Slac2-a/Melanophilin, the Missing Link between Rab27 and Myosin Va

IMPLICATIONS OF A TRIPARTITE PROTEIN COMPLEX FOR MELANOSOME TRANSPORT*

Mitsunori Fukuda‡§, Taroho S. Kuroda‡, and Katsuhiko Mikoshiba‡§

From the ‡Laboratory for Developmental Neurobiology, Brain Science Institute, RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako, Saitama 351-0198, Japan and the ¶Division of Molecular Neurobiology, Department of Basic Medical Science, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

Myosin Va is a member of the unconventional class V myosin family, and a mutation in the myosin Va gene causes pigment granule transport defects in human Griscelli syndrome and dilute mice. How myosin Va recognizes its cargo (i.e. melanosomes), however, has remained undetermined over the past decade. In this study, we discovered Slac2-a/melanophilin to be the “missing link” between myosin Va and GTP-Rab27A present in the melanosome. Deletion analysis and site-directed mutagenesis showed that the N-terminal Slp (synaptotagmin-like protein) homology domain of Slac2-a specifically binds Rab27A/B isoforms and that the C-terminal half directly binds the globular tail of myosin Va. The tripartite protein complex (Rab27A/Slac2-a/myosin Va) in melanoma cells was further confirmed by immunoprecipitation. The discovery that myosin Va indirectly recognizes its cargo through Slac2-a, a novel Rab27A/B effector, should shed light on molecular recognition of its specific cargo by class V myosin.

The synaptotagmin-like protein (Slp) family is classified as a subfamily of C-terminal-type tandem C2 proteins (1–7) and was originally defined as an N-terminal Slp homology domain (SHD) (5) and C-terminal tandem C2 domains, putative Ca2+ binding motifs (known as the C2A domain and C2B domain) (8). To date, four members of the Slp family (Slp1/Jfc1, Slp2-a, Slp3-a, and Slp4/granuphilin-a) have been described in the mouse and human (4, 5, 9, 10), and several alternatively splicing isoforms have been identified in Slp2, Slp3, and Slp4 (5, 10).

The Slp family contains two conserved domains at the N terminus, referred to as SHD1 and SHD2 (5). The SHD1 and SHD2 of Slp3-a and Slp4 are separated by a sequence containing two zinc-finger motifs, whereas Slp1 and Slp2-a lack such motifs, and their SHD1 and SHD2 are linked together (5). The SHD has also been found in other proteins, including Slac2-a (Slp homologue lacking C2 domains-a) and Slac2-b/KIAA0624 (11), suggesting a general role of the SHD in cellular signaling.

Two very recent important discoveries have been made concerning the functional relationship between the SHD and Rab27A, one of the small GTP-binding proteins believed to be essential for membrane trafficking in eukaryotic cells (12). The first was our discovery that the SHD of the Slp family and Slac2-a directly interact with the GTP-bound form of Rab27A both in vitro and in intact cells (13). Since mutations in the rab27A gene cause hemophagocytic syndrome (Griscelli syndrome), an uncontrolled T lymphocyte and macrophage activation syndrome in humans (14, 15), and defects in granule exocytosis in cytotoxic T lymphocytes and melanosome transport in ashen mice (16–19), we hypothesized that the Slp family and Slac2 are involved in such membrane trafficking (13). The second very recent important discovery is the identification of Slac2-a as melanophilin and that a mutation in the mlph gene causes defects in melanosome transport in leaden mice (20). Interestingly ashen mice, which carry a mutation in the rab27A gene, and dilute mice, which carry a mutation in the myosin Va gene, which encodes one of the actin-based motor proteins (21, 22), have shown similar 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 similarly lighter coat color (14, 16, 19, 23–25). In addition, genetic analysis has shown that these three proteins function in the same or overlapping transport pathways (20), but the functional relationships between these three molecules, Rab27A, Slac2-a/ melanophilin, and myosin Va, in melanosome transport remained to be clarified (25).

In this study, we report on two domain structures of Slac2-a, the N-terminal SHD, which specifically interacts with the Rab27A and Rab27B isoforms, and the C-terminal domain, which directly interacts with the globular tail of myosin Va, and we discuss the role of the tripartite protein complex in melanosome transport based on our findings.

