Melanophilin directly links Rab27a and myosin Va through its distinct coiled-coil regions

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Abstract Rab GTPases regulate the membrane transport pathways by recruiting their specific effector proteins. Melanophilin, a putative Rab effector, has recently been identified as a gene that is mutated in *leaden* mice, in which peripheral localization of melanosomes is impaired in melanocytes. Genetic studies suggest that three coat-color mutation genes, *dilute* (*MyoVa*^{d}), *ashen* (*Rab27a*^{ash}), and *leaden* (*Mlph*^{ln}), act in the same or overlapping pathways. Here we have cloned and characterized a human melanophilin homolog, which belongs to the rabphilin3/granuphilin-like Rab effector family. Cosedimentation assays using recombinant proteins reveal that melanophilin directly binds to Rab27a and myosin Va through its N-terminal and its first C-terminal coiled-coil region, respectively. Moreover, we show that Rab27a, melanophilin, and myosin Va form a ternary complex in the human melanocyte cell line HMV-II. These findings suggest that melanophilin has a role in bridging Rab27a on melanosomes and myosin Va on actin filaments during melanosome transport. We also propose that the Rab-binding region conserved in a novel rabphilin3/granuphilin-like Rab effector family constitutes an α-helix-based coiled-coil structure.

Key words: Rab effector; Rab27a; Myosin Va; Melanosome; Coiled-coil

1. Introduction

The protein families important for vesicle trafficking have been characterized and their functions are becoming well defined. Rab proteins, which represent the largest family of small GTPases, are believed to function in regulating the processes of vesicle targeting and fusion [1]. Rab proteins cycle between active (GTP-bound) and inactive (GDP-bound) conformation, and exert their function in the active GTP-bound form by recruiting a variety of downstream effectors.

Rabphilin3, one of the mammalian Rab effector proteins, directly interacts with the GTP-bound form of Rab3. Association with Rab3 requires an amino-terminal region including a cysteine-rich zinc-finger motif [2,3]. In the crystal structure, the α-helix region just upstream of a zinc-finger domain, and the SGAWFF structural motif, constitute the main interfaces of rabphilin3 with Rab3a in their complex [4]. Both rabphilin3 and Rab3 are expressed in neurons and neuroendocrine cells and appear to play a regulatory role in the exocytosis of synaptic vesicles. However, the phenotype of mice lacking rabphilin3 is distinct from that of Rab3a-deficient mice, and actually do not exhibit obvious physiological impairments [5]. Therefore, their precise function on the regulated exocytosis remains unclear.

Recently, we identified and characterized a novel set of Rab and its effector, Rab27a/granuphilin, which is localized on the membrane of insulin granules in pancreatic beta cells [6,7]. Granuphilin shows homology to rabphilin3 in domain structure that includes a zinc-finger motif at the amino-terminus and C2 domains at the carboxyl-terminus. Like rabphilin3, the N-terminal domain of granuphilin specifically binds to an active GTP-bound form of Rab27a [7]. Although the molecular mechanisms are undefined, they may function in regulating exocytosis of insulin-containing secretory granules.

Another rabphilin3/granuphilin-like protein, melanophilin encoded by the *leaden* locus, was recently identified by positional cloning [8]. In three independent coat-color mutations, *dilute* (*MyoVa*^{d}), *ashen* (*Rab27a*^{ash}), and *leaden* (*Mlph*^{ln}), the polarized transport of melanosomes is impaired and leads to abnormal accumulation of melanosomes at the perinuclear area of the cells rather than at the periphery [9]. Because the phenotype of all three mutants is rescued by an unidentified gene, *dilute suppressor* (*dsu*) [10], myosin Va, Rab27a, and melanophilin likely function in the same or overlapped melanosome transport pathway. Myosin Va, one of unconventional myosins, is an actin-based and organelle-associated motor [11]. Melanophilin has a putative Rab-binding sequence at the N-terminus, which is homologous to those of rabphilin3 and granuphilin, although its physiological interaction with Rab protein has not been visualized.

In the present study, we demonstrate that human melano-

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philin indeed binds to Rab27a directly and nucleotide-dependently through its N-terminal region. We further show that the critical binding region constitutes an α-helix-based coiled-coil domain, which is commonly seen for other melanophilin-like proteins found in a cDNA database. In addition, we show that melanophilin directly binds to myosin Va at one of its C-terminal coiled-coil domains that are unique to melanophilin. Our findings regarding the physical interaction of these three proteins in human melanocytes provide a biochemical basis for their physiological interactions that are suggested by previous genetic analyses.

