Missense mutations in the globular tail of myosin-Va in \textit{dilute} mice partially impair binding of Slac2-a/melanophilin

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Summary

The well-known coat-color mutant mouse \textit{dilute} exhibits a defect in melanosome transport, and although various mutations in the \textit{myosin-Va} gene, which encodes an actin-based motor protein, have been identified in \textit{dilute} mice, why missense mutations in the globular tail of myosin-Va, a putative cargo-binding site, cause the \textit{dilute} phenotype (i.e. lighter coat color) has never been elucidated. In this study we discovered that missense mutations (I1510N, M1513K and D1519G) in the globular tail of myosin-Va partially impair the binding of Slac2-a/melanophilin, a linker protein between myosin-Va and Rab27A on the melanosomes in dilute mutant mice. The myosin-Va-GT-binding site in Slac2-a was mapped to the region (amino acids 147-240) adjacent to the N-terminal Rab27A-binding site, but it is distinct from the myosin-Va-exon-F-binding site (amino acids 320-406). The myosin-Va-GT·Slac2-a interaction was much weaker than the myosin-Va-exon-F·Slac2-a interaction.

The missense mutations in the GT found in \textit{dilute} mice abrogated only the myosin-Va-GT·Slac2-a interaction and had no effect on the myosin-Va-exon-F·Slac2-a interaction. We further showed that expression of green fluorescence protein-tagged Slac2-a lacking the myosin-Va-GT-binding site (ΔGT), but not the wild-type Slac2-a, severely inhibits melanosome transport in melan-a cells, especially at the melanosome transfer step from microtubules to actin filaments (i.e. perinuclear aggregation of melanosomes). On the basis of our findings, we propose that myosin-Va interacts with Slac2-a Rab27A complex on the melanosome via two distinct domains, both of which are essential for melanosome transport in melanocytes.

Key words: Slac2-a/melanophilin, Myosin-Va, Rab27A, Melanosome transport, Griscelli syndrome

Introduction

Genetic analysis of coat-color mutant mice (\textit{dilute, ashen} and \textit{leaden}) (Moore et al., 1988; Mercer et al., 1991; Wilson et al., 2000; Matesic et al., 2001; Hume et al., 2002; Provance et al., 2002) and patients with Griscelli syndrome (Pastural et al., 1997; Menasché et al., 2000; Bahadoran et al., 2001; Menasché et al., 2003; Bahadoran et al., 2003), as well as recent biochemical studies of their gene products (myosin-Va, Rab27A, and Slac2-a (synaptotagmin-like protein (Slp) homologue lacking C2 domains-a/melanophilin, respectively) (Fukuda et al., 2002; Wu et al., 2002a; Strom et al., 2002; Menasché et al., 2003; Bahadoran et al., 2003), have provided evidence that a tripartite protein complex formed by myosin-Va, Slac2-a and Rab27A is essential for melanosome transport from the perinuclear region of melanocytes to their actin-rich cell periphery (for a review, see Marks and Seabra, 2001; Hammer and Wu, 2002; Fukuda, 2002a). Slac2-a simultaneously binds the GTP-bound form of Rab27A on the melanosome, via the N-terminal Slp homology domain (referred to as SHD or RBD27) (Fukuda and Mikoshiba, 2001; Fukuda et al., 2001a; Fukuda et al., 2002; Wu et al., 2002a; Strom et al., 2002; Kuroda et al., 2002a; Kuroda et al., 2002b; Fukuda, 2002a; Fukuda, 2002b; Fukuda, 2003), and myosin-Va, an actin-based motor protein, via the large C-terminal domain (Fukuda et al., 2002; Wu et al., 2002a; Strom et al., 2002). The interaction between Slac2-a and myosin-Va has been shown to be regulated by a melanocyte (MC)-specific alternative splicing in the tail domain of myosin-Va (Seperack et al., 1995; Huang et al., 1998; Wu et al., 2002a; Wu et al., 2002b): Slac2-a strongly interacts with the MC-type myosin-Va containing the MC-specific exon F (– exon B, + exon D and + exon F), and weakly with a brain-type myosin-Va lacking exon F (+ exon B, – exon D, and – exon F) (Fukuda et al., 2002; Wu et al., 2002a; Fukuda and Kuroda, 2002) (see also Fig. 2C).

