Slac2-c (Synaptotagmin-like Protein Homologue Lacking C2 Domains-c), a Novel Linker Protein that Interacts with Rab27, Myosin Va/VIIa, and Actin*

Received for publication, April 22, 2002, and in revised form, August 23, 2002
Published, JBC Papers in Press, September 7, 2002, DOI 10.1074/jbc.M203862200

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Molecular Cloning of Mouse Slac2-c cDNAs—cDNAs encoding the novel region of Slac2-c were amplified from Marathon-Ready adult mouse brain cDNA (Clontech Laboratories, Inc.; Palo Alto, CA) by 5′-rapid amplification of cDNA ends (RACE) as described previously (20). First 5′-RACE reactions were carried out using the adapter primer 1 (5′-CCATCCTAATACGACTCACTATAGGGC-3′) and Slac2-c-stop primer (5′-TTAGTACATCACAGCTGACT-3′; termination codon is shown in boldface letters) designed on the basis of rat and mouse EST sequences (GenBank™ accession numbers BF287121 and BG869374). Second RACE reactions were carried out using the internal adapter primer 2 (5′-ACTCCTAATACGACTCACTATAGGGC-3′) and Slac2-c C1 primer (5′-GCGTTGAGCGGGATTAGTTACGGC-3′). The purified PCR products were inserted directly into the pGEM-T Easy vector (Promega; Madison, WI), and both strands were sequenced completely with a Hitachi SQ-5500 DNA sequencer as described previously (20). cDNAs

Recent studies have suggested that several Rabs (i.e. Rab6, Rab11, and Rab25) are involved in the movement of transport vesicles from their site of formation to their site of fusion, because these Rabs have been found to interact directly with specific microtubule- or actin-based motor proteins (see Refs. 7–9, and reviewed in Ref. 10). For instance, Rab6 interacts with the C-terminal domain of Rabkinetsin-6 (7), whereas Rab11 and Rab25 interact with the C-terminal domain of the myosin Vb tail (8). Very recently, another type of Rab-motor protein interaction has been discovered in melanosomes transport (11–14) and plasma membrane recycling systems (15); myosin Va indirectly recognizes Rab27A on melanosomes via Slac2-c (synaptotagmin-like protein (Slp)1 homologue lacking C2 domains-a) (also called melanophilin), a linker protein that interacts specifically and directly with the GTP-bound form of Rab27A and myosin Va (11, 12, 16, 17), whereas myosin Vb interacts with Rab11 (and Rab25), as well as Rab11-FIP2 (18). Although five members of the Rab11-FIP family have been described to date (15, 18, 19), no information is available for the existence of a Slac2-a homologue (or Slac2 family). Because Rab27A and myosin Va are known to be expressed in tissues other than melanocytes, we hypothesized that other linker protein(s) must exist in the body.

In this report, we report on a novel Slac2-a homologue (named Slac2-c) that interacts specifically with Rab27A/B and myosin Va/VIIa by means of EST database searches and biochemical experiments. We also discovered that the most conserved C-terminal region of Slac2 functions as a novel actin-binding site. Based on our findings, we discuss the role of Slac2 in Rab27A-dependent membrane trafficking.

EXPERIMENTAL PROCEDURES

The Rab family, one of the small GTP-binding protein superfamilies (1), is thought to control intracellular membrane trafficking in eukaryotic cells (reviewed in Refs. 2–4). More than 60 distinct Rab proteins have been identified in humans (5, 6), and they seem to regulate various steps of membrane trafficking (e.g. vesicle formation, docking, tethering, and fusion) (2–4). Although Rab proteins are generally believed to act with specific effector molecule(s) that preferentially bind the GTP-bound form of Rab27A/B both in vitro and in intact cells, and the C-terminal domain of Slac2-c interacts with myosin Va and myosin VIIa. In addition, we discovered that the most C-terminal conserved region of Slac2-a (amino acids 400–580) and Slac2-c (amino acids 670–856), which is not essential for myosin Va binding, directly binds actin and that expression of these regions in PC12 cells and melanoma cells colocalized with actin filaments at the cell periphery, suggesting a novel role of Slac2-a/c in capture of Rab27-containing organelles in the actin-enriched cell periphery.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB083782–3.

