

# Activation of Myosin Va Function by Melanophilin, a Specific Docking Partner of Myosin Va\*

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**It is known that melanophilin is a myosin Va-targeting molecule that links myosin Va and the cargo vesicles in cells. Here we found that melanophilin directly activates the actin-activated ATPase activity of myosin Va and thus its motor activity. The actin-activated ATPase activity of the melanocyte-type myosin Va having exon-F was significantly activated by melanophilin by 4-fold. Although Rab27a binds to myosin Va/melanophilin complex, it did not affect the melanophilin-induced activation of myosin Va. Deletion of the C-terminal actin binding domain and N-terminal Rab binding domain of melanophilin resulted in no change in the activation of the ATPase by melanophilin, indicating that the myosin Va binding domain (MBD) is sufficient for the activation of myosin Va. Among MBDs, the interaction of MBD-2 with exon-F of myosin Va is critical for the binding of myosin Va and melanophilin, whereas MBD-1 interacting with the globular tail of myosin Va plays a more significant role in the activation of myosin Va ATPase activity. This is the first demonstration that the binding of the cargo molecule directly activates myosin motor activity. The present finding raises the idea that myosin motors are switched upon their binding to the cargo molecules, thus avoiding the waste of ATP consumption.**

Myosins are motor proteins that interact with actin filaments and convert energy from ATP hydrolysis into mechanical force. In addition to well characterized conventional, filament-forming myosin-II of muscle and non-muscle cells, a number of unconventional myosins have been discovered. The myosin superfamily is currently organized into 18 classes based upon phylogenetic sequence comparisons of the motor domain (1–4).

Class V myosin is considered one of the oldest classes of myosin, which distributes from low eukaryotic, such as yeast, to vertebrate cells (4). In vertebrates, there are three distinct subclasses of myosin V, named myosin Va, Vb, and Vc. Myosin Va consists of two identical heavy chains that dimerize through the formation of several parallel coiled-coil regions to form a homodimer. The N-terminal motor domain contains the ATP- and actin-binding sites, which is followed by a neck consisting of six IQ motifs with the consensus of IQXXXRXXXXR, the binding sites for CaM<sup>1</sup> and myosin light chains. The next ~500

amino acids are predicted to form a series of coiled-coils separated by more flexible regions. The last ~400 amino acids form a globular tail domain (5). This globular tail domain, in conjunction with a portion of the coiled-coil region, mediates myosin Va binding to specific membrane-bound organelles such as melanosome (6, 7).

Evidence has accumulated that myosin Va is a cargo-transporting motor in cells, which moves cargo molecules on actin cables to the specific destination (for review see Ref. 8). Supporting this notion, it has been shown that myosin Va is a processive motor that travels on an actin filament for a long distance without dissociating from actin (9–12). A critical question is how myosin Va motor activity is regulated. Recently we, and other groups, found that the ATPase activity of full-length myosin Va is well regulated by Ca<sup>2+</sup> and a large conformational transition is accompanied with the activation (13–15). On the other hand, the truncated myosin Va, such as myosin Va HMM and S1, are constitutively active and not regulated by Ca<sup>2+</sup> (16–18). Sedimentation analysis showed that the *s*<sub>20,w</sub> of myosin Va undergoes a Ca<sup>2+</sup>-induced conformational transition from 14 to 11 S. Electron microscopy revealed that, in physiological ionic conditions, myosin Va shows an extended conformation in high Ca<sup>2+</sup>, whereas it forms a folded shape in the presence of EGTA, in which the tail domain was folded back toward the head-neck junction. A critical finding is that Ca<sup>2+</sup>-induced conformational transition is closely correlated to the actin-activated ATPase activity. Furthermore, we found that the motor domain of myosin Va folds back to the neck domain in Ca<sup>2+</sup>, whereas the head-neck domain is more extended in EGTA (13). The following model was proposed based on the above findings. The binding of the tail domain to the head-neck of myosin Va inhibits the mechanoenzymatic activity of myosin Va. Upon Ca<sup>2+</sup> binding to CaM at the neck domain, the tail domain dissociates from the head-neck, and the inhibition of the mechanoenzymatic activity of myosin Va is reversed.