MATERIALS AND METHODS

Molecular Cloning of Mouse Rab27B, Rab34, and Myosin Va cDNAs—cDNA encoding a full open reading frame of mouse Rab27B, Rab34, and myosin Va was amplified from Marathon-Ready adult brain cDNA (CLONTECH) by reverse transcriptase PCR as described previously (26). The purified PCR products were directly inserted into the pGEM-T Easy vector (Promega, Madison, WI). Both strands were completely sequenced using a Hitachi SQ-5500 DNA sequencer. We identified one deletion as a result of alternative splicing (deletion of amino acids 1387–1411) and several amino acid differences (R695A, D904E, K905N, Q911R, R912K, V913I, R917K, E941K, R942W, K947R, R951Q, R953W, and F954L).

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‡ To whom correspondence should be addressed. Tel.: 81-48-467-9745; Fax: 81-48-467-9744; E-mail: mnfukuda@brain.riken.go.jp.

§ The abbreviations used are: Slp, synaptotagmin-like protein; GST, glutathione S-transferase; GT, globular tail; HRP, horseradish peroxidase; SHD, Slp homology domain; Slac2-a, Slp homologue lacking C2 domains.
and V905L) compared with the reported myosin Va sequences (24), and addition of the FLAG tag (or T7 tag) to the N terminus of Rab54 (pEF-FLAG-Rab54) or to the N terminus of myosin Va (pEF-T7- or FLAG-myosin Va) and construction of the plasmids was performed as described previously (26, 29). pEF-T7-Slac2-a, -FLAG-Slac2-a, and other -FLAG-Rabs were prepared as described previously (13).

Construction of Deletion Mutants of Slac2-a and Myosin Va and Site-directed Mutagenesis—Deletion mutants of Slac2-a (pEF-T7-Slac2-a-H9004 and -H9004-1146) and of myosin Va (pEF-FLAG-myosin Va-GT (globular tail)) were essentially constructed by conventional PCR as described previously (28, 30) using the following oligonucleotides with restriction enzyme sites (underlined) or stop codons (in bold): 5'-CCG-CATTCCGAGGTGGATCTGAGCC-3' (Slac2-a-H9004 primer; sense), 5'-GGCAGTATCGTCAGATCCTCCACCATCC-3' (Slac2-a-H9004 3'-primer), and 5'-GCGATGCAAGAACAGGATAAACTGTT-3' (myosin Va-H11417 primer; sense). pEF-T7-Slac2-a-A41 carrying a SLEWY-to-ALEA substitution (Fig. 1A) was constructed by two-step PCR techniques as described previously using the following mutagenic oligonucleotides (31): 5'-GGCGGCCGCCAGACCCGCTTCCATCC-3' (A4 primer 1; antisense) and 5'-GGCGGCCGCCAGACCCGCTTCCATCC-3' (A4 primer 2; sense).

Direct Interaction between Slac2-a and Myosin Va—pEF-T7-GST-Slac2-a-H9004, -T7-GST-Slac2-a, -T7-Rab27/27A, -T7-GST-myosin Va-GT, and pEF-T7-GST vectors were constructed by PCR essentially as described elsewhere. 2 COS-7 cells (5 10^6 cells) were lysed by sonication for 10-30 s in 0.5 ml of a buffer containing 50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin and 10 mM pepstatin A in a glass-Teflon Potter homogenizer with 10 strokes at 900-1000 rpm. After solubilization with 1% Triton X-100, insoluble material was removed by centrifugation at 15,000 rpm for 10 min. Extracts were mixed with each GST fusion protein and affinity-purified on glutathione-Sepharose beads (wet volume, 20 ml; Amersham Biosciences) to remove the thrombin. Protein concentrations were estimated by 10% SDS-PAGE or determined with a Bio-Rad protein assay kit using bovine serum albumin as a reference.

The purified FLAG-myosin Va-GT protein and Rab27/27A were incubated with glutathione-Sepharose beads (wet volume, 20 ml) either coupled with T7-GST-Slac2-a or T7-GST alone in 50 ml HEPES-KOH, pH 7.2, 100 mM NaCl, 1 mM MgCl2, and 0.2% Triton X-100 for 1 h at 4 °C. After washing three times with 1 ml of the binding buffer without recombinant proteins, proteins trapped with the beads were analyzed by 10% SDS-PAGE followed by immunoblotting with horseradish (HRP)-conjugated anti-FLAG tag antibody (Sigma) and anti-Rab27 mouse monoclonal antibody (Transduction Laboratories, Lexington, KY) as described previously (26, 30).