2. Materials and methods

2.1. DNA construction

A full-length human melanophilin cDNA clone (MAMMA1000395, accession number: AK022207) provided by Helix Research Institute (Kisarazu, Japan) was amplified by polymerase chain reaction (PCR) using primers 5′-GGAATTCATGGGGAAGAAACTGGATCT-3′ and 5′-GGTCGACGAAAGCACAGAGCCAATGAG-3′, and subcloned into the *Eco*RI and *Xho*I sites of pcDNA3-HA [12] (pcDNA3-HA-exph3). Mutagenesis of melanophilin was performed using the following primers: 5′-CGGGATCCGTGGGTCTCGTTCAGATGGGC-3′ and 5′-GCGGATCCCATCCGGCCAGAGTCGTGAAG-3′ for ΔZinc-finger, 5′-GGAATTCATGGGGAAGAAACTGGATCTTTCCAAGCTCACTGATGAAGCGGC-3′ for E14A, 5′-CTTGGAAGTTGTTCAAGCAGATT-3′ and 5′-TCGGAGGTCAAAATCTGCTTGAA-3′ for R24A, 5′-TTGACCTCCGAAGGAAAGCAGAG-3′ and 5′-CTCTAGCCGTTCCTCTGCTTTC-3′ for E32A, 5′-GAAGATCGGCTCACTGGAGTCGTACT-ATG-3′ and 5′-CACATGCTCATAGTACGACTCCAGTGA-3′ for W120S, and 5′-AAGATCGGCTCACTGGAGGCGGCCTATGAG-3′ and 5′-TTTCACATGCTCATAGGCCGCCTCCAGTGA-3′ for W120A/Y121A. The PCR products were cloned into restriction enzyme sites of pcDNA3-HA-exph3. Truncated fragments of melanophilin were constructed by PCR. The DNA fragment that encodes residues 1106–1580 of mouse myosin Va was amplified from B16 melanoma cell cDNAs using primers 5′-GGAATTCCCTAAGCCAGGACACAAGAG-3′ and 5′-GAGAGCCAGAAGGAGACAGT-3′. This fragment coding a melanocyte-medial tail region [13] was ligated with the DNA fragment that encodes residues 1468–1877 of mouse myosin Va originally provided by Dr. N.A. Jenkins (National Cancer Institute, Frederick, MD, USA) [14]. The composite clone that codes the C-terminal tail region (1106–1877 amino acids) of melanocyte-form myosin Va was subcloned into pcDNA3.1/HisC (Invitrogen, Carlsbad, CA, USA) or pGEX4T-1 vector (Amersham Pharmacia Biotech, Buckinghamshire, UK). The residue numbers of mouse myosin Va in this work represent those of the melanocyte-form [13]. All the subcloned PCR products were directly sequenced. Plasmids bearing Rab27a cDNA were described previously [7].

2.2. Cell culture and transfection

The HMV-II human melanoma cell line, established by T. Kasuga (Tokyo Medical and Dental Univ.) [15], was provided by the RIKEN Cell Bank (Tsukuba, Japan). HMV-II cells were grown in Ham's F-12 medium supplemented with 10% fetal calf serum. Transfections were performed using FuGENE6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

2.3. Antibodies

Rabbit anti-melanophilin antibodies were raised against glutathione *S*-transferase (GST)-fused N-terminal human melanophilin protein (amino acids 1–301), and specifically recognize human melanophilin but not mouse melanophilin. Anti-myosin Va antibodies were produced against GST-fused mouse myosin Va (1468–1877), and specifically recognize both human and mouse myosin Va. Anti-Rab27a and anti-Rab4 mouse monoclonal antibodies were purchased from BD Transduction Laboratories (Lexington, KY, USA). Anti-Xpress mouse and anti-hemagglutinin (HA) rat (clone 3F10) monoclonal antibodies were purchased from Invitrogen and Roche Diagnostics, respectively.