We and others have recently mapped the binding site for MC-type myosin-Va to the middle region of Slac2-a (Strom et al., 2002; Fukuda and Kuroda, 2002; Nagashima et al., 2002) (see Fig. 1A, shaded box), but little is known about the binding site for brain-type myosin-Va in Slac2-a (i.e. the interaction between globular tail (GT) of myosin-Va and Slac2-a). Because the MC-specific exon F alone is insufficient for melanosome recognition by myosin-Va (Wu et al., 2002b; da Silva Bizario et al., 2002), an additional region (i.e. the globular tail) must also be required for melanosome transport. Moreover, several missense mutations have been found in the GT of myosin-Va in \textit{dilute} mice (Huang et al., 1998), indicating a crucial role of the GT of myosin-Va in melanosome transport. Why such mutations cause a defect in melanosome transport, however, has never been elucidated at the molecular level, nor
Fig. 1. Mapping of the site in Slac2-a responsible for the binding of the globular tail of myosin-Va. (A) Deletion mutants of Slac2-a. Slac2-a consists of four distinct domains: the N-terminal SHD (SHD1 = RBD27, two Cys-based Zn$^{2+}$-finger motifs, and SHD2; black boxes) (Fukuda et al., 2001a; Fukuda, 2002a; Fukuda, 2002b; Kuroda et al., 2002a), a myosin-Va-GT-binding site (MyoVa-GT; cross-hatched box), a myosin-Va-exon-F-binding site (MyoVa-F; gray box) (Strom et al., 2002; Fukuda and Kuroda, 2002; Nagashima et al., 2002) and an actin-binding site (hatched box) (Fukuda and Kuroda, 2002; Kuroda et al., 2003). The solid lines represent the deletion constructs of T7-tagged Slac2-a. The myosin-Va-GT-binding activity or the myosin-Va-exon-F-binding activity of each mutant is indicated after its name (+ or –) (see Fig. 1B). Bold lines indicate the minimal myosin-Va-GT-binding site of Slac2-a (amino acid residues 147-240), which is completely different from the myosin-Va-exon-F-binding site (shaded box) (Strom et al., 2002; Fukuda and Kuroda, 2002; Nagashima et al., 2002). The sequences at the bottom represent the myosin-Va-GT-binding site of human and mouse Slac2-a. Residues in the sequences that are conserved and similar are shown against a black background and a shaded background, respectively. The amino acid numbers are indicated at the right-hand side of each sequence. (B) Mapping of the site in Slac2-a responsible for the binding of the GT of myosin-Va. Purified T7-Slac2-a mutants coupled with anti-T7 tag antibody-conjugated agarose (Fukuda and Kuroda, 2002) were incubated with COS-7 cell lysates containing FLAG-myosin-Va-GT, and proteins trapped with the beads were analyzed by immunoblotting with HRP-conjugated anti-FLAG tag antibody (1/10,000 dilution) (top 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-a mutants had been loaded (bottom panel; Blot, anti-T7; IP, anti-T7). Note that myosin-Va-GT bound amino acids 147-240 of Slac2-a, adjacent to the SHD. Although the apparent molecular mass of the T7-Slac2-a mutants almost corresponded to their calculated molecular weight, several additional bands with higher molecular mass were observed in Slac2-a-Δ146Δ321 mutant (asterisks in lane 4). These bands were probably produced by certain post-translational modifications caused by the truncation of Slac2-a protein. One of the possible modifications is fatty-acylation, which often contributes to the formation of a SDS-insensitive oligomer on SDS-polyacrylamide gel (Fukuda et al., 2001b). In addition, Slac2-a-Δ400 and –Δ240 mutants contained some degradation products (lanes 5 and 6). The positions of the molecular mass markers ($\times 10^{-3}$) are shown on the right. (C) Rab27A enhanced myosin-Va-GT binding to Slac2-a. The purified T7-Slac2-a coupled with the beads was incubated with recombinant FLAG-myosin-Va-GT in the presence and absence of HA-Rab27A. The positions of the molecular mass markers ($\times 10^{-3}$) are shown on the left.
has the effect of dilute mutations on Slac2-a binding activity or on the GT-binding site in Slac2-a ever been determined.

