Synaptotagmin-like Protein (Slp) Homology Domain 1 of Slac2-a/Melanophilin Is a Critical Determinant of GTP-dependent Specific Binding to Rab27A*

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The N-terminal synaptotagmin-like protein (Slp) homology domain (SHD) of the Slp and Slac2 families has recently been identified as a specific Rab27A-binding domain (Kuroda, T. S., Fukuda, M., Ariga, H., and Mikoshiba, K. (2002) J. Biol. Chem. 277, 9212–9218; Fukuda, M., Kuroda, T. S., and Mikoshiba, K. (2002) J. Biol. Chem. 277, 12432–12438). The SHD consists of two conserved α-helical regions (SHD1 and SHD2) that are often separated by two zinc finger motifs. However, the structural basis of Rab27A recognition by the SHD (i.e. involvement of each region (SHD1, zinc finger motifs, and SHD2) in Rab27A recognition and critical residue(s) for Rab27A/SHD interaction) had never been elucidated. In this study, systematic deletion analysis and Ala-based site-directed mutagenesis showed that SHD1 of Slac2-a/melanophilin alone is both necessary and sufficient for high affinity specific recognition of the GTP-bound form of Rab27A. By contrast, the zinc finger motifs and SHD2 are not an autonomous Rab27A-binding site and seem to be important for stabilization of the structure of the SHD or higher affinity Rab27A binding. In addition, chimeric analysis of Rab3A and Rab27A showed that the specific sequence of the switch II region of Rab27 isoforms (especially Leu-84, Phe-88, and Asp-91 of Rab27A), which is not conserved in the Rab3 or Rab8 isoforms, is essential for recognition by the Slac2-a SHD. Based on these findings, I propose that SHD1 of the Slp and Slac2 families be referred to as RBD27 (Rab-binding domain specific for Rab27A isoforms).

The Rab family belongs to the small GTP-binding protein Ras superfamily and is distinguished from the Ras, Rho, and Arf families by its primary sequences (1). It is widely believed to be a key player in the control of 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 types of intracellular membrane trafficking as well as various steps in membrane trafficking (2–4). The physiological importance of Rab proteins is supported by the fact that dysfunction of certain Rab proteins has been linked to human diseases (reviewed in Ref. 7).

One well known disease associated with Rab genes, human type I Griscelli syndrome (8), and ashen mice (i.e. mutation in the rab27a gene) (9) exhibit defects in melanosome transport in melanocytes and loss of cytotoxic killing activity (i.e. loss of Ca²⁺-regulated granule exocytosis) by cytotoxic T lymphocytes (8–13). The recent discovery of the specific Rab27A effector domain, the synaptotagmin-like protein (Slp)₁ homology domain (SHD) of the Slp family (Slp1/Jfc1, Slp2-a, Slp3-a, Slp4/ granophilin, and Slp5) (14–21) and the Slac2 family (Slp homolog lacking C2 domains; Slac2-a/melanophilin, Slac2-b, and MyRIP/Slac2-c) (22–25),² has greatly improved our understanding of the molecular mechanism of melanosome transport. Genetic and biochemical analyses have indicated that the leaden gene product, Slac2-a/melanophilin (26), directly binds Rab27A on the melanosomes via its N-terminal SHD (16, 22, 23, 25) and the actin-based motor protein myosin Va via its C-terminal domain (22, 23, 25) and that the formation of a tripartite protein complex is required for melanosome transport (22, 23, 25, 27–29). By contrast, however, little is known about the involvement of the Slp and Slac2 families in granule exocytosis in immune systems.

The SHD of the Slp and Slac2 families basically consists of two conserved regions that are predicted to form an α-helix (SHD1 and SHD2) (15), and these regions are often separated by two zinc finger motifs (15, 16), the same as in rabphilin, a Rab3A effector (16, 30, 31). Although the specific interaction between the SHD and Rab27A/B has been well demonstrated (16, 17, 22, 24, 25), the structural basis of the molecular recognition of Rab27A by the SHD (i.e. critical residue(s) for Rab27A (or SHD) recognition and involvement of the three regions (SHD1, zinc finger motifs, and SHD2) in Rab27A binding) had never been elucidated. This information is crucial, however, to understanding the different downstream cascades between Rab3A and Rab27A at the molecular level.