2.4. Immunoprecipitation

Cell extracts were prepared in lysis buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, and the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of aprotinin, 10 μg/ml of pepstatin A, and 10 μg/ml of leupeptin. Immunoprecipitation and immunoblot analyses were performed as described previously [7]. Briefly, cell extracts (3 mg each) were incubated with anti-Rab27a, anti-Rab4, or anti-Xpress antibodies. Immunoprecipitates and an aliquot of the original cell extracts (15 μg) were analyzed by immunoblotting with antibodies against melanophilin or myosin Va.

2.5. In vitro binding assay with GST fusion proteins

GST fusion proteins were produced as described previously [6]. In vitro translation of HA- or Xpress-tagged proteins was performed using the TNT coupled reticulocyte lysate system (Promega, Madison, WI, USA). Purified GST-fused proteins (1 μg) immobilized on 20 μl of glutathione-Sepharose beads were incubated with either HMV-II cell extracts (1 mg total proteins) or various normalized amounts of in vitro-translated proteins (6–10 μl) in binding buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.1% Nonidet P-40, and the protease inhibitors described above) at 4°C for 2 h. The beads were washed three times and the bound proteins were subjected to SDS–polyacrylamide gel electrophoresis and immunoblotting.

3. Results and discussion

3.1. Rabphilin3/granuphilin-like Rab effectors

We searched for rabphilin3/granuphilin-like proteins in a cDNA expression database and found three independent genes, FLJ20163 (accession number: AK000170), MAMMA1000395 (AK022207), and KIAA0624 (AB014524). The predicted proteins of FLJ20163 and MAMMA1000395 were human homologs of recently reported Slp2-a (synaptotagmin-like protein 2-a) and melanophilin/Slac2, respectively [16]. In addition, we found three other genes in a mouse EST database, two of which were identical to Slp-1 and Slp-3. Rabphilin3, granuphilin, and all proteins encoded by those novel genes have highly conserved amino acid sequences (27–50%) at their N-termini including the SGAWFF motif that in rabphilin3 may play a critical role in binding to Rab3a-GTP [4]. Some of them apparently lack tandem C2 domains at their C-termini. Thus, this novel protein family likely acts as a Rab effector rather than a Ca^{2+} sensor protein, such as synaptotagmin. Because rabphilin3 and granuphilin bind the exocytotic Rab proteins, Rab3 and Rab27a, respectively, these putative Rab effectors may also function in similar exocytotic pathways. Thus, we termed them exophilins (exocytosis-associated rabphilin3/granuphilin-like proteins) (Fig. 1A). Exophilin4/Slp2-a, exophilin5, and exophilin7/Slp1 lack a series of conserved cysteine residues that form a zinc-finger motif, in their putative Rab-binding regions (Fig. 1A, B), which suggests that this motif is dispensable for Rab binding in some exophilins. In fact, granuphilin mutant without its zinc-finger region still appears to bind to Rab27a in vivo (S. Torii, unpublished data), although mutation of zinc-coordinating cysteine residues in rabphilin3 severely impairs Rab3a binding [2,3].

3.2. Melanophilin binds to Rab27a through the N-terminal coiled-coil domain

Among the novel exophilins, a partner of Rab protein is genetically estimated for melanophilin, since both Rab27a encoded by *ashen* and melanophilin encoded by *leaden* play essential roles in the peripheral localization of melanosomes [8,17], although their interaction has not been defined at a