In this study we determined the minimal essential binding site for the GT of myosin-Va in Slac2-a and discovered that missense mutations in the GT of myosin-Va observed in dilute mice (i.e. 11510N, M1513K and D1519G) impair binding to Slac2-a. We also found that expression of green fluorescence protein (GFP)-tagged Slac2-a lacking the myosin-Va-GT-binding site causes perinuclear aggregation of melanosomes in melan-a cells. The physiological importance of the GT of myosin-Va in melanosome transport is discussed on the basis of our findings.

### Materials and Methods

**Construction of deletion mutants of Slac2-a and myosin-Va**

Deletion mutants of Slac2-a (pEF-T7-Slac2-a-Δ146/Δ241, pEF-T7-Slac2-a-ΔGT (Δ153-235) and pEGFP-C1-Slac2-a-ΔGT) and of myosin-Va (pEF-FLAG-myosin-Va-GT-ΔAf6 and -myosin-Va-GT-Af6) were essentially constructed by conventional PCR as described previously (Fukuda et al., 1994). Mutant myosin-Va plasmids carrying an Ile-to-Asn substitution at amino acid position 1510 (I1510N), M1513K, D1519G or S1650E (S1650E primer 2). The mutant myosin-Va-GT fragments were subcloned into the pEF-FLAG-tag expression vector as described previously (Fukuda et al., 1999; Fukuda and Mikoshiba, 2000; Kuroda et al., 2003). Transfection of pEGFP-C1-Slac2-a-ΔGT and -myosin-Va-GT-ΔAf6 was achieved with FuGENE 6 (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s instructions. Two days after transfection, cells were fixed in 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, stained with anti-Rab27A mouse monoclonal antibody (BD Transduction Laboratories, Lexington, KY) and anti-myosin-Va rabbit polyclonal antibody (Fukuda et al., 2002), followed by anti-rabbit Aalexa Fluor 633 IgG and anti-mouse Aalexa Fluor 568 IgG (Molecular Probes, Eugene, OR), and then examined for fluorescence and by bright-field images with a confocal fluorescence microscope (Fluoview; Olympus, Tokyo, Japan) as described previously (Kuroda et al., 2003). The pigment distribution of the transfected cells (‘dispersed’ = normal peripheral distribution of melanosomes shown in Fig. 6E or ‘aggregated’ = accumulation of...
melanosomes in the perinuclear regions shown in Fig. 6J) was evaluated as described previously (Strom et al., 2002; Kuroda et al., 2003). More than 50 cells transfected with GFP-Slac2-a mutants in one dish were counted for each construct, and the same experiments were independently repeated three times (n>150).

Miscellaneous procedures

Transfection of plasmids into COS-7 cells (7.5×10^5 cells/10 cm dish, the day before transfection) was performed as described previously (Fukuda et al., 2001b). Proteins were solubilized at 4°C for 1 hour with a buffer containing 1% Triton X-100, 250 mM NaCl, 1 mM MgCl_2, 50 mM HEPES-KOH, pH 7.2, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin and 10 μM pepstatin A. T7-Slac2-a mutants were immunoprecipitated with anti-T7 tag antibody-conjugated agarose (Novagen, Madison, WI) as described previously (Fukuda et al., 1999; Fukuda and Mikoshiba, 2000). SDS-PAGE and immunoblotting analyses with horseradish peroxidase (HRP)-conjugated anti-FLAG tag (Sigma Chemical Co.; St Louis, MO), anti-HA tag (Roche Molecular Biochemicals) and anti-T7 tag antibodies (Novagen) were also performed as described previously (Fukuda et al., 1999; Fukuda and Mikoshiba, 2000). The intensity of the bands on x-ray film or gels was quantified with Basic Quantifier Software (version 1.0) (BioImage) or Lane Analyzer (version 3.0) (ATTO, Tokyo, Japan) as described previously (Fukuda and Mikoshiba, 2000). The protein concentration of Slac2-a (or myosin-Va-tail) in the SDS-polyacrylamide gel was then estimated using bovine serum albumin as a reference. The statistical analyses and curve fitting were performed with a GraphPad PRISM computer program (version 4.0). The blots and gels shown in this paper are representative of at least two or three independent experiments.