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† The abbreviations used are: Slp, synaptotagmin-like protein; BR, brain; GST, glutathione S-transferase; GT, globular tail; HRP, horse-radish peroxidase; MC, melanocyte; RACE, 5′-rapid amplification of cDNA ends; RT, reverse transcriptase; SHD, Slp homology domain; EST, expressed sequence tag; GFP, green fluorescent protein.

* This work was supported in part by grants from the Science and Technology Agency to Japan (to M. F.) and Grant 15780624 from the Ministry of Education, Science, and Culture of Japan (to M. F.). 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.

** The Journal of Biological Chemistry Vol. 277, No. 45, Issue of November 8, pp. 43096–43103, 2002
Printed in U.S.A.
encoding the open reading frame of the mouse Slac2-c were amplified by reverse transcriptase (RT)-PCR from Marathon-Ready adult mouse brain cDNA (5′-GGATCCATGGGGGAGGAGCTGAC-3′; BamHI site is underlined) and Slac2-c stop primers. The purified PCR products were subcloned into the pGEM-T Easy vector (Promega) and verified. DNA sequencing was performed on Slac2-c cDNA using the T7 tag to the N terminus of Slac2-c and construction of the mammalian cell expression vector (named pEF-T7-Slac2-c) were performed as described previously (20–22). The human Slac2-c cDNA was determined by database searching (standard BLAST search) using the mouse Slac2-c as a query.

Molecular Cloning of Mouse Myosin VIIa and Melanocyte (MC)-type Myosin Va cDNAs—cDNA encoding a tail domain of mouse myosin VIIa (23) or MC-type myosin Va (− exon B, + exon D, and + exon F) (24, 25) was amplified from Marathon-Ready adult mouse brain cDNA or spleen cDNA from mouse MTC Panel I (Clontech Laboratories, Inc.), respectively, by RT-PCR as described previously (11, 16). The following oligonucleotides were used for amplification of the mouse myosin VIIa cDNA: 5′-CGGATCCGGGTGTGATACCCCGCGG-3′ (myosin VIIa-N1 primer, sense), 5′-GATGTGGTCAATTGACGCCG-3′ (myosin VIIa-N2 primer, sense), 5′-CTCAAGTATCATGGGGCAGCTA-3′ (myosin VIIa-N3 primer, sense), 5′-GGCTGGAATTGTGAAGCTT-3′ (myosin VIIa-C1 primer, antisense), 5′-CTCAAAAGCTTCTGACGGTA-3′ (myosin VIIa-C2 primer, antisense), and 5′-TCACCTCCCCTGCCTGGTAGTGTGTGTGT-3′ (myosin VIIa-stop primer, antisense). The purified PCR products were inserted directly into the pGEM-T Easy vector and were sequenced completely. We identified one amino acid difference (L1156F) compared with the reported myosin VIIa sequences (GenBankTM accession No. H11032) previously (11). Brain (BR)-type myosin Va cDNA (myosin Va-F-GT (MC-specific exon F

Molecular Cloning and Characterization of Slac2-c—Slac2-a, -Slac2-a-SHD, and -Slac2-a-CTD cDNAs were amplified by PCR using primer pairs as described previously (11, 16). Oligonucleotides with restriction enzyme sites (underlined) or stop codons (in bold): 5′-GGATCCCATGGGGGAGGAGCTGAC-3′ (Slac2-a-D240 primer, sense), 5′-GGATCCATGGGGGAGGAGCTGAC-3′ (Slac2-a-D400 primer, sense), 5′-GCACTCTGATGCTACGG-3′ (Slac2-a-D400 primer, sense), 5′-GCACTCATGATGCTACGG-3′ (Slac2-a-D400 primer, sense), 5′-GCACTCATGATGCTACGG-3′ (Slac2-a-D240 primer, sense), 5′-GCACTCATGATGCTACGG-3′ (Slac2-a-D240 primer, sense). The purified PCR products were subcloned into the pGEM-T Easy vector and were sequenced completely. We identified one amino acid difference (L1156F) compared with the reported myosin VIIa sequences (GenBankTM accession No. H11032) previously (11, 16). Brain (BR)-type myosin Va cDNA (+ exon B, − exon D, and − exon F) (pEF-FLAG-BR-myosin Va) was prepared as described previously (11).