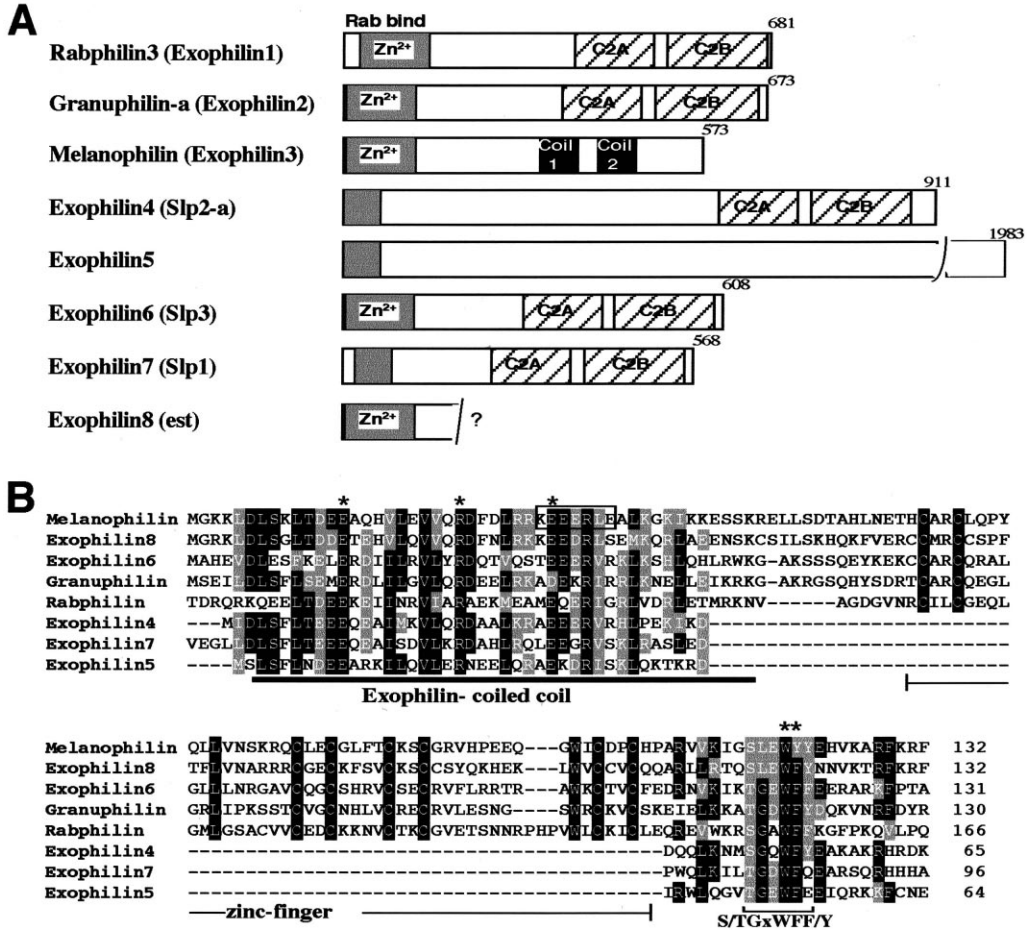

Fig. 1. Structural comparison of exophilins. A: Schematic representation of primary structures of exophilin proteins. Human melanophilin (exophilin3), exophilin4, and exophilin5 are encoded by the human cDNA clones MAMMA1000395, FLJ20163, and KIAA0624, respectively. Exophilin8 is estimated by the sequence of a mouse EST clone (BB653357). Gray box, N-terminal homologous Rab-binding region; Zn^{2+}, zinc-finger motif; dashed box, C2 domain; black box, coiled-coils. B: Alignment of the N-terminal region of exophilins. The putative Rab-binding coiled-coil sequences of exophilins are shown. Residues conserved in at least five members are shown in dark boxes. Asterisks indicate the positions of the amino acids mutated in this study. Amino acids in human melanophilin in a box correspond to those of mouse melanophilin that are deleted in *leaden*.

molecular level. We therefore biochemically examined whether melanophilin interacts with Rab27a. First, extracts of the human melanocyte cell line HMV-II were incubated with bacterially expressed GST-fused Rab27a protein that had been immobilized on glutathione-Sepharose beads and preloaded with either GDP or GTPγS. Bound melanophilin was then detected by immunoblotting with anti-melanophilin antibodies. Melanophilin specifically bound to GTPγS-loaded GST–Rab27a, but not to GDP-bound Rab27a or GST alone (Fig. 2A), indicating that the interaction is regulated in a manner dependent on the nucleotide state of Rab27a.

We next analyzed whether the interaction is direct using recombinant proteins. GST–Rab27a–GTPγS directly bound to HA-tagged melanophilin translated in vitro (Fig. 2B). The region within melanophilin required for binding to Rab27a was mapped to the N-terminal portion. To analyze critical amino acids in the N-terminus of melanophilin for binding to Rab27a, various melanophilin mutants were produced and directed to in vitro assay. Among these, three melanophilin mutants, ΔZinc-finger (64–107 amino acids-deleted), W120S, and W120A/Y121A, bound to GST-fused Rab27a as similarly as a wild type (Fig. 2C; data not shown). These results indicate that the tryptophan-based motif in melanophilin is unnecessary for binding to Rab27a, in contrast to granuphilin (S. Torii, unpublished data). In addition to the SGAWFF structural element, the N-terminal α-helical region of rabphilin3 also forms the main interface with Rab3a and contacts with its switch I and switch II regions in the crystal structure [4]. This α-helix region of rabphilin3 alone indeed binds to Rab3a specifically but with low affinity [18]. We introduced three distinct point mutations in the corresponding region of melanophilin that may affect the charged clusters separated by hydrophobic amino acids (asterisks in Fig. 1B). E14A and E32A mutants had no significant binding activities to Rab27a, whereas R24A showed a lower activity compared with the wild type (Fig. 2C). These results suggest that the N-terminal α-helical region of melanophilin is essential for the binding of Rab27a. It should be noted that the seven consecutive amino acids deleted in *leaden* are located in this region (Fig. 1B, boxed), suggesting that loss of function of melanophilin in *leaden* is due to its defect in binding to Rab27a.

