slac2-a, Slac2-b, and the RBD of rabphilin-3 (see “Experimental Procedures” for details). To our surprise, all the SHDs interacted with Rab27A. The SHD of Slp1, Slp2-a, and Slac2-b specifically interacted with Rab27A but not with other Rabs (Fig. 2, C, D, and H). The SHD of Slp3-a and Slac2-a preferentially interacted with Rab27A and weakly with Rab10 (Fig. 2, E and G). The SHD of Slp4

FIG. 2. Specific interaction of the SHD of the Slp family and Slac2 with Rab proteins. A, FLAG-tagged Rab proteins were expressed in COS-7 cells, and the proteins expressed were solubilized with 1% Triton X-100. B–I, GST pull-down assay was performed as described under “Experimental Procedures.” The proteins trapped with the beads were subjected to 12.5% SDS-PAGE and transferred to the PVDF membrane. The blots were first probed with HRP-conjugated anti-FLAG tag antibody (upper panels in B–I). The same blot was then stripped (46) and reprobed with HRP-conjugated anti-GST antibody to ensure that the same amounts of GST-fused proteins were loaded. A, expression of recombinant Rab proteins with FLAG tag in the reaction mixtures (input). B, glutathione-Sepharose coupled with GST alone; C, GST-Slp1-SHD; D, GST-Slp2-a-SHD; E, GST-Slp3-a-SHD; F, GST-Slp4-SHD; G, GST-Slac2-a-SHD; H, GST-Slac2-b-SHD; and I, GST-rabphilin-3-RBD. Note that all the SHDs of Slp and Slac2 interacted with Rab27A. Asterisks indicate the degradation products of GST fusion proteins. The results shown are representative of at least two or three independent experiments. Molecular weight markers (×10−9) are shown at the right.
 preferentially interacted with Rab8 and Rab27A and weakly with Rab3A (Fig. 2F). By contrast, the RBD of rabphilin-3 interacted with Rab3A, Rab8, and Rab27A with almost similar affinity (Fig. 2F). Under our experimental conditions, none of the Rab proteins were trapped with the beads coupled with GST alone (Fig. 2B), indicating that the interaction of the SHD with Rab27A is not a nonspecific interaction with GST or beads alone. It should be noted that Rab3A, Rab8, and Rab27A are phylogenetically similar and form a small branch on the phylogenetic tree (Fig. 3I).

To further investigate whether the Slp family and Slac2s interact with specific Rab proteins in intact cells, we performed a co-transfection assay in COS-7 cells. Briefly, T7-tagged Slp or T7-Slac2 and FLAG-tagged Rabs were co-transfected into COS-7 cells, and associations of T7- and FLAG-tagged proteins were evaluated by the immunoprecipitation method (27, 30). As shown in Fig. 3, T7-Slp1, Slp2-a, Slp3-a, Slac2-a, and Slac2-b specifically co-immunoprecipitated with Rab27A but not with Rab3A, Rab8, or Rab10, whereas T7-Slp4 and T7-rabphilin-3 specifically co-immunoprecipitated with Rab3A, Rab8, and Rab27A, but not with Rab10, indicating that the Slp family and Slac2 interact with specific Rab proteins in intact cells as well. The association of the full-length Slps and Slac2 with Rab in intact cells was more specific than observed in the in vitro GST pull-down assay. For example, the GST-SHD of Slp3-a and Slac2-a preferentially interacted with Rab27A, and weakly with Rab10 (Fig. 2, E and G), whereas the full-length Slp3-a and Slac2-a specifically interacted with Rab27A, but not with Rab10 (Fig. 3, C and E). This discrepancy was probably attributable to the nonspecific interaction of the degradation product of GST-Slp3-a-SHD and -Slac2-a-SHD with Rab10 (Fig. 2, E and G, asterisks).

The Slp Family and Slac2 Directly Interact with the GTP-Bound Form of Rab27A—Next we investigated whether the interaction between Slp or Slac2 and Rab proteins occurs directly to rule out the possibility that additional proteins in the COS-7 cell lysates form a link between Slp (or Slac2) and Rab27A, and purified recombinant proteins (FLAG-Rab27A and GST-SHDs) were used for binding assay (see “Experimental Procedures” for details). Briefly, recombinant FLAG-Rab27A protein coupled with the anti-FLAG antibody-conjugated agarose was incubated with the GST-fused SHD of Slp1, Slp3-a, or Slac2-a, and proteins bound to Rab27A-beads (or beads alone, as a control) were detected by immunoblotting. As expected, the SHD of Slp1, Slp3-a, and Slac2-a interacted with the Rab27A-beads but not with the beads alone (Fig. 4A), thereby demonstrating direct interaction of the Slp family or Slac2 with Rab27A.

The small G protein superfamily, including the Rab proteins, is generally activated in a GTP-bound form and inactivated in the GDP-bound form, and exchange between the GTP/GDP forms is an essential step in expression of the function of small G proteins. A variety of Rab effector molecules (e.g. Rab-GAP,
Rab-GEF, and Rab-GDI) specific for the GTP or GDP forms of Rab proteins have been reported (24). Because it is important to determine whether the SHD functions as an effector domain for GTP- or GDP-Rab27A to understand the physiological function of Rab27A binding to the SHD, we investigated the guanine nucleotide-selective interaction of Rab27A with the Slp family or Slac2. Recombinant FLAG-Rab27A coupled with agarose beads was first incubated with 0.5 mM GTP or GDP and then with TT-Slp1, -Slp3-a, or -Slac2-a in the presence of GTP or GDP. The proteins bound to the beads were analyzed by immunoblotting with HRP-conjugated anti-T7 tag antibody. As shown in Fig. 4B, Slp1, Slp3-a, and Slac2-a preferentially associated with GTP-Rab27A rather than GDP-Rab27A (Fig. 4B). These results support the idea that the Slp family and Slac2 function as a Rab27A effector by directly interacting with the GTP-bound active form of Rab27A via the SHD.