This raises the possibility that the tail domain not only functions as a cargo binding site, but also serves as a regulatory component of myosin Va. In other words, myosin Va may become active upon cargo binding. In the present study, we addressed this possibility by studying the effect of melanophilin binding on myosin Va motor function. Melanophilin or Slac2-a, the organelle receptor for myosin Va in mouse melanocyte, has been identified by genetic and biochemical approaches (7, 19–22). Myosin Va binds to melanosome by interacting with a receptor-protein complex containing Rab27a and melanophilin. The melanophilin bound to Rab27a associated with melanosome recruits myosin Va. The melanophilin binding site of

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<sup>1</sup> The abbreviations used are: CaM, calmodulin; Mc, melanocyte;

McM5, melanocyte-type myosin Va; McM5ΔF, melanocyte-type myosin Va with exon-F deletion; ABD, actin-binding domain; BSA, bovine serum albumin; CaMKII, Ca<sup>2+</sup>/CaM-dependent protein kinase II; DTT, dithiothreitol; GTPγS, guanosine 5′-3-O-(thio)triphosphate; MBD, myosin Va-binding domain; RBD, Rab27a-binding domain.

myosin Va is encoded by a melanocyte (Mc)-specific exon-F and melanophilin strongly interacts with the Mc-type myosin Va containing exon-F, but weakly interacts with brain-type myosin Va lacking exon-F (23). Despite the intensive investigation of the interactions between myosin Va and melanophilin, as well as Rab27a, it is unknown if the interaction with melanophilin regulates the activity of myosin Va. In this study, we found that melanophilin directly stimulates the actin-activated ATPase activity of myosin Va.

#### EXPERIMENTAL PROCEDURES

**Materials**—Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA), unless indicated otherwise. Actin was prepared from rabbit skeletal muscle acetone powder according to Spudich and Watt (24). Recombinant CaM of *Xenopus* oocyte (25) was expressed in *Escherichia coli* as described previously (26). Nickel-nitrilotriacetic acid-agarose was purchased from Qiagen (Hilden, Germany). Anti-FLAG M2 antibody, Anti-FLAG M2 affinity gel, FLAG peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Lys), phosphoenolpyruvate, 2,4-dinitrophenylhydrazine, and pyruvate kinase were from Sigma.

**Myosin Va Expression Vectors**—Murine melanocyte-type myosin Va (McM5) was subcloned into pFastBac (Invitrogen) as described before (13). McM5 lacking exon-F (McM5ΔF) was constructed by overlap extension PCR technique by using PfuUltra high fidelity DNA polymerase (#600380, Stratagene, La Jolla, CA) and plasmid of McM5/pFastBac as a template. The 5' fragment, which begins in 5' of unique natural HindIII site and ends with a sequence that skips exon-F and contains the 25 bp that follow exon-F, was amplified using following 5' and 3' primers: CTTTGAGAAGCCCCGCATGT (M5RM-S544+) and 5'-GCTTTTCAAGTTGTTCCATCAGATCCAGGTTTTCA-TTGGTCAGCCGGG-3' (M5-DelF-). The 3' fragment, which begins with a sequence containing the 21 bp preceding exon-F and then skips exon-F, and ends at the stop codon of McM5 (nucleotide of 5634; 5562 in GenBank™ accession number: X57377), was amplified using following 5' and 3' primers: 5'-CGGCTGACCAATGAAAACCTGGATCTGATGGAACAACCTGAAAAGCAG-3' (M5-DelF+) and 5'-ATATCTC-GAGTCAGACCCGTGCGATGAAGCCAGG-3' (M5ARV1835-). Those two fragments were purified, mixed, and amplified using the outside primers. The resulting product was digested with HindIII and BstEII and used to replace the corresponding HindIII/BstEII fragment in McM5/pFastBac, generating McM5ΔF/pFastBac. McM5-S1650A/pFastBac was produced by mutating AGT (Ser-1675) (nucleotide 5020–5022 in McM5; nucleotide 4948–4950 (Ser-1650) in X57377) into GCT (Ala) or GAG (Glu). Note: for easy comparison with previous publications, we used the nucleotide number of X57377, which contains exon-B and -F, but lacks exon-D. The melanocyte type myosin Va contains exon-D and -F, but lacks exon-B.