In this study, systematic deletion analyses combined with Ala-based site-directed mutagenesis showed that SHD1 of Slac2-a is both necessary and sufficient for recognition of the GTP-bound form of Rab27A, and the zinc finger motifs were shown to be important for protein stability of the SHD and SHD2 for higher affinity binding to Rab27A (16). Based on these findings, I discuss how the SHD specifically recognizes Rab27A isoforms, but not Rab3 or Rab8 isoforms.

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¹ The abbreviations used are: Slp, synaptotagmin-like protein; SHD, Slp homology domain; GST, glutathione S-transferase; HA, hemagglutinin; HRP, horseradish peroxidase; MyRIP, myosin-VIIa and Rab-interacting protein.

² Fukuda, M., and Kuroda, T. S. (September 7, 2002) J. Biol. Chem. DOI 10.1074/jbc.M203862200.

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**EXPERIMENTAL PROCEDURES**

**Construction of Deletion Mutants of the Slac2-a SHD**—Deletion mutants of the Slac2-a SHD (pEF-T7-GST-Slac2-a-SHD1, pEF-T7-GST-Slac2-a-SHD2, pEF-T7-Slac2-a-SHD1, and pEF-T7-Slac2-a-SHD2) were essentially constructed by conventional PCR as described previously (22, 35–37) using the following oligonucleotides with restriction enzyme sites (underlined) or stop codons (boldface): Slac2-a-SHD1 primer (sense), 5'-CGGATCCAGGAGAATCCAGAGGAGG-3'; Slac2-a-SHD1-3' primer (antisense), 5'-GTCGCAGCTGTCCATGGCACC-3'; Slac2-a-SHD2 primer (antisense), 5'-TAAGTCTGCTGGCAGTGCCAGG-3'; and Slac2-a-SHD2-5' primer (sense), 5'-CGGATCCAGGAGAATCCAGAGGAGG-3'. pEF-HA-Rab27A was similarly constructed by PCR using the following oligonucleotide: HA-Rab27A primer (sense), 5'-CCACATGGGCTTACCTGGATGACAGG-3'.

**Rab27A-binding Determinants of Slac2-a**—The SHD of Slac2-a consists of two SHD repeats (SHD1 and SHD2) (16) that are predicted to form an α-helix. The SHD1 repeats are predicted to be located in the amino-terminal region of Slac2-a and to form a hydrophobic core that is essential for recognition of the GTP-bound form of Rab27A (15). SHD2 is predicted to be located in the carboxyl terminus of Slac2-a and to form a hydrophobic core that is essential for recognition of the GDP-bound form of Rab27A (15). The SHD of Slac2-a consists of two SHD repeats (SHD1 and SHD2) (16) that are predicted to form an α-helix. The SHD1 repeats are predicted to be located in the amino-terminal region of Slac2-a and to form a hydrophobic core that is essential for recognition of the GTP-bound form of Rab27A (15). SHD2 is predicted to be located in the carboxyl terminus of Slac2-a and to form a hydrophobic core that is essential for recognition of the GDP-bound form of Rab27A (15).

**Site-directed Mutagenesis**—Mutant Slac2-a plasmids carrying an E14A, V18A, V21A, R24A, E32A, L39A, or CA (C64A/C67A/C81A/C84A) were produced as described previously (32, 37). The phospholipid binding activity of each mutant (or -2) is indicated. The amino acid positions are indicated on both sides. B, interaction of Slac2-a mutants with Rab27A. pEF-T7-Slac2-a mutants and pEF-FLAG-Rab proteins were co-transfected into COS-7 cells. The proteins were expressed and solubilized with 1% Triton X-100 and immunoprecipitated with anti-T7 tag antibody-conjugated agarose (32). The amino acid positions are indicated on both sides. B, interaction of Slac2-a mutants with Rab27A. pEF-T7-Slac2-a mutants and pEF-FLAG-Rab proteins were co-transfected into COS-7 cells. The proteins were expressed and solubilized with 1% Triton X-100 and immunoprecipitated with anti-T7 tag antibody-conjugated agarose (32). The amino acid positions are indicated on both sides. B, interaction of Slac2-a mutants with Rab27A. pEF-T7-Slac2-a mutants and pEF-FLAG-Rab proteins were co-transfected into COS-7 cells. The proteins were expressed and solubilized with 1% Triton X-100 and immunoprecipitated with anti-T7 tag antibody-conjugated agarose (32). The amino acid positions are indicated on both sides. B, interaction of Slac2-a mutants with Rab27A. pEF-T7-Slac2-a mutants and pEF-FLAG-Rab proteins were co-transfected into COS-7 cells. The proteins were expressed and solubilized with 1% Triton X-100 and immunoprecipitated with anti-T7 tag antibody-conjugated agarose (32).