Construction of Deletion Mutants of Slac2-a and Slac2-c—Deletion mutants of Slac2-a (pEF-T7-Slac2-a-Δ146/481, Δ146/321, Δ400, and Δ240) and of Slac2-c (pEF-T7-Slac2-c-Δ136/907, Δ136/494, Δ495/856, and pEF-T7-GST-Slac2-c-Δ136/907) were constructed essentially by conventional PCR as described previously (11, 26) using the following oligonucleotides with restriction enzyme sites (underlined) or stop codons (in bold): 5′-GGATCCCATGGGGGAGGAGCTGAC-3′ (Slac2-a-D240 primer, sense), 5′-GGATCCATGGGGGAGGAGCTGAC-3′ (Slac2-a-D400 primer, sense), 5′-GCACTCATGATGCTACGG-3′ (Slac2-a-D400 primer, sense), 5′-GCACTCATGATGCTACGG-3′ (Slac2-a-D240 primer, sense), 5′-GCACTCATGATGCTACGG-3′ (Slac2-a-D240 primer, sense). The purified PCR products were subcloned into the pGEM-T Easy vector and were sequenced completely. We identified one amino acid difference (L1156F) compared with the reported myosin VIIa sequences (GenBankTM accession No. H11032) previously (11, 16).

Cell Culture, Transfections, and Immunocytochemistry—PC12 cells were cultured on glass-bottom dishes (35-mm dish; MatTek Corp., Ashland, MA) as described previously (31). A 4 μg/ml amount of pEF-T7-Slac2-c-Δ136/907, pEF-T7-Slac2-c-Δ136/907, or pEF-T7-GST-Slac2-c-Δ136/907 plasmid DNA was transfected into PC12 cells by using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Exposure to nerve growth factor (Merck KGaA, Darmstadt, Germany) was performed as described previously (32). Melanoma cell line B16-F1 was cultured as described previously (16). pEGFP-C1-Slac2-a was transfected into melanoma cells by using FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Three days after transfection, cells were fixed in 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 for 2 min, incubated for 1 h at room temperature with anti-TAG-7 (1/4000 dilution; Novagen, Madison, WI) or anti-Rab27A mouse monoclonal antibody (1/250 dilution; Transduction Laboratories; Lexington, KY), and visualized by a second antibody (1/10000 dilution; anti-rabbit Alexa 488 antibodies; Molecular Probes, Inc.; Eugene, OR) or Texas Red-conjugated phalloidin (1/200 or 1/500 dilution; Molecular Probes, Inc.) as described previously (31, 32). The cells were then examined with a confocal fluorescence microscope (Fluoview; Olympus, Tokyo, Japan). Images were pseudo-colored and superimposed with Adobe Photoshop software (version 5.0).

Miscellaneous Procedures—Cotransfection of pEF-T7-Slac2-c and pEF-FLAG-Rabs (or -FLAG-myosin Va) into COS-7 cells (7.5 × 10⁵ cells, the day before transfection/10-cm dish) was performed as described previously (26). Proteins were solubilized with a buffer containing 1% Triton X-100, 250 mM NaCl, 1 mM MgCl₂, 50 mM HEPES-KOH, pH 7.2, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10 μM pepstatin A at 4 °C for 1 h. T7-Slac2-c was immunoprecipitated with anti-TAG-7 antibody-conjugated agarose (Novagen) as described previously (27). Direct interaction of myosin Va-GT with Slac2-c and in vitro formation of the tripartite protein complex (Slac2-c-Rab27A-Amyosin Va-F-GT) were also performed as described previously (11). SDS-PAGE and immunoblotting analyses with HRP-conjugated anti-FLAG tag (Sigma) and anti-T7 tag antibodies (Novagen) were also performed as described previously (20, 26). The blots and gels shown are representative of at least two or three independent experiments.
RESULTS