Results

Globular tail and exon F of myosin-Va bind distinct domains of Slac2-a/melanophilin

A systematic deletion analysis was performed as described previously (Fukuda and Kuroda, 2002) to determine the minimal essential domain required for the binding of myosin-Va-GT to Slac2-a. As shown in Fig. 1A,B, myosin-Va-GT bound a previously uncharacterized region adjacent to SHD2 (amino acids 147-240; cross-hatched box in Fig. 1A). By contrast, myosin-Va-F-GT (i.e. exon F) has been shown to bind the middle region of Slac2-a (amino acids 320-406; shaded box in Fig. 1A) (Strom et al., 2002; Fukuda and Kuroda, 2002; Nagashima et al., 2002; Kuroda et al., 2003). Interestingly, twice the amount of myosin-Va-GT bound to Slac2-a-Rab27A-beads as bound to Slac2-a-beads alone (i.e. without Rab27A) (Fig. 1C, top panel, compare lanes 1 and 2), suggesting that myosin-Va-GT preferentially interacts with the Rab27A-Slac2-a complex rather than with Slac2-a alone.

The interaction between Slac2-a and myosin-Va-GT was less stable (Fig. 2) and much weaker (Fig. 3) than the interaction between Slac2-a and myosin-Va-F-GT. As shown in Fig. 2A, the Slac2-a-myosin-Va-GT interaction was highly sensitive to ionic strength (mild concentrations of NaCl), whereas the Slac2-a-myosin-Va-F-GT (+ exon F) interaction was resistant to high NaCl concentrations (up to 750 mM NaCl) (Fig. 2A,C). Similar results were obtained when the entire tail domain of brain-type myosin-Va and MC-type myosin-Va were used (Fig. 2B). Slac2-a was also found to bind the brain myosin-Va-tail...
with much lower affinity than the MC myosin-Va-tail (top panels in Fig. 3A). Because the dose-dependence curve of the MC myosin-Va-tail (open circles in Fig. 3B) was almost saturated, we used the curve-fitting program to calculate the EC50 (myosin-Va-tail concentration at half maximal binding = 0.15) and Bmax values (0.74 μg). Interestingly, under the maximal binding conditions, one molecule of Slac2-a (0.25 μg; approximately 3.6 pmol) was estimated to bind approximately two molecules of MC myosin-Va-tail (0.74 μg; approximately 6.7 pmol), which is consistent with the fact that myosin-Va functions as a dimer (Fig. 2C). The dose-dependence curve of brain myosin-Va-tail (closed circles in Fig. 3B), however, was not saturated under our experimental conditions, and only 0.15 μg of brain myosin-Va-tail bound Slac2-a at the highest concentrations of myosin-Va-tail (lane 7 in the upper right panel), suggesting that Slac2-a binds brain myosin-Va with more than ten times lower affinity than it binds MC myosin-Va (Fig. 3B, closed and open circles, respectively). Consistent with these findings, brain-type myosin-Va has been shown not to bind Slac2-a after extensive washing of the myosin-Va-beads (Wu et al., 2002a; Wu et al., 2002b).

**Effect of dilute missense mutations in the GT of myosin-Va on Slac2-a binding**

Because the GT of mouse class V myosins (myosin-Va, Vb and Vc) consists of two parts, a first part and a second part, which contains an Af6 homology domain (Zhao et al., 1996; Hock et al., 1998; Reck-Peterson et al., 2000; Rodriguez and Cheney, 2002) (see boxed in Fig. 4A), we attempted to identify the
domain(s) responsible for binding to Slac2-a. As shown in Fig. 4B (middle panel), Slac2-a was found to bind the first part of GT (myosin-Va-GT-Af6) but not the Af6 homology domain (myosin-Va-GT-Af6). It should be noted that several missense mutations were found in the first part of the myosin-Va-GT in *Xenopus* eggs (I1510N, M1513K and D1519G; asterisks in Fig. 4A) (Karcher et al., 1998), as well as the Ca2+/calmodulin-dependent protein kinase II phosphorylation site (Ser-1650; # in Fig. 4A) (Karcher et al., 2001), all of which are highly conserved among mouse class V myosins. Because the S1650E mutant of the mouse myosin-Va (mimics the phosphorylated form of myosin-Va) does not recruit to Rab27A and FLAG-brain myosin-Va-tail, and proteins trapped with the beads were analyzed by immunoblotting with HRP-conjugated anti-T7 tag antibody (1/10,000 dilution) (third panel; Blot: anti-T7, IP: anti-T7) and HRP-conjugated anti-HA tag antibody (1/10,000 dilution) (fourth panel; Blot: anti-HA, 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-a mutant proteins had been loaded (bottom panel; Blot: anti-T7, IP: anti-T7). Input means 1/80 volume of the reaction mixture (top and second panels). Note that the Slac2-a-ΔGT mutant specifically impaired brain myosin-Va-tail binding activity (lanes 1 and 2), but that it normally interacted with Rab27A and MC myosin-Va-tail (lanes 4 and 5).