**RESULTS**

**SHD1 of Slac2-a Functions as a Critical Determinant of Specific GTP-Rab27A Binding**—The SHD of Slac2-a consists of three regions, SHD1, zinc finger motifs, and SHD2 (Fig. 1A) (15), and SHD1 and SHD2 are predicted to form an α-helix by Chou and Fasman secondary structure analysis (DNASIS Version 3.6, Hitachi Software Engineering Co., Ltd.), the same as the Rab3A-binding domain of rabphilin (31). The SHD1 repeats are predicted to be located in the amino-terminal region of Slac2-a and to form a hydrophobic core that is essential for recognition of the GTP-bound form of Rab27A (15). SHD2 is predicted to be located in the carboxyl terminus of Slac2-a and to form a hydrophobic core that is essential for recognition of the GDP-bound form of Rab27A (15). The SHD of Slac2-a consists of two SHD repeats (SHD1 and SHD2) (16) that are predicted to form an α-helix. 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Fig. 2. SHD1 of Slac2-a determines Rab27A-binding specificity. A–E, pEF-T7-Slac2-a mutants and pEF-FLAG-Rab3A, pEF-FLAG-Rab8A, pEF-FLAG-Rab10, or pEF-FLAG-Rab27A were cotransfected into COS-7 cells. The proteins expressed were immunoprecipitated with anti-T7 tag antibody-conjugated agarose as described previously (32). Co-immunoprecipitated FLAG-Rab proteins were first detected with HRP-conjugated anti-FLAG tag antibody (1:10,000 dilution) (B–E, upper panels). The same blots were then stripped and reprobed with HRP-conjugated anti-T7 tag antibody to ensure that the same amounts of mutant T7-Slac2-a proteins had been loaded (B–E, lower panels). Total expressed FLAG-Rab proteins (0.0125 volumes of reaction mixtures; input) used for immunoprecipitation are shown in A. Note that SHD1 alone is sufficient for specific binding to Rab27A (D, lane 4). The T7-Slac2-a-SHD proteins often migrated as doublet bands upon SDS-PAGE, and they were probably produced by protein degradation or post-translational modification(s). The position of a molecular mass marker (in kilodaltons) is shown on the left.

Fig. 3. SHD1 of Slac2-a functions as a GTP-Rab27A-binding site. A–D, pEF-T7-Slac2-a mutants and pEF-FLAG-Rab27A(Q78L) or pEF-FLAG-Rab27A(T23N) were cotransfected into COS-7 cells. The proteins expressed were immunoprecipitated with anti-T7 tag antibody-conjugated agarose as described previously (22, 32). Co-immunoprecipitated FLAG-Rab proteins were first detected with HRP-conjugated anti-FLAG tag antibody (1:10,000 dilution) (B–D, upper panels). The same blots were stripped and reprobed with HRP-conjugated anti-T7 tag antibody to ensure that the same amounts of mutant T7-Slac2-a proteins had been loaded (B–D, lower panels). Total expressed FLAG-Rab27A proteins (0.0125 volumes of reaction mixtures; input) used for immunoprecipitation are shown in A. Note that SHD1 alone is sufficient for recognition of Rab27A(Q78L), a dominant-active form that mimics the GTP-bound form (lane 1), but not Rab27A(T23N), a dominant-negative form that mimics the GDP-bound form (lane 2).

The initial focus on six highly conserved amino acids in SHD1 (Glu-14, Val-18, Val-21, Arg-24, Glu-32, and Leu-39 of Slac2-a) (Fig. 4A, arrowheads). However, mutation to an Ala residue never greatly altered the a-helical propensity of SHD1 by secondary structure prediction (data not shown). As shown in Fig. 4B, Glu-14, Val-18, Val-21, and Glu-32 were found to be essential for Rab27A binding, whereas mutation of Arg-24 and Leu-39 had little effect on Rab27A binding. It should be pointed out that a small deletion (amino acids 31–37 of Slac2-a) in SHD1 has been found in leaden mice, which exhibit a lighter coat color (i.e. accumulation of melanosomes in the perinuclear region) due to defects in melanosome transport (Fig. 4A, solid line) (26). Because the deletion contains one of the critical residues for Rab27A binding (Glu-32), I hypothesized that Slac2-a in leaden mice is unable to recognize Rab27A on the melanosome. As expected, neither Slac2-a-SHD(leaden) nor full-length Slac2-a(leaden) bound Rab27A (Fig. 4, C and D, middle panels), whereas both wild-type and mutant Slac2-a(leaden) did bind myosin Va (Fig. 4D, upper panel). These results indicate that the defect in melanosome transport observed in leaden mice is attributable to lack of Rab27A binding (i.e. inability to form the tripartite protein complex (Rab27A-Slac2-a-myosin Va) essential for melanosome transport) (22, 23, 25).