**Melanophilin and Rab27a Expression Vectors**—Murine melanocyte melanophilin (GenBank™ accession number: NM\_053015) was cloned from mouse kidney by RT-PCR using following primers: 5'-GCTAGC-ATGGGGAGGAAAGCTGGACCTGTCGGGTCTGACC and GATGGCTG-TTGGGTCAAGGCATGTCGTGTACG, and subcloned into p21TOPO vector (Invitrogen). Full-length melanophilin cDNA was excised by NheI/XhoI digestion and subcloned into a modified pFastBac plasmid (Invitrogen), pFastBacHis6, which has a C-terminal Tag (VSAHAGT-KHHHHHH) containing a His tag. Truncation of melanophilin was produced by PCR and subcloned into pFastBacHTb (Invitrogen). All truncated melanophilin constructs contain an N-terminal tag (MSYY-HHHHHHDYDIPTTENLYFQGGAMGIRNSKYVDELTS) containing a His tag.

Murine Rab27a (GenBank™ accession number: AB046693) was cloned from mouse brain by RT-PCR using primers 5'-CGGATCCGATGTCG-GATGGAGATTACGAT and 5'-ATGCCTCGAGTCAACAGCCACACAAC-CCCT, and subcloned into the BamHI and XhoI sites of pFastBacHTe (Invitrogen). A constitutive active form of Rab27a was produced by site-directed mutation of CAG (Glu-78) to TTG (Leu). Rab27a constructs contain an N-terminal tag (MSYYHHHHHHHDYDIPTTENLYFQGGAM-GIR) containing a His tag. All PCR-derived subcloned fragments were sequenced to confirm that no errors had been inadvertently introduced.

**Expression and Purification of Myosin Va, Melanophilin, and Rab27a Constructs**—Production of recombinant baculoviruses containing myosin Va heavy chains (McM5, McM5ΔF, McM5-S1650A, or McM5-S1650E), and expression and purification of recombinant myosin Va were described as previously (13), except the purified myosin Va samples were dialyzed against 5 mM Tris-HCl (pH 7.5 at 20 °C), 0.1 M

NaCl, 0.1 mM EGTA, and 1 mM DTT. Similar procedures were used to produce recombinant baculoviruses containing full-length or truncated melanophilin and Rab27a. Sf9 cells, infected with recombinant baculovirus containing melanophilin, were harvested by centrifugation and washed with 4 mM EGTA in Tris-buffered saline (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl). Cell pellets were then lysed with sonication in 50 mM Tris-HCl, pH 7.5, 15 mM imidazole-HCl, pH 7.5, 0.3 M NaCl, 1 mM EGTA, 0.02% NaN<sub>3</sub>, 5 mM ATP, 5 mM 2-mercaptoethanol, 10 μg/ml leupeptin, 0.2 mg/ml Trypsin inhibitor (egg), and 0.5% Triton X-100. After centrifugation at 120,000 × g for 30 min, the supernatant was incubated with 1.0 ml of nickel-nitrilotriacetic acid-agarose affinity resin in a 50-ml conical tube on a rotating wheel in a cold room for 1 h. The resin suspension was then loaded on a column (1 × 10 cm) and washed with 30 ml of solution A (50 mM Tris-HCl, pH 7.5, 15 mM imidazole-HCl, pH 7.5, 0.3 M NaCl, 0.2 mM EGTA, 2 μg/ml leupeptin, and 5 mM 2-mercaptoethanol). Melanophilin bound to resin was eluted with 0.25 M imidazole in solution A. The collected melanophilin was dialyzed overnight on ice against 1 liter of 5 mM Tris-HCl (pH 7.5 at 20 °C), 0.2 M NaCl, 0.1 mM EGTA, and 1 mM DTT.