Using the COILS prediction program [19], we found that this α-helical region of melanophilin may be capable of forming coiled-coils. Analysis of the corresponding regions in other exophilin members also predicted similar coiled-coil struc-

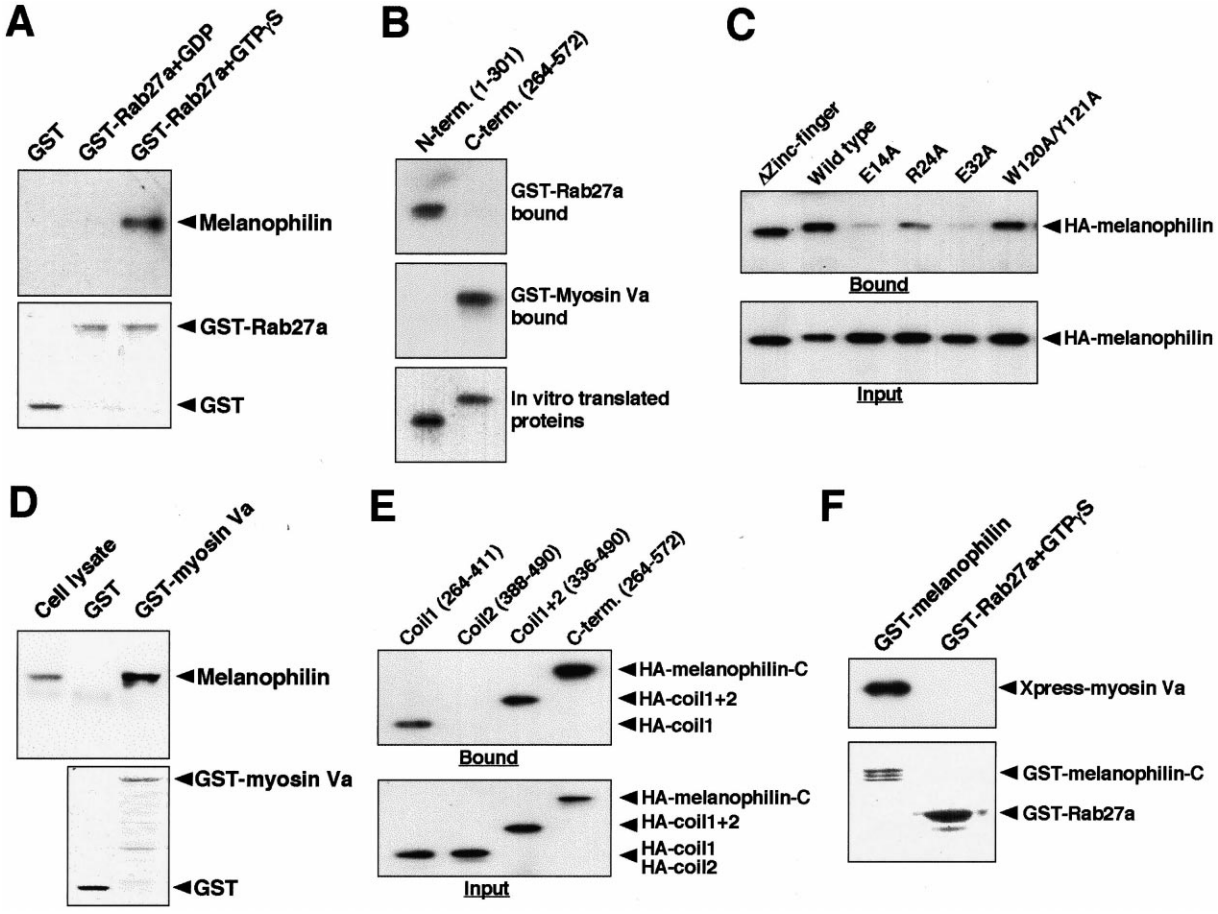

Fig. 2. In vitro binding assays among melanophilin, Rab27a, and myosin Va. A: Aliquots of glutathione beads containing either GST alone or GST-fused Rab27a preloaded with GDP or GTPγS were incubated with HMV-II cell extracts and then washed three times. Proteins that bound to the GST fusion proteins were analyzed by immunoblotting with anti-melanophilin antibodies (upper panel). Total GST protein levels were visualized by Coomassie blue staining (lower panel). B: GTPγS-loaded GST–Rab27a or GST-fused myosin Va C-terminal fragment (1106–1877 amino acids) were incubated with in vitro-translated, HA-tagged N-terminal fragment (1–301) or C-terminal fragment (264–572) of melanophilin. Bound proteins as well as 10% of input proteins (bottom panel) were analyzed by immunoblotting with anti-HA antibodies. C: GTPγS-loaded GST–Rab27a was incubated with in vitro-translated, HA-tagged wild-type melanophilin, zinc-finger-deleted melanophilin (ΔZinc-finger), or melanophilin point mutants (E14A, R24A, E32A, or W120A/Y121A). Bound proteins (upper panel) as well as 10% of input proteins (lower panel) were analyzed as in (B). D: GST alone or GST-fused myosin Va C-terminal fragment were incubated with HMV-II cell extracts, and then bound melanophilin was detected by immunoblotting as in A (upper panel). Total GST protein levels were visualized (lower panel). E: GST–myosin Va was incubated with in vitro-translated, HA-tagged fragments of melanophilin; Coil1 (264–411), Coil2 (388–490), Coil1+2 (336–490), or C-term. (264–572). Bound proteins (upper panel) as well as 10% of input proteins (lower panel) were analyzed as in (B). F: GST–melanophilin (264–572) or GTPγS-loaded GST–Rab27a were incubated with in vitro-translated, Xpress-tagged myosin Va (1106–1877). Proteins that bound to the GST fusion proteins were analyzed by immunoblotting with anti-Xpress antibodies (upper panel). Total GST protein levels were visualized (lower panel).