Role of the Zinc Finger Motifs of Slac2-a in Stabilization of the SHD—Although zinc finger motifs themselves are not essential for Rab27A binding, they are often found between SHD1 and SHD2 in the Slp and Slac2 families and thus should have an important role in the SHD. Interestingly, the zinc finger motifs of the SHD exhibited weak homology to the FYVE domain of early endosome antigen-1 (Fig. 5A, asterisks), a recently identified phosphatidylinositol 3-phosphate-binding
Two independent phospholipid binding assays were performed (liposome sedimentation and dot-blot assays) to determine whether the zinc finger motifs of the Slac2-a SHD function as a phosphatidylinositol 3-phosphate-binding domain, but no significant phosphatidylinositol 3-phosphate-binding activity of the Slac2-a SHD was observed (data not shown).

Because the protein expression level of the Slac2-a-SHD(CA) mutant was always much lower than that of the wild-type protein, as noted above, I next evaluated the protein stability property of the Slac2-a-SHD(CA) mutant by limited proteolysis (Fig. 5B). To do so, both the wild-type and mutant SHDs were first purified with anti-T7 tag antibody-conjugated agarose (16). At low trypsin concentrations (5 ng/ml), both wild-type and mutant T7-Slac2-a-SHD (Fig. 5B, arrows) were resistant to trypsin (lane 2). At 10 ng/ml trypsin, wild-type T7-Slac2-a-SHD was still resistant; and an ~16–17-kDa tryptic fragment of the Slac2-a SHD was protected (Fig. 5B, upper panel, lanes 3 and 4, asterisk), but it was not detected at 100–500 ng/ml trypsin. By contrast, the Slac2-a-SHD(CA) mutant was rapidly degraded at 10 ng/ml trypsin (Fig. 5B, lower panel, lane 3). These results indicate that the zinc finger motifs of the Slac2-a SHD are involved in the stability property of the SHD rather than in Rab27A recognition.

Identification of Determinants on the Rab27A-SHD Complex in Rab27A—Finally, I attempted to determine structural determinants on the Rab27A-SHD complex in Rab27A. Because rabphilin recognizes Rab3A, Rab8A, and Rab27A, whereas Slac2-a recognizes only Rab27 isoforms (16, 22), the amino acid sequences of Rab3A/B/C/D, Rab8A/B, and Rab27A/B were first compared (Fig. 6A). Several amino acids were found to be conserved only in the Rab27 isoforms, and not in the Rab3 or Rab8 isoforms (e.g. Glu-48, Asp-91, Cys-123, and Cys-188 in Rab27A). Among these residues, I especially focused on the amino acids present in the switch I and switch II regions because, in the rabphilin-Rab3A complex,
Higher affinity Rab27A binding possibly by stabilizing Rab27A bound to SHD1 rather than recognition of Rab27A itself. The accessory role of SHD2 of Slac2-a in high affinity Rab27A binding seems to be critical under physiological conditions because the expression level of ΔSHD2 protein is very low in COS-7 cells (see above), and all of the Rab27A-binding proteins reported thus far contain both SHD1 and SHD2 (15–19, 24). Moreover, a search of the human (and mouse) genome and expressed sequence tag data bases failed to retrieve any other protein(s) containing SHD1 alone without SHD2 (data not shown). The zinc finger motifs are essential for the stability property of the whole SHD of Slac2-a and are not involved in Rab27A binding. Therefore, even without the zinc finger motifs, the SHD of Slp1, Slp2-a, and Slac2-b recognizes Rab27A with high affinity (16).