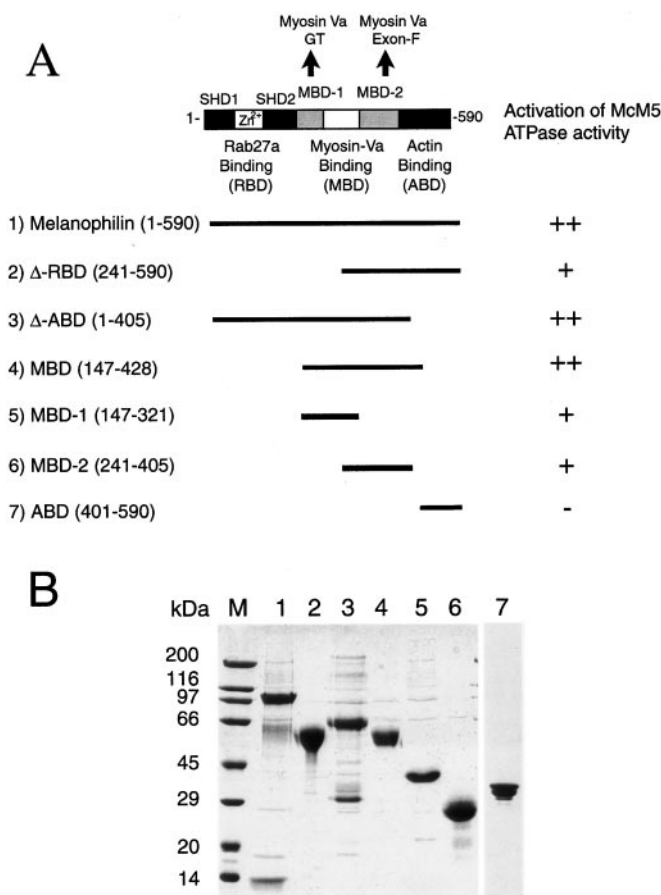
Protein concentration of Myosin Va was determined by Coomassie Brilliant Blue R250 staining of SDS-PAGE (7.5–20%) using smooth muscle myosin heavy chain as a standard as described previously (13). The concentrations of Rab27a and full-length and truncated melanophilin were measured by a Coomassie Plus Protein Assay Reagent (Pierce) using BSA as standard. The molecular masses of 66.7 kDa (full-length melanophilin), 42.6 kDa (Δ-RBD), 49.7 kDa (Δ-ABD), 35.0 kDa (MBD), 24.4 kDa (MBD-1), 22.5 kDa (MBD-2), 25.1 kDa (ABD), and 28.6 kDa (Rab27a) were used for calculation of molar concentration.

**Other Assays**—The ATPase activity of myosin Va was measured using an ATP regeneration system at 25 °C as described before (13). Reaction solution, except ATP, was mixed and preincubated at 25 °C for 10 min before adding ATP to start reaction. Coimmunoprecipitation of His-tagged melanophilin and FLAG-tagged McM5 was performed in the condition similar to ATPase assay. 85 nM McM5 and 255 nM melanophilin were incubated in washing solution composed of 20 mM MOPS-KOH (pH 7.0), 1 mM MgCl<sub>2</sub>, 0.25 mg/ml BSA, 1 mM DTT, 12 μM CaM, 0.2 M KCl, 1 mM EGTA (EGTA condition) or 1 mM CaCl<sub>2</sub> and 1.031 mM EGTA (pCa5 condition) at 25 °C for 10 min. 228 μl of the above mixture was mixed with 10 μl of Anti-FLAG M2 gel, and the mixture was incubated at room temperature for 10 min, spun down, and washed twice with 250 μl of corresponding washing solution. After carefully removing the supernatant, 20 μl of 0.3 mM FLAG peptide in washing solution was added to the precipitation. After mixing and spinning down, supernatant was carefully removed and subjected to SDS-PAGE (7.5–20%) and melanophilin-His was detected by mouse monoclonal antibody recognizing penta-His (#344660, Qiagen Inc., Germany).

#### RESULTS

**Expression and Purification of Melanophilin Constructs**—A full-length murine melanophilin (or slac2-a) and various truncated constructs were expressed in sf9 cells and purified by nickel-nitrilotriacetic acid-agarose affinity chromatography and their ability to stimulate the ATPase activity of murine melanocyte-type myosin Va (McM5) was examined. As shown in Fig. 1A, melanophilin is composed of three distinct domains, i.e. Rab27a binding domain, myosin Va binding domain, and actin binding domain. We produced seven constructs of melanophilin, i.e. full-length, Δ-RBD, Δ-ABD, MBD, MBD-1, MBD-2, and ABD. To facilitate purification, a histidine tag was attached to the C terminus of the full-length or the N terminus of the truncated melanophilin. Purified melanophilin constructs are shown in Fig. 1B. The apparent molecular masses of the purified melanophilin constructs estimated by SDS-PAGE are larger than the calculated molecular mass based on their amino acid sequences. For example, the calculated molecular mass of full-length melanophilin with C-terminal His-tag is 66.7 kDa, which is much lower than the apparent molecular mass of 90 kDa. The higher apparent molecular mass may be due to post-translational modification, or the unique amino acid composition of melanophilin. A similar result has been found for mammalian cell expressed melanophilin (27, 28).