Identification of Slac2-c, A Novel Homologue of Slac2-a—
Slac2 was identified originally as a protein that contains an N-terminal SHD without tandem C2 domains (29, 35–37). Two forms of Slac2 (Slac2-a and Slac2-b/KIAA0624) have been reported in humans, but they do not show any significant homology except for the SHD (16). Recently, Slac2-a was identified as melanophilin, and a mutation in the \text{mlph} \> gene was discovered to cause defects in melanosome transport in \text{leaden} mice exhibiting a lighter coat color (i.e. accumulation of melanosomes in the perinuclear region) (38). Slac2-a/melanophilin has been shown to function as a linker protein that bridges between Rab27A on melanosomes (39–42) and myosin Va (43–45), an actin-based motor protein, and formation of the tripartite protein complex (Rab27A/\text{H18528}Slac2-a/\text{H18528}myosin Va) is essential for melanosome transport (11–14). To determine whether Slac2-a is the only protein that links Rab27 with myosin Va, we searched for novel linker protein(s) in the mouse, rat, and human EST databases by standard blastn (www.ddbj.nig.ac.jp/E-mail/homology-j.html) using Slac2-a cDNA sequence as a query. We found several EST clones in the mouse, rat, and human EST databases that encode a novel protein exhibiting a significant homology to the C terminus of Slac2-a (GenBank™ accession numbers BG869374 (mouse), BF287121 (rat), and BE550329 (human)). cDNA cloning of this putative linker protein by 5’-RACE revealed that the 2571-base sequence comprises a single open reading frame encoding 856 amino acids. Interestingly, the corresponding protein also contained the highly conserved SHD with two zinc-finger motifs at the N terminus (more than 40% identity to Slac2-a), but not tandem C2 domains (Fig. 1), indicating that it should be classified as a third member of the Slac2 family, and we designated it as Slac2-c. The C terminus of this protein does not contain any known protein motifs and did not exhibit homology to any known proteins reported thus far except the putative myosin Va-binding site of the Slac2-a C terminus (about 25% identity) (Fig. 1), suggesting that Slac2-c functions as a linker protein between Rab27 and myosin Va.

Database searching in the human genome draft sequences revealed that the human ortholog of mouse Slac2-c consists of 859 amino acids (79.7% identity), and the highest homology between the mouse and human Slac2-c was observed in the SHD (90.3% identity). The human \text{slac2-c} \> gene was mapped to 3p21.33–32, whereas the human \text{slac2-a/\text{mlph}} \> and \text{slac2-b} \> were mapped to 2q37.3 and 11q23.1, respectively. The locations of mouse \text{slac2-a/\text{mlph}, slac2-b,} \> and \text{slac2-c} \> were predicted to be on mouse chromosomes 1, 9, and 9, respectively, by Human-Mouse Homology Map analysis (www.ncbi.nlm.nih.gov/Homology/). The exon-intron boundaries of the \text{slac2-c} \> and \text{slac2-a genes} were exactly the same in the SHD and similar in the most C-terminal region but not in the middle of the C-terminal domain, suggesting that the \text{slac2-a and slac2-c genes} may have evolved from the same ancestor.
RT-PCR analysis revealed the highest expression of Slac2-c mRNA in brain, lung, and testis, weak expression in heart, skeletal muscle, and kidney, and absence of expression in spleen and liver (Fig. 2, top panel). By contrast, Slac2-a mRNA was expressed almost ubiquitously in all tissues tested (Fig. 2, middle panel) (38). It should be noted that the mRNAs of Slac2-a and the Slp1–3 were expressed highly at embryonic day 7 (E7) (29), whereas the Slac2-c mRNA was absent completely at this stage (Fig. 2, top and middle panels).