Expression of GFP-Slac2-a-ΔGT inhibits melanosome transport in melan-a cells

To investigate further the physiological significance of the myosin-Va-GT-Slac2-a interaction, we prepared a deletion mutant of Slac2-a lacking the myosin-Va-GT-binding site (named Slac2-a-ΔGT) (Fig. 5A). Because the mutant Slac2-a-ΔGT protein still contained both the Rab27A-binding site (amino acid residues 1-146) and the myosin-Va-exon-F-binding site (amino acid residues 320-406), the mutant protein formed a tripartite protein complex with HA-Rab27A and FLAG-MC myosin-Va-tail, the same as the wild-type protein (Fig. 5B, lane 5 at third and fourth panels), but it did not interact with FLAG-brain myosin-Va-tail (Fig. 5B, lane 2 at third panel). The mutant Slac2-a-ΔGT bound the MC myosin-Va-tail with lower affinity than the wild-type Slac2-a, probably because of the lack of myosin-Va-GT-Slac2-a interaction, but it completely lacked brain myosin-Va-tail binding activity (Fig. 3B, open squares and closed squares, respectively).

If the myosin-Va-GT-binding site was essential for melanosome transport in vivo, expression of the Slac2-a-ΔGT in melan-a cells should inhibit melanosome transport (Wu et al., 2002a; Strom et al., 2002), and therefore it should not recruit to Rab27A and decrease the immunoreactivity of endogenous myosin-Va (Kuroda et al., 2002a; Strom et al., 2002), the same as the Slac2-a(EA) mutant lacking the MC myosin-Va-binding site (Kuroda et al., 2003). As expected, almost all of the cells expressing the Slac2-a-ΔGT protein exhibited ‘aggregated’ melanosomes in their perinuclear region (Fig. 6J) (91.6±3.0% (mean±s.e.) cells exhibiting aggregated melanosomes, n=169). Slac2-a-ΔGT-expressing cells exhibited segregation of endogenous Rab27A from myosin-Va (Fig. 6I, Rab27A in green and myosin-Va in red) and decreased immunoreactivity of endogenous myosin-Va (compare panels C and H in Fig. 6). Similar attenuation of myosin-Va-immunoreactivity was observed in ashen- and leaden-mouse-derived melanocytes (Provance et al., 2002) as
Two forms of myosin-Va binding to Slac2-a

Discussion

We and others previously showed that Slac2-a functions as a receptor for myosin-Va in melanocytes and that formation of a tripartite protein complex composed of Rab27A, Slac2-a and myosin-Va is essential for normal melanosome distribution (Fukuda et al., 2002; Wu et al., 2002a; Strom et al., 2002; Kuroda et al., 2003). In the present study we discovered two forms of myosin-Va binding to Slac2-a: weak interaction between GT and Slac2-a (amino acids 147-240) and a strong interaction between exon F and the middle region of Slac2-a (Fig. 1A). The dilute missense mutations (I1510N, M1513K and D1519G) in the GT of myosin-Va impair only the former interaction, not the latter (Fig. 4), indicating that the Slac2-a×myosin-Va-GT interaction should be physiologically relevant. The physiological significance of the latter interaction is evident on the basis of the following observations: (1) deletion of exon F by mutations in the alternative splicing sites causes the dilute phenotype (i.e. perinuclear aggregation of melanosomes) (Seperack et al., 1995; Huang et al., 1998); (2) expression of brain myosin-Va lacking an exon F cannot rescue the phenotype of dilute-derived melanocytes (Wu et al., 2002b), and expression of MC myosin-Va-tail, but not of brain myosin-Va-tail, induces perinuclear aggregation of melanosomes (Wu et al., 2002b; da Silva Bizario et al., 2002; Westbroek et al., 2003); and (3) Slac2-a mutants lacking the exon-F-binding site cannot support normal melanosome distribution in melan-a cells (Kuroda et al., 2003). We therefore concluded that both interaction sites in Slac2-a identified in this study, the myosin-Va-GT-binding site and the myosin-Va-exon-F-binding site, are essential for melanosome transport in melanocytes.