Antibody Production and Immunoprecipitation—cDNA encoding the C terminus of myosin Va (amino acids 401-590) was amplified by conventional PCR and subcloned into the pGEX-4T-3 vector (named pGEX-4T-3 myosin Va). The plasmid was linearized with NotI and transcribed in vitro using T7 RNA polymerase. The resulting RNA was translated in reticulocyte lysate, and the resulting proteins were affinity-purified by glutathione-Sepharose and detected by subsequent immunoblotting.

RESULTS AND DISCUSSION

The SHD of Slac2-a Specifically Interacts with Rab27A/B Isoforms—We recently discovered that the SHD of Slac2-a/b directly binds the GTP-bound form of Rab27A in vitro and in intact cells (13). However, it remained undetermined whether the full-length Slac2-a protein specifically recognizes the Rab27A molecule and whether Rab27A interacts with the SHD alone or also with the large Slac2-a C-terminal domain with unknown function. To address these issues, we first investigated the specific interaction of full-length Slac2-a with various 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 by co-expression assay (30, 35). In brief, FLAG-tagged Slac2-a and each of the FLAG-tagged Rabs were co-expressed in COS-7 cells, and T7-Slac2-a protein was immunoprecipitated with the anti-T7 antibody-conjugated agarose (26, 30). As expected, T7-Slac2-a protein specifically co-immunoprecipitated with the FLAG-Rab 27A and -Rab27B isoforms but not with other Rabs (Fig. 1B, lane 18, and D, lane 1). The faint signal observed in Fig. 1B, lane 20, was probably attributable to nonspecific interaction of Rab34 with the agarose beads.

Next we investigated the possible involvement of the C terminus of Slac2-a in Rab27A/B binding in the same co-transfection assay. The SHD domain alone efficiently co-immunoprecipitated with both the Rab27A and Rab27B isoforms (Fig. 1, C and D, lane 2), but the C-terminal domain lacking the SHD (A146) did not (Fig. 1, C and D, lane 3). In addition, the Slac2-a mutant carrying the SLEWY-to-ALEA substitution in SHD2 (referred to as Slac2-a(A4)), see Fig. 1A) dramatically reduced the Rab27A/B binding activity (Fig. 1, C and D, lane 4). Consistent with this, crystallographic analysis has shown the corresponding sequence (SGAWFF) in rabphilin-3 directly interacts with Rab3A (36). These findings indicated that the SHD of Slac2-a specifically binds Rab27A/B isoforms but that the large C-terminal domain is not involved in the recognition of Rab27A/B molecules and might have different functions.

Two Domain Structures of Slac2-a: the N-terminal SHD Responsible for Rab27A/B Binding and the C-terminal Domain Responsible for Myosin Va Binding—The results of a genetic analysis comparing dilute, ashen, and leader mice have indicated that myosin Va, Rab27A, and Slac2-a function in the same or overlapping transport pathways in melanosome transport (20). Consistent with this, myosin Va in extracts from melanocytes has been shown to co-immunoprecipitate with Rab27A (19). However, when FLAG-Rab27A and T7-myosin Va were co-expressed in COS-7 cells, no Rab27A/myosin Va complex was detected in the cell lysates (Fig. 2A, lane 8), indicating that an additional protein must link Rab27A and myosin Va.

2 M. Fukuda, manuscript in preparation.
Since the SHD of Slac2-a specifically binds Rab27A (Fig. 1), we hypothesized that Slac2-a is the missing link between Rab27A and myosin Va in melanosome transport. To test this hypothesis, three proteins (FLAG-Rab27A, T7-Slac2-a, and T7-myosin Va) were co-expressed in COS-7 cells, and their associations were analyzed by immunoprecipitation as described above (26, 35). As expected, in the presence of full-length T7-Slac2-a, T7-myosin Va co-immunoprecipitated with FLAG-Rab27A (Fig. 2A, lane 5), whereas in the absence of T7-Slac2-a, T7-myosin Va was undetectable in the anti-FLAG antibody immunoprecipitants (Fig. 2A, lane 8). Interestingly the SHD alone (Slac2-a-SHD) or the C-terminal half alone (Slac2-aΔ146) failed to mediate co-immunoprecipitation of T7-myosin Va with FLAG-Rab27A (Fig. 2A, lanes 6 and 7), suggesting that different domains of Slac2-a may be involved in Rab27A binding and myosin Va binding. Similar results were obtained when FLAG-Rab27B was used instead of FLAG-Rab27A (data not shown).