**Slac2-c Functions as a Linker Protein between Rab27 and Myosin Va**—In the next set of experiments, we investigated whether Slac2-c functions as a linker protein between Rab27 and myosin Va both in vitro and in intact cells. We first investigated the specific interaction of full-length Slac2-c with 22 different Rab proteins (Rab1, Rab2, Rab3A, Rab4A, Rab5A, Rab6A, Rab7, Rab8, Rab9, Rab10, Rab11A, Rab17, Rab18, Rab20, Rab22, Rab23, Rab25, Rab27A, Rab27B, Rab28, Rab34, or Rab37) in intact cells as described previously (11, 16). As expected, T7-tagged Slac2-c protein coimmunoprecipitated specifically with the FLAG-tagged Rab27A and Rab27B isoforms but not with any of the other Rabs tested (Fig. 2, top panel). Coimmunoprecipitated (IP) FLAG-Rabs were first detected at the leftmost lane (1/80 volume of the reaction mixture) used for immunoprecipitation. The asterisk indicates the degradation products of T7-Slac2-c. The N-terminal SHDs (SHD1 and SHD2, black boxes) are separated by two zinc-finger motifs (29). The Rab27A/B-binding activity of each mutant is indicated after its name (+ or −). B, specific interaction of Slac2-c with Rab27A and Rab27B. pEF-T7-Slac2-c and pEF-FLAG-Rab were cotransfected into COS-7 cells. The proteins expressed in the presence of exon F, a melanocyte-specific exon, in myosin Va. G3PDH mRNA in brain, lung, and testis, weak expression in heart, skeletal muscle, kidney, and testis and on embryonic days 7 (E7), 11, 15, and 17. The SHD of Slac2-c interacted specifically with Rab27A (lane 3 in Fig. 3C) but not with any of the other Rabs tested (lanes 18 and 19 in Fig. 3B). Deletion mutant analysis further indicated that the SHD alone coimmunoprecipitated efficiently with Rab27A (lane 2 in Fig. 3C) but that the C-terminal domain lacking the SHD (ΔSHD) did not (lane 3 in Fig. 3C). In addition, the SHD of Slac2-c bound a Rab27A(Q78L) mutant (dominant active form that mimics GDP-bound form) the same as the SHD of Slac2-a, Slac2-b, Slp1, Slp2-a, Slp3-a, and Slp5 (11, 16, 46).

We then investigated the interaction between the Slac2-c C terminus with full-length myosin Va in intact cells (11, 12) to determine whether Slac2-c functions as a linker protein between Rab27 and myosin Va. The same as Slac2-a, the full-length Slac2-c and Slac2-c-ΔSHD, but not Slac2-c-ΔSHD, interacted with BR-type myosin Va (Fig. 4B, lanes 1–3). Although the binding of Slac2-c to myosin Va was enhanced greatly by the presence of exon F, a melanocyte-specific exon, in myosin Va (MC-myosin Va) (12), Slac2-c can interact with BR-myosin Va (+ exon B, − exons D and F) (24, 25) (Fig. 4E, compare lanes 2 and 3 in the middle panel). Direct interaction of GST-Slac2-c-ΔSHD with FLAG-myosin Va-GT was also demonstrated by in vitro binding experiments using the purified proteins (Fig. 4C). Formation of the tripartite protein complex (Rab27A-Slac2-c-myosin Va-GT) from the purified components in vitro.
vitro was demonstrated further in Fig. 4D. These results indicated strongly that Slac2-c functions as a linker protein that bridges between Rab27 and myosin Va, the same as Slac2-a.

While this manuscript was being reviewed, Slac2-c has also been identified as MyRIP (myosin-VIIa- and Rab-interacting protein), which may be involved in transport of retinal melanosomes to the actin cytoskeleton (47). Consistent with this finding, Slac2-c interacted with the tail domain of myosin VIIa (Fig. 4E, lanes 1–3). By contrast, however, Slac2-a interacted only with the tail domain of myosin Va but not myosin VIIa (Fig. 4E, lanes 4 and 5).