tures, though with different probabilities (data not shown). We suggest that these exophilin-specific coiled-coil sequences based by α-helices may be involved in their binding to Rab proteins.

3.3. Melanophilin binds to myosin Va through the C-terminal coiled-coil domain

Myosin Va encoded by *dilute* is also genetically shown to be essential for the transport of melanosomes [14]. Furthermore, Rab27a and myosin Va are colocalized on melanosomes and form a complex in melanocytes [20–23]. Using a similar cosedimentation assay described above, we showed that melanophilin specifically bound to the GST-fused carboxyl-terminal tail region of myosin Va (1106–1877), but not to GST alone, in HMV-II cell extracts (Fig. 2D). The interaction is direct and specific because GST–myosin Va bound to HA-tagged melanophilin translated in vitro (Fig. 2B, E), but not to Xpress-tagged Rab27a similarly produced (data not shown). Furthermore, in vitro-translated myosin Va-tail protein was coprecipitated with GST-fused melanophilin, but not with GTPγS-loaded GST–Rab27a (Fig. 2F). These findings indicate that melanophilin directly mediates the interaction between Rab27a and myosin Va.

The region within melanophilin required for binding to myosin Va was mapped to the C-terminal portion (Fig. 2B). Unlike rabphilin3 and granuphilin, the C-terminal region of melanophilin lacks C2 domains, but instead possesses two tandem coiled-coils at the residues 345–381 (Coil1) and 429–468 (Coil2) (Fig. 1A). We constructed melanophilin fragments to determine whether these coiled-coil structures are involved in the binding to myosin Va. As shown in Fig. 2E, the first coiled-coil region (Coil1) was capable of and sufficient for binding to GST-fused myosin Va, indicating that the Coil1 region constitutes the specific binding domain.