We next sought to identify the myosin Va-binding site in Slac2-a by dual tag (T7 and FLAG) co-expression assay. When T7-Slac2-a deletion mutants and FLAG-myosin Va were co-expressed in COS-7 cells, T7-Slac2-aΔ146, but not T7-Slac2-aΔHID, co-immunoprecipitated with FLAG-myosin Va (Fig. 2B), indicating the two domain structures of Slac2-a: the N-terminal SHD involved in the GTP-bound form of Rab27A binding and the C-terminal domain involved in myosin Va binding.
and Amido Black staining (lower panel) FLAG-myosin Va-GT visualized by anti-FLAG antibody (upper panel) Va-GT (1/80 volume of the reaction mixture) used for immunoprecipitation—acted with both FLAG-myosin Va-GT (and Rab27A) visualized by anti-FLAG antibody (top panel) tripartite protein complex from purified components (GST-Slac2-a, open box) including the IQ motif, and a proximal tail, medial tail (shaded boxes), and globular tail (black box) (see also Fig. 4 and Ref. 21). The position of the PEST sequence is indicated by PEST.

Since Slac2-a is expressed in various tissues, including the brain (20), the Slac2-a-Rab27A-myosin Va complex may be involved in other types of membrane trafficking. For instance, Slac2-a-Rab27B-myosin Va may be involved in endoplasmic reticulum transport to dendrites in neurons because the inositol 1,4,5-trisphosphate receptor on the endoplasmic reticulum 

**Fig. 3.** Mapping of the domain responsible for Slac2-a binding in myosin Va. A, schematic representation of mouse myosin Va. Myosin Va consists of an N-terminal head (motor) domain (hatched box), a neck domain (open box) including the IQ motif, and a proximal tail, medial tail, and FLAG (open arrowhead) co-expression assay, and as shown in Fig. 3B, Slac2-aΔ146 interacted with the globular tail of myosin Va in intact cells. We used purified recombinant proteins (GST-Slac2-a (or -Δ146), Rab27A, and FLAG-myosin Va-GT) for an in vitro binding assay to further confirm the direct interaction between the C terminus of Slac2-a and the globular tail of myosin Va as well as the in vitro formation of a tripartite protein complex from purified components. As expected, FLAG-myosin Va-GT bound GST-Slac2-aΔ146 but not GST alone (Fig. 3C, lane 2, arrow), and full-length Slac2-a bound both FLAG-myosin Va-GT and Rab27A (Fig. 3D, arrow).

The Tripartite Protein Complex (Rab27A-Slac2-a-Mycosin Va) in Melanoma Cells—Lastly, immunoprecipitation analysis was performed to investigate whether the tripartite protein complex (Rab27A-Slac2-a-myosin Va) is formed at physiological conditions. As shown in Fig. 3F, both myosin Va and Rab27A were co-immunoprecipitated with anti-Slac2-a-specific antibody (Fig. 3E), but not control IgG, from melanoma cell lysates. Thus, the tripartite protein complex (Rab27A-Slac2-a-myosin Va) demonstrated by in vitro binding experiments should be physiologically relevant.

**Conclusions**—The results of a recent biochemical analysis have suggested that the tail domain of myosin Va (or Vb) recognizes its cargo by directly binding to the proteins present in the cargo (e.g., Rab11, Rab25, and synaptobrevin-synaptophysin complex) (37–39). However, since myosin Va did not directly recognize Rab27A in the melanosomes, an additional linker protein was proposed to assist melanosome recognition in melanosome transport (25). In the present study, we discovered that Slac2-a is a missing link between Rab27A and myosin Va in melanosome transport and demonstrated how myosin Va recognizes its specific cargo (i.e., melanosomes) by its globular tail domain. The possible role of the tripartite protein complex (Slac2-a, Rab27A, and myosin Va) in melanosome capture in actin-rich cell periphery is summarized in Fig. 4. The SHD of Slac2-a specifically binds the GTP-Rab27A in the melanosomes, and the C terminus of Slac2-a binds the globular tail of myosin Va, which binds actin filament via the head domain. Since Slac2-a is expressed in various tissues, including the brain (20), the Slac2-a-Rab27a-myosin Va complex may be involved in other types of membrane trafficking. For instance, Slac2-a-Rab27B-myosin Va may be involved in endoplasmic reticulum transport to dendrites in neurons because the inositol 1,4,5-trisphosphate receptor on the endoplasmic reticulum.
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does not migrate to the postsynaptic spines in dilate mice (40, 41). Further work is necessary to define the universality and/or specialty of the tripartite protein complex, Slac2-a, Rab27A/B, and myosin Va in membrane trafficking.

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