The C Terminus of Slac2 Interacts Directly with Actin—Although the minimal essential myosin Va-binding site in Slac2-a was mapped to the middle of the Slac2-a molecule (amino acid residues 241–400) (Fig. 5, A and C, bottom panel) (a minimal determinant of myosin Va-binding site has recently been mapped to amino acid residues 367–400 of Slac2-a; see Ref. 48), the C-terminal 200 amino acids of Slac2-a were also conserved in Slac2-c. Accordingly, in the final set of experiments we attempted to determine the function of the most C-terminal region of Slac2-a/c. First, to investigate its possible function as a protein-protein interaction site, recombinant T7-tagged Slac2-a C terminus (T7-Slac2-a/H9004400) was coupled to anti-T7 tag antibody-conjugated agarose beads, and the COS-7 cell lysates were loaded. After washing the beads extensively, proteins that bound the beads were analyzed by 10% SDS-PAGE and Amido Black staining. We found a single protein with an apparent molecular mass of 45,000 daltons (named Fig. 4. The C terminus of Slac2-c, but not the SHD, interacts with myosin Va and myosin VIIa. A, schematic representation of deletion mutants of Slac2-c. The myosin Va-binding activity of each mutant is indicated after its name (+ or −). B, pEF-T7-Slac2-c deletion mutants and pEF-FLAG-BR-myosin Va were cotransfected into COS-7 cells. Coimmunoprecipitated (IP) FLAG-BR-myosin Va and immunoprecipitated T7-Slac2-c are shown in the upper panel (Blot: anti-FLAG, IP: anti-T7) and lower panel (Blot: anti-T7, IP: anti-T7), respectively. The far left lane in the upper panel indicates the total FLAG-BR-myosin Va expressed (1/80 volume of the reaction mixture) used for immunoprecipitation. Note that the C terminus of Slac2-c interacted with BR-myosin Va (lane 3), but the N-terminal SHD did not (lane 2). C, direct interaction of GST-Slac2-c-ΔSHD with FLAG-myosin Va-GT visualized by anti-FLAG tag antibody (top panel) and Amido Black staining (bottom panel). GST-Slac2-c-ΔSHD (open arrowhead), but not GST alone (closed arrowhead), interacted directly with FLAG-myosin Va-GT (closed arrow). D, in vitro formation of the tripartite protein complex from purified components (T7-Slac2-c, FLAG-myosin Va-GT, and Rab27A) visualized by anti-FLAG tag antibody (top panel), anti-Rab27A antibody (middle panel), and Amido Black staining (bottom panel), respectively. E, interaction of Slac2-a/c with myosin Va and myosin VIIa. pEF-T7-Slac2-c (or -Slac2-a) and pEF-FLAG-MC-myosin Va-tail (or -FLAG-myosin VIIa-tail) were cotransfected into COS-7 cells. The proteins expressed were immunoprecipitated by anti-T7 tag antibody-conjugated agarose (11, 20). Coimmunoprecipitated (IP) FLAG-myosins were detected first with HRP-conjugated anti-FLAG tag antibody (1/10,000 dilution) (middle panel; Blot: anti-FLAG, IP: anti-T7). The same blots were then stripped and reprobed with HRP-conjugated anti-T7 tag antibody (1/10,000 dilution) to ensure that the same amounts of T7-Slac2 proteins had been loaded (bottom panel; Blot: anti-T7, IP: anti-T7). The upper panel indicates the total expressed FLAG-myosins (1/80 volume of the reaction mixture) used for immunoprecipitation. Note that Slac2-c bound both myosin Va and VIIa (lanes 1–3), whereas Slac2-a bound only myosin Va (lane 5). The positions of the molecular weight markers (×10^3) are shown on the left.
p45) that was enriched specifically and dramatically by the C terminus of Slac2-a column (Fig. 5C, lane 5, top panel), and use of a specific anti-actin antibody subsequently showed that the p45 protein was actin (Fig. 5C, second panel). Similar results were obtained for the C terminal of Slac2-c (Slac2-c-495/856) (Fig. 5D, top and middle panels, lanes 2 and 4). Direct interaction of the C terminus of Slac2-a (GST-Slac2-a–C400) with purified G-actin and F-actin in vitro was demonstrated in Fig. 5E, indicating that Slac2-a binds both filamentous and globular actin. By contrast, GST alone hardly interacted with both G- and F-actin beads under our experimental conditions (Fig. 5E, lanes 3 and 4). Because the myosin Va-binding site (Fig. 5C, bottom panel) and actin-binding site (Fig. 5C, top panel) were different, Slac2-a bound F-actin and MC-myosin Va C terminus (Fig. 5F) simultaneously. In vitro interaction between Slac2-a and actin in melanoma cells was confirmed further by immunoprecipitation with anti-Slac2-a-SHD antibody (Fig. 5G).