3.4. Rab27a, melanophilin, and myosin Va interact together in melanocytes

To explore whether these interactions exist in melanocytes under physiologic conditions, coimmunoprecipitation experiments were performed within lysates from HMV-II cells. Rab27a immunoprecipitates contained a significant amount of myosin Va and melanophilin, whereas those of Rab4 did not (Fig. 3A). These findings indicate that the ternary complex consisting of Rab27a, melanophilin, and myosin Va forms in melanocytes, which is consistent with a previous report showing the existence of the Rab27a–myosin Va complex in melanocytes [21]. Furthermore, our immunofluorescence analysis suggests that these three proteins partially colocalize with melanosomes (data not shown). These observations combined with the previous findings suggest that melanophilin associates on melanosomes presumably through a direct interaction with Rab27a, which in turn directly recruits the actin-based motor protein myosin Va. It is notable that granuphilin, another Rab27a effector in the pancreatic beta cell, was not expressed in the melanocyte cell lines tested [7].

Finally, we defined the interaction domain of myosin Va against melanophilin using a coimmunoprecipitation analysis. Previous studies suggest that the C-terminal tail domain (1258–1877) is involved in the association with melanosomes [20]. Xpress-tagged C-terminal truncation constructs of myosin Va were transfected into HMV-II cells, and the cell extracts were then immunoprecipitated with anti-Xpress antibodies. Myosin Va-tail consists of amino acids 1106–1877 (melanocyte-medial and globular tails) coprecipitated melanophilin, whereas the globular tail alone (1468–1877) failed to bind melanophilin (Fig. 3B). Tissue-specific alternative splicing within the exons B–F (amino acid; 1284–1435) of myosin Va gene generates a brain-specific form, a melanocyte-specific form, and other forms [13]. The brain form does not localize on melanosomes when expressed in melanocytes [24], suggesting that variable regions from melanocyte-specific exon D (1318–1334) and/or exon F (1411–1435) are important for myosin Va association with melanosomes. Taken together, the binding activity of myosin Va to melanophilin highly correlates with its specific localization on melanosomes.

In summary, the present study showed the structural features of the rabphilin3/granuphilin-like Rab effector proteins, termed exophilins. They have a highly conserved N-terminal domain that is capable of forming coiled-coils to directly bind to Rabs. We first characterized melanophilin, which is defective in *leaden*, and demonstrated that it directly binds to both Rab27a and myosin Va. Further characterization of other exophilins and identification of their Rab partners will help identify their function on related secretory pathways.

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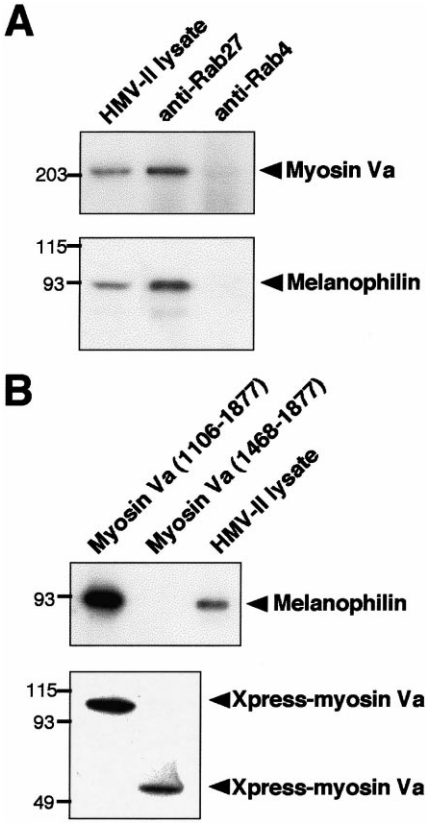

Fig. 3. Immunoprecipitation analysis of melanophilin, Rab27a, and myosin Va. A: HMV-II cell extracts (3 mg each) were incubated with either anti-Rab27a or anti-Rab4 monoclonal antibodies. Each immunoprecipitate and an aliquot of the original cell lysates were analyzed by immunoblotting with antibodies against melanophilin and myosin Va. B: HMV-II cells were transiently transfected with plasmids encoding Xpress-tagged myosin Va containing either globular tail region alone (1468–1877) or medial and globular tail regions (1106–1877). Immunoprecipitates of anti-Xpress antibodies and an aliquot of the untransfected cell lysates were analyzed by immunoblotting with anti-melanophilin antibodies (upper panel). The expression level of each transfection was assessed by immunoblotting with anti-Xpress antibodies (lower panel).

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