How does the SHD of Slac2-a specifically recognize the Rab27 isoforms? Based on the following evidence, I inferred that the fundamental structure of the Rab27A-SHD complex is similar to that of the rabphilin-Rab3A complex previously reported (31). First, Rab27A is the Rab that is phylogenetically closest to the Rab3 isoforms (6), and the SHD of Slac2-a exhibits significant homology to the Rab3a-binding domain of rabphilin and shares the same domain structures (a1, two zinc motifs, and a2) (16, 26, 31). Second, the N-terminal a-helical region (a1) of Rim1 and rabphilin, corresponding to SHD1, but not the zinc finger motifs or a2, is the minimal essential domain for Rab3a binding (48, 49), the same as SHD1 being critical for Rab27A binding. Third, the a1-region of rabphilin directly interacts with the switch I and switch II regions of Rab3A (Fig. 6A) (31). Similarly, SHD1 of Slac2-a presumably recognizes specific amino acids in the switch II region of Rab27A (e.g. Leu-84, Phe-88, and Asp-91) because mutation(s) in the switch II region in Rab27A or SHD1 of Slac2-a completely abrogated Rab27A/Slac2-a interaction (Figs. 4 and 6). All of these similarities between rabphilin-Rab3A and Rab27A/Slac2-a complexes suggest that the Rab3a-binding domain of rabphilin and Rim1 and the SHD of the Slp and Slac2 families may be derived from a common ancestor and have acquired different Rab-binding specificity during evolution as a result of changes in the nature of the a1-region. Consistent with this, the SHD of the Slp and Slac2 families forms a branch distinct from the Rab3-binding domain of Rim1, Rim2, rabphilin, and Noc2 in the phylogenetic tree (16).

One critical determinant of Rab27A recognition by the Slac2-a SHD is a Gly-to-Asp variation in the switch II region of Rab27A (Fig. 6A) because the Rab27A(D91G) mutant hardly bound to the Slac2-a SHD. It should be noted that the corresponding position is occupied by Gly in most other Rab proteins, including Rab3A/B/C/D and Rab8/A/B (data not shown). However, the Gly-to-Asp variation in the switch II region should not be sufficient for specific Rab27A recognition by the SHD, and the surrounding sequence around the Gly-to-Asp variation must also be important for its specific recognition. For instance, the Gly-to-Asp variation (boldface) in the switch II region was also found in Rab6 (GQERFRSLIPSYRDST) and Rab37 (GQERFRSVTHAYYRDQ), which do not interact with the Slac2-a SHD (16); but the surrounding sequence in the switch II region of these Rab proteins is different from that in Rab27A (e.g. di-Phe residues are missing in Rab6 and Rab37, and YI or YY residues occupy the corresponding positions (underlined above)). Therefore, the SHD of Slac2-a is likely to recognize a specific sequence in the switch II region of the Rab27 isoforms (i.e. Leu-84, Phe-88, and Asp-91 of Rab27A/B). Additional three-dimensional analysis is necessary to determine the involvement of each of the amino acids in the switch II region of Rab27A in the recognition by the SHD. In summary, I have demonstrated that...
Rab27A(L84I/F88Y/D91G) mutant did not interact with Slac2-a (lane 6). The positions of the molecular mass markers (in kilodaltons) are shown on the left. The upper panel indicates total expressed FLAG-Rab (0.0125 volume of the reaction mixture; input) used for immunoprecipitation. Note that the amino acid substitutions are indicated below in pEF-T7-rabphilin (tated (31). The amino acid substitutions are indicated below in parentheses. Amino acid numbers are indicated on the right. B, pEF-T7-Slac2-a (left panels) or pEF-T7-rabphilin (right panels) and pEF-FLAG-Rab27A mutants or pEF-FLAG-Rab3A were cotransfected into COS-7 cells. Co-immunoprecipitated IP–FLAG-Rab27A and immunoprecipitated T7-Slac2-a-SHD proteins are shown in the middle and lower panels, respectively. The upper panel indicates total expressed FLAG-Rab (0.0125 volume of the reaction mixture; input) used for immunoprecipitation. Note that the Rab27A(L84I/F88Y/D91G) mutant did not interact with Slac2-a (lane 6), but it did interact with rabphilin (lane 9), a Rab3A-, Rab8A-, and Rab27A-binding protein (16). The positions of the molecular mass markers (in kilodaltons) are shown on the left.

SHD1 of Slac2-a, but not the zinc finger motifs or SHD2, is both necessary and sufficient for high affinity specific recognition of the GTP-bound form of Rab27A, and I propose that SHD1 of the Slp and Slac2 families be referred to as RBD27 (Rab-binding domain specific for Rab27 isoforms).

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