Expression of the Slac2-a/C C Terminus Inhibits Neurite Outgrowth of PC12 Cells—To investigate further whether the C terminus of Slac2-a/c functions as an actin-binding site in intact cells, we expressed either GFP-tagged Slac2-a–C400 or T7-tagged Slac2-a–C400 in nerve growth factor-differentiated PC12 cells. As shown in Fig. 6, most of the Slac2-a/C400 (A–C) and Slac2-c–C400 proteins (D–F) were present at the cell periphery and colocalized closely with actin filaments. It should be noted that PC12 cells abundantly expressing the Slac2-a/C C terminus hardly extended any neurites compared with the control cells and PC12 cells expressing the N-terminal domain of Slac2-a (Slac2-a–SHD) (Fig. 6, G–I). The inhibitory effect of the most C-terminal region of Slac2-a/c on neurite outgrowth may be attributable to inhibition of actin filament remodeling during neurite outgrowth. By contrast, the Slac2-a–SHD proteins were present in the cell body, as well as the tips of neurites, where Rab27A was enriched (i.e. dense-core vesicles (49, 50); see arrowheads in Fig. 6F). Interestingly, when Slac2-a–SHD was expressed in PC12 cells, Rab27A signals

Fig. 5. Direct interaction of the most C-terminal region of Slac2-a/c with actin. A and B, schematic representation of deletion mutants of Slac2-a and Slac2-c, respectively. The hatched boxes indicate the conserved region between Slac2-a and Slac2-c, and they are responsible for actin binding (see below). The shaded box indicates the minimal myosin Va-binding site (see bottom panel in C). C and D, the C-terminal 200 amino acids of Slac2-a and -c interact with actin. pEF-T7-Slac2-a/c deletion mutants were transfected into COS-7 cells, and the T7-tagged proteins expressed were immunoprecipitated with anti-T7 tag antibody-conjugated agarose (11, 20). Copurified proteins were visualized by Amido Black staining (top panels) or anti-actin antibody (1/200 dilution; second panels). The immunoprecipitated T7-Slac2-a/c mutants were visualized by HRP-conjugated anti-T7 tag antibody (third panel in C and bottom panel in D), because they were not detected by Amido Black staining. The bottom panel in C indicates the mapping of myosin Va-F-GT-binding site. Minimal MC-myosin Va-binding site was determined to be amino acids 241–400 of Slac2-a (see also Ref. 48). E, direct interaction between the GST-Slac2-a–C400 and purified G- and F-actin. The purified GST-Slac2-a–C400 (lanes 1 and 2) or GST alone (lanes 3 and 4) were incubated with either G-actin beads or F-actin beads for 1 h. After washing five times with the G-buffer (or F-buffer), proteins trapped with the beads were analyzed by 10% SDS-PAGE. Slac2-a, actin, and myosin Va were visualized with Amido Black staining, anti-actin (1/200 dilution), and HRP-conjugated anti-GST antibody (1/10,000 dilution), respectively. The same blots were then reprobed with anti-actin antibody to ensure that the same amounts of actin had been conjugated to the beads (bottom panel; Blot: anti-actin, IP: anti-actin). Input means 1/80 volume of the reaction mixture used for immunoprecipitation (top panel; Blot: anti-GST). F, direct and simultaneous interaction of Slac2-a with F-actin and FLAG-myosin Va-F-GT (lane 3). The purified FLAG-MC-myosin Va-F-GT and F-actin were incubated for 1 h with glutathione-Sepharose beads coupled with T7-GST-Slac2-a–Δ146 in the F-buffer. After washing three times with the F-buffer without recombinant proteins, proteins trapped with the beads were analyzed by 10% SDS-PAGE. Slac2-a, actin, and myosin Va were visualized with Amido Black staining, anti-actin (1/200 dilution), and HRP-conjugated anti-Flag tag antibodies (1/10,000 dilution), respectively. Input means 1/20 volume of the reaction mixture. G, in vitro interaction between Slac2-a and actin in melanoma cells. Note that both myosin Va (top panel) and actin (middle panel) coimmunoprecipitated with Slac2-a (bottom panel) from the melanoma cell lysates. The positions of the molecular weight markers (×10–5) are shown on the left.
The actin-binding site of Slac2-a/c did not show any significant homology to the known actin-binding motifs; it should be classified as a novel actin-binding motif. Further work is necessary to determine critical residue(s) responsible for actin binding.