Do the GT and exon F of myosin-Va function differently, synergistically or sequentially in melanosome transport? Although a previous analysis of yeast myosin V mutants showed that the tail domain has two distinct functions (Catlett et al., 2000), the GT and exon F of mammalian myosin-Va probably function synergistically in melanosome transport, given the following observations. First, neither the GT nor exon F alone (GST-myosin-Va-exon-F) recognized Slac2-a or melanosomes in melanocytes (data not shown) (Wu et al., 2002b), indicating that both domains are required for recognition of melanosomes in vivo. Second, both the mutant Slac2-a lacking the myosin-Va-GT-binding site and the mutant lacking the exon-F-binding site failed to mediate melanosome transport (Fig. 6J) (Kuroda et al., 2003). Third, the phenotype induced by expression of Slac2-a-ΔGT or Slac2-a(EA) lacking exon-F-binding activity in melanocytes is identical, indicating that both sites function at the same step of melanosome
transport (Fig. 6J) (Kuroda et al., 2003). Both mutants induced melanosome aggregation in the perinuclear region of the melanocytes (i.e., inhibition of the melanosome transition from microtubules to actin filaments rather than actin-based transport itself) (Fig. 6J) and induced segregation of myosin-Va from Rab27A (Fig. 6I) and attenuation of myosin-Va immunoreactivity (Fig. 6H). Because deletion of the GT-binding site of Slac2-a slightly reduced the binding affinity to MC myosin-Va (Fig. 3B), we speculate that both the GT- and exon-F-binding sites of Slac2-a are essential for higher affinity or stable recognition of myosin-Va in vivo. Alternatively, the interaction between myosin-Va-GT and Slac2-a may be a prerequisite for starting unidirectional actin-based transport driven by the myosin-Va motor.

In summary, we have identified two distinct myosin-Va-binding sites in Slac2-a, the GT-binding site (amino acids 147-240) and the exon-F-binding site (amino acids 320-406), both of which are essential for melanosome transfer from microtubules to actin filaments. Because missense mutations in the GT of myosin-Va selectively impair the former interaction, the recognition mechanism of myosin-Va by Slac2-a is not so simple as previously thought. Three-dimensional structural analysis of the Slac2-a-MC myosin-Va complex will be necessary to fully understand the role of the tripartite protein complex in the melanosome transfer step at the molecular level.

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References
Bahadoran, P., Aberdam, E., Mantoux, F., Buscà, R., Bille, K., Yamanashi, Yamanashi, Japan) for kindly donating melan-a cells, and Eiko Kanno and Yukie Ogata for expert technical assistance. This work was supported in part by Grants-in-Aid for Young Scientists (A) (15689006) from the Ministry of Education, Culture, Sports and Technology of Japan (to M.F.), and grants from Uehara Memorial Foundation (to M.F.).

Fukuda, M. (2003). Distinct Rab binding specificity of Rim1, Rim2, Rabphilin, and Noc2: Identification of a critical determinant of Rab3a/Rab27A recognition by Rim2. J. Biol. Chem. 278, 15373-15380.

Fukuda, M. and Kuroda, T. S. (2002). Slac2-c (synaptotagmin-like protein homologue lacking C2 domains-c), a novel linker protein that interacts with Rab27, myosin Va/VIIa, and actin. J. Biol. Chem. 277, 43096-43103.

Fukuda, M. and Mikoshiba, K. (2000). Distinct self-oligomerization activities of synaptotagmin family: unique calcium-dependent oligomerization properties of synaptotagmin VII. J. Biol. Chem. 275, 28180-28185.

Fukuda, M. and Mikoshiba, K. (2001). Synaptotagmin-like protein 1-3: a novel family of C-terminal-type tandem C2 proteins. Biochem. Biophys. Res. Commun. 281, 1226-1233.

Fukuda, M., Aruga, J., Niinobe, M., Aimoto, S. and Mikoshiba, K. (1994). Inositol-1,3,4,5-tetrakisphosphate binding to C2B domain of IP3R/synaptotagmin II. J. Biol. Chem. 269, 29206-29211.