**Slp1 and Slp2 Localize in the Melanosome Transport Machinery in Melanoma Cells**—Genetic mapping in the coat color-mutated mouse and in human disease characterized by an immune deficiency and a partial albinism (Griscelli syndrome) indicated that Rab27A is a key player in melanosome transport (22, 23, 39–42). To investigate the possible involvement of the Slp family in melanosome transport in concert with Rab27A, immunofluorescence analyses of Slp1 and Slp2 in the B16-F1 melanoma cell line derived from the wild-type mouse (35) were performed using rabbit polyclonal antibodies against the C2B domains of Slp1 and Slp2. These antibodies were highly specific for each antigen and did not cross-react with other Slp proteins (data not shown). Slp1 and Slp2 proteins were highly expressed in the melanoma cells and closely colocalized with Rab27A (Fig. 5, A–C, and data not shown). Note that both Slp2 and Rab27A were distributed to the peripheral region of the wild-type melanoma cells (Fig. 5C, shown in yellow). The localization of Slp2 in melanosomes was further confirmed by co-immunostaining with anti-TRP-1 (a melanosome-resident protein) (Fig. 5E, shown in white). High magnification images revealed that Slp2 and Rab27A were distributed to melanosomes especially at the dendrite tips (Fig. 5, A–E, insets).

Myosin Va, a product of *MyoVa* (dilute), is known to function in the actin-based transport of melanosomes to the cell periphery, and recent cellular and molecular analyses revealed the synergistic role of Rab27A and myosin Va in the melanosome transport mechanism (39–43). The S91/Cloudman melanoma cell line was derived from the *dilute* mouse (36), which shows a defect in melanosome transport as a result of mutation in *MyoVa*, and the mislocalization of TRP-1 (the perinuclear clustering of melanosomes) was observed in this cell line (Fig. 5I). If Slp1 and Slp2 were an *in vivo* rab27A effector, localization of Slp1 and Slp2 should be different in the S91/Cloudman melanoma cells from the wild-type cells (Fig. 5, A–E). As expected, Slp2 also accumulated around the nucleus and did not show any peripheral or dendritic localization in contrast to the wild-type mouse-derived B16-F1 cells (Fig. 5, F–J). Similar contrast distribution between wild-type mouse-derived and *dilute* mouse-derived melanoma cells was observed in Slp1 (data not shown). These observations, together with biochemical evidences, strongly indicate that the SHD of the Slp family is indeed an *in vivo* rab27A binding domain and suggest that at least Slp1 and Slp2 are involved in the melanosome transport mechanism probably by functioning as effector molecules of Rab27A.

**Possible Roles of the Slp Family and Slac2s in Rab27A-Dependent Melanosomal Transport**—If the Slp family and Slac2 function as Rab27A effector molecules, how do they function in the transport of melanosomes? While this manuscript was being prepared, Slac2-a was identified as a melanophilin in which a mutation causes a defect in melanosome transport in *leaden* mice (44). It is noteworthy that *ashen* mice carrying a Rab27A mutation and *dilute* mice carrying a myosin Va mutation showed the same defects in pigment granule transport (*i.e.*, clumping of melanosomes in the perinuclear region), and as a result *ashen, leaden*, and *dilute* mice all exhibit a similar lighter coat color (22, 23, 39–45). In addition, genetic analysis has shown that these three proteins function in the same or overlapping transport pathways (44), although the functional relationships between the three molecules (Rab27A, Slac2-a/ melanophilin, and myosin Va) remain to be clarified. Thus, our finding of a specific interaction between Rab27A and the SHD of Slac2-a (Fig. 2G) should be physiologically relevant and implies that their interaction is an essential step in melanosomal transport.

Although recent studies indicated that Rab27A enables myosin Va to capture melanosomes by recruiting myosin Va to melanosomes (40–42), Rab27A did not directly interact with myosin Va in our preliminary experiment, suggesting that one

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or more additional “linker proteins” must be required for functional interaction between Rab27A and myosin Va. Thus, it is highly possible that Slac2-a and the Slp family function as an unidentified linker: The N-terminal SHD binds Rab27A, and the large C-terminal domain may interact with myosin Va. Indeed, some C2 domains are now known to function as a protein interaction site (3–5). Another possible function of the Slp family is that it functions as the “anchoring” protein of Rab27A to melanosomes through the SHD domain and the phospholipid binding C2 domains. These two hypotheses can explain why the subcellular localization of Slp2 (or Slp1) and Rab27A was different between wild-type and melanosome transport-defective melanoma cells and why Slp2 (or Slp1) colocalized with Rab27A independent of myosin Va (Fig. 5). Because Slp2 (or Slp1) was localized at the dendritic tip in B16-F1 melanoma cells (Fig. 5, A–E, insets), it is also possible that the Slp family is involved in the melanosome transfer from melanocytes into keratinocytes, although the mechanism of melanosome transfer itself remains unclear. Further work is necessary to determine the specific step or steps of melanosome transport in which the Slp family is involved.

In summary, we have demonstrated by GST pull-down assays that the SHD of the Slp family and Slac2 function as a novel GTP-Rab27A binding domain. The interaction of the Slp family and Slac2 with Rab27A was confirmed in intact cells. Co-localization of Slp2 (or Slp1), Rab27A, and the melanosome was further demonstrated in the peripheral region of the B16-F1 (wild-type) cell line or in the perinuclear region of S91/Cloudman (defect in melanosome transport) cell line. Our discovery should greatly accelerate understanding of the molecular mechanisms of Rab27A-dependent melanosome transport.

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