Are the functions of Slac2-c and Slac2-a redundant? If not, what is the cargo of the Rab27-Slac2-c/melanophilin (or VIIa) a tripartite protein complex? Genetic analysis of coat color mutation in mouse has indicated that mutations in rab27A (ashen mice; see Ref. 41), slac2-almlph (leaden mice; see Ref. 38), and myoVa (dilute mice; see Ref. 45) resulted in the same lighter coat color (i.e., defects in melanosome transport to the actin-enriched cell periphery) but that their phenotypes, other than in melanocytes, are different. For instance, dilute mice and type II Griscelli syndrome patients (mutation in myoVa gene) exhibit neurological defects (e.g., transport of endoplasmic reticulum to dendrites in the cerebellum) (51, 52), whereas ashen mice and type I Griscelli syndrome patients (mutation in rab27A gene) do not. By contrast, Rab27A-deficient T cells exhibited reduced cytotoxicity and cytolytic granule exocytosis, whereas myosin Va-defective T cells did not (39, 53, 54). These observations suggest that, at least in some tissues, closely related isoforms (Rab27B, myosin Vb, Vc, VIIa, and Slac2-c) may compensate for the function of Rab27A, Slac2-a, or myosin Va (55–58). Because the expression of Slac2-c mRNA was highest in brain, and myosin Va and Rab27B proteins, but not Rab27A protein, were expressed abundantly in brain (59), it is tempting to speculate that the Rab27B-Slac2-c/melanophilin complex functions in specific membrane transport in brain (e.g., in endoplasmic reticulum membrane that contains inositol 1,4,5-trisphosphate receptor) (51, 52, 60). Further work is necessary to identify the specific cargo of Slac2-c-containing vesicles/or- ganelles. An attempt to identify cargoes is now under way in our laboratory.

In summary, we have identified a novel linker protein (named Slac2-c) that bridges between a small GTP-binding protein, Rab27, and a motor protein, myosin Va (or VIIa). We also showed that the most C-terminal region of Slac2-a and Slac2-c directly binds actin and that it may be involved in the tethering of cargo (e.g., melanosomes) in actin-enriched cell periphery.

Acknowledgments—We thank Eiko Kanno and Yukie Ogata for expert technical assistance.

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11. We and others showed recently that Slac2-a/melanophilin is a linker protein that bridges between the GTP-bound form of Rab27A and myosin Va and that the tripartite protein complex (Rab27A-Slac2-amyosin Va) regulates melanosome transport to the actin-enriched cell periphery (11, 12). In the present study, we isolated a novel homologue of Slac2-a, named Slac2-c, that was distributed differently from Slac2-a in mouse tissues (Fig. 2). Slac2-c contains the N-terminal SHD that specifically binds the GTP-bound activated form of Rab27A/B isoforms (Fig. 3), the same as other Slac2 proteins and the Slp family (11, 16, 17, 46), and the C-terminal domain directly binds myosin Va and myosin VIIa (Fig. 4) (47). These results strongly support the idea that Slac2-c also functions as a linker protein between Rab27A/B and myosin Va/VIIa and that Rab27-Slac2-