Fukuda, M., Kojima, T., Aruga, J., Niinobe, M. and Mikoshiba, K. (1995). Functional diversity of C2 domains of synaptotagmin family: mutational analysis of inositol high polyphosphate binding domain. J. Biol. Chem. 270, 26523-26527.

Fukuda, M., Kanno, E. and Mikoshiba, K. (1999). Conserved N-terminal cysteine motif is essential for homo- and heterodimer formation of synaptotagmins III, V, VI, VII, and X. J. Biol. Chem. 274, 31421-31427.

Fukuda, M., Saegusa, C. and Mikoshiba, K. (2001a). Novel splicing isoforms of synaptotagmin-like proteins 2 and 3: identification of the Slp homology domain. Biochem. Biophys. Res. Commun. 283, 513-519.

Fukuda, M., Kanno, E., Ogata, Y. and Mikoshiba, K. (2001b). Mechanism of the SDS-resistant synaptotagmin clustering mediated by the cysteine cluster at the interface between the transmembrane and spacer domains. J. Biol. Chem. 276, 40319-40325.

Fukuda, M., Kuroda, T. S. and Mikoshiba, K. (2002). Slac2-a/melanophilin, the missing link between Rab27 and myosin Va: implications of a tripartite protein complex for melanosome transport. J. Biol. Chem. 277, 12432-12436.

Hammer, J. A., III and Wu, X. S. (2002). Rabs grab motors: defining the connection between Rab GTases and motor proteins. Curr. Opin. Cell Biol. 14, 69-75.

Hock, B., Bohme, B., Karu, T., Yamamoto, T., Kaibuchi, K., Holtrich, U., Holland, S., Watson, T., Rubsam-Waigmann, H. and Strebehart, K. (1998). PDZ-domain-mediated interaction of the Eph-related receptor tyrosine kinase EphB3 and the ras-binding protein AF6 depends on the kinase activity of the receptor. Proc. Natl. Acad. Sci. USA 95, 9779-9784.

Huang, J. D., Merrall, V., Strobel, M. C., Russell, L. B., Mosoexer, M. S., Copeland, N. G. and Jenkins, N. A. (1998). Molecular genetic dissection of mouse unconventional myosin-Va: tail region mutations. Genetics 148, 1963-1972.

Hume, A. N., Collinson, L. M., Hopkins, C. R., Strom, M., Barral, D. C., Bossi, G., Griffiths, G. M. and Seabra, M. C. (2002). The leaden gene product is required with Rab27a to recruit myosin Va to melanosomes in melanocytes. Traffic 3, 193-202.

Karcher, R. L., Roland, J. T., Zappacosta, F., Huddleston, M. J., Annan, R. S., Carr, S. A. and Gelfand, V. I. (2001). Cell cycle regulation of myosin-V by calcium/calmodulin-dependent protein kinase II. Science 293, 1317-1320.

Kuroda, T. S., Fukuda, M., Ariga, H. and Mikoshiba, K. (2002a). The Slp homology domain of synaptotagmin-like proteins 1 and 2 functions as a novel Rab27 binding domain. J. Biol. Chem. 277, 9212-9218.

Kuroda, T. S., Fukuda, M., Ariga, H. and Mikoshiba, K. (2002b). Synaptotagmin-like protein 5: a novel Rab27a effector with C-terminal tandem C2 domains. Biochem. Biophys. Res. Commun. 293, 899-906.

Kuroda, T. S., Ariga, H. and Fukuda, M. (2003). The actin-binding domain of Slac2-a/melanophilin is required for melanosome distribution in melanocytes. Mol. Cell. Biol. 23, 5245-5255.

Marks, M. S. and Seabra, M. C. (2001). The melanosome: membrane dynamics in black and white. Nat. Rev. Mol. Cell. Biol. 2, 738-748.

Matesic, L. E., Yip, R., Reuss, A. E., Swing, D. A., O'Sullivan, T. N., Fletcher, C. F., Copeland, N. G. and Jenkins, N. A. (2001). Mutations in Mph, encoding a member of the Rab effector family, cause the melanosome transport defects observed in leaden mice. Proc. Natl. Acad. Sci. USA 98, 10238-10243.

Menasché, G., Pastural, E., Feldmann, J., Certain, S., Ersoy, F., Dupuis, S., Wulfraat, N., Bianchi, D., Fischer, A., le Deist, F. et al. (2000). Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. Nat. Genet. 25, 173-176.
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Menasché, G., Feldmann, J., Houdusse, A., Desaymard, C., Fischer, A., Goud, B. and de Saint Basile, G. (2003). Biochemical and functional characterization of Rab27a mutations occurring in Griscelli syndrome patients. Blood 101, 2736-2742.

Mercer, J. A., Seperack, P. K., Strobel, M. C., Copeland, N. G. and Jenkins, N. A. (1991). Novel myosin heavy chain encoded by murine dilute coat colour locus. Nature 349, 709-713.

Moore, K. J., Seperack, P. K., Strobel, M. C., Swing, D. A., Copeland, N. G. and Jenkins, N. A. (1988). Dilute suppressor dss acts semidominantly to suppress the coat color phenotype of a deletion mutation, d174o, of the murine dilute locus. Proc. Natl. Acad. Sci. USA 85, 8131-8135.

Nagashima, K., Torii, S., Yi, Z., Igarashi, M., Okamoto, K., Takeuchi, T. and Izumi, T. (2002). Melanophilin directly links Rab27a and myosin Va through its distinct coiled-coil regions. FEBS Lett. 517, 233-238.

Pastural, E., Barrat, F. J., Dufourcq-Lagelouse, R., Certain, S., Sanal, O., Jabado, N., Seger, R., Griscelli, C., Fischer, A. and de Saint Basile, G. (1997). Griscelli disease maps to chromosome 15q21 and is associated with mutations in the myosin-Va gene. Nat. Genet. 16, 289-292.

Provance, D. W., Jr, James, T. L. and Mercer, J. A. (2002). Melanophilin, the product of the leaden locus, is required for targeting of myosin-Va to melanosomes. Traffic 3, 124-132.

Reck-Peterson, S. L., Provance, D. W., Jr, Mooseker, M. S. and Mercer, J. A. (2000). Class V myosins. Biochim. Biophys. Acta 1496, 36-51.

Rodriguez, O. C. and Cheney, R. E. (2002). Human myosin-Vc is a novel class V myosin expressed in epithelial cells. J. Cell Sci. 115, 991-1004.

Seperack, P. K., Mercer, J. A., Strobel, M. C., Copeland, N. G. and Jenkins, N. A. (1995). Retroviral sequences located within an intron of the dilute gene alter dilute expression in a tissue-specific manner. EMBO J. 14, 2326-2332.

Strom, M., Hume, A. N., Tarafder, A. K., Barkagianni, E. and Seabra, M. C. (2002). A family of Rab27-binding proteins. Melanophilin links Rab27a and myosin Va function in melanosome transport. J. Biol. Chem. 277, 25423-25430.

Westbroek, W., Lambert, J., Bahadoran, P., Buscá, R., Herteleer, M. C., Smit, N., Mommaas, M., Ballotti, R. and Naeyaert, J. M. (2003). Interactions of human myosin Va isoforms, endogenously expressed in human melanocytes, are tightly regulated by the tail domain. J. Invest. Dermatol. 120, 465-475.

Wilson, S. M., Yip, R., Swing, D. A., O’Sullivan, T. N., Zhang, Y., Novak, E. K., Swank, R. T., Russell, L. B., Copeland, N. G. and Jenkins, N. A. (2002). A mutation in Rab27a causes the vesicle transport defects observed in ashen mice. Proc. Natl. Acad. Sci. USA 97, 7933-7938.

Wu, X. S., Rao, K., Zhang, H., Wang, F., Sellers, J. R., Matesic, L. E., Copeland, N. G., Jenkins, N. A. and Hammer, J. A., III (2002a). Identification of an organelle receptor for myosin-Va. Nat. Cell Biol. 4, 271-278.

Wu, X., Wang, F., Rao, K., Sellers, J. R. and Hammer, J. A., III (2002b). Rab27a is an essential component of melanosome receptor for myosin Va. Mol. Biol. Cell 13, 1735-1749.

Zhao, L. P., Koslovsky, J. S., Reinhard, J., Bahler, M., Witt, A. E., Provance, D. W., Jr and Mercer, J. A. (1996). Cloning and characterization of myr 6, an unconventional myosin of the dilute/myosin-V family. Proc. Natl. Acad. Sci. USA 93, 10826-10831.