**Melanophilin Stimulates the ATPase of Melanocyte-type Myosin Va (McM5)**—To examine whether the binding of mela-



**FIG. 1. Melanophilin and its truncation mutants.** *A*, schematic representation of three functional domains of mouse melanophilin and its deletion mutants used in this study. The Rab27a-binding domain (RBD) is composed of two potential  $\alpha$ -helical regions (SHD1 and SHD2) separated by two zinc finger motifs. The myosin Va-binding domain (MBD) is composed of two binding sites, MBD-1 and MBD-2, which bind to globular tail domain (GT) and exon-F region of myosin V, respectively. The C terminus is an actin-binding domain (ABD). The activities of each mutants (1  $\mu$ M) to stimulate the actin-activated ATPase activity of McM5 are indicated as “++,” >100% stimulation; “+,” 5–50% stimulation; and “-,” <2% stimulation. *B*, SDS-PAGE of purified melanophilin and its truncation mutants. Lane M is the molecular weight marker; lanes 1–7 correspond to the schematic structures in A.

nophilin to myosin Va affects the function of myosin Va, we measured the actin-activated ATPase of myosin Va in the presence of melanophilin. As shown in Fig. 2A, melanophilin significantly stimulated the actin-activated ATPase activity of melanocyte-type myosin Va (McM5) in the presence of EGTA. The stimulation by melanophilin follows Michaelis-Menten equation, with  $V_{\max}$  of  $5.39 \text{ s}^{-1}\text{head}^{-1}$  and  $K_m$  of  $0.15 \mu\text{M}$  melanophilin. The maximal stimulation of actin-activated ATPase activity of McM5 by melanophilin is about four times that in the absence of melanophilin (Table I). It should be mentioned that no significant ATPase activity was detected from the melanophilin sample alone. The activation was only observed for the actin-activated ATPase activity, and there was no significant activation of the basal ATPase activity of McM5 by melanophilin (not shown).

It has been shown that C terminus of melanophilin interacts with actin (29). One possibility for the activation of myosin Va is that melanophilin increases the affinity between myosin Va and actin. Therefore, we measured the ATPase activity of McM5 as a function of actin to determine the effect of melanophilin on the apparent  $K_{\text{actin}}$  and  $V_{\max}$  of the McM5 ATPase activity. As shown in Fig. 3A and Table II, although apparent  $K_{\text{actin}}$  was not significantly changed, the  $V_{\max}$  was increased by

3-fold by melanophilin in EGTA condition. The results indicate that the activation of myosin Va by melanophilin is not due to increase in the affinity between myosin Va and actin. Contrast to EGTA condition, there is no significant change of  $V_{\max}$  of actin-activated ATPase of McM5 in pCa5 condition by melanophilin, although the apparent  $K_{\text{actin}}$  was decreased by 2-fold by melanophilin (Fig. 3B and Table II). Coimmunoprecipitation of melanophilin and McM5 showed that melanophilin binds to McM5 in both EGTA and pCa5 condition (Fig. 3C).

Fig. 4 shows the melanophilin-induced activation of the actin-activated ATPase of McM5 as a function of ionic strength. In the absence of melanophilin, the activity increased from 0.15 to  $0.4 \text{ M}$  KCl. Previously, it was shown that myosin Va changes its conformation from a folded to an extended conformation, and this is reflected by the ATPase activity (13–15).  $\text{Ca}^{2+}$  and ionic strength affect the change in the conformation, and high ionic strength favors the extended active conformation (13–15). Therefore, it is thought that the increase in McM5 ATPase activity by ionic strength is due to the change in the conformation of McM5. Melanophilin activated the ATPase activity of McM5 at wide range of ionic conditions, but the ratio of the ATPase activity in the presence and absence of melanophilin reached the maximal at  $0.2 \text{ M}$  KCl. Similar results were found for truncated melanophilin constructs (see below). Therefore, all ATPase activity was measured in the presence of  $0.2 \text{ M}$  KCl.

**Domain Analysis of the Activation of McM5 ATPase Activity by Melanophilin**—Melanophilin consists of three distinct domains, *i.e.* Rab27a binding domain (RBD) at N terminus, myosin Va binding domain (MBD) in the middle region, and actin-binding domain (ABD) at C terminus (29). We produced various deletion constructs of melanophilin to determine the domain essential for the activation of myosin Va ATPase activity. As shown in Fig. 2,  $\Delta$ -ABD activated the actin-activated ATPase activity of McM5 similar to wild type melanophilin, suggesting that the actin-binding domain of melanophilin is not critical for the activation of McM5. Further, truncation of RBD did not predominantly attenuate the maximum activation of the ATPase activity, although a higher concentration was required for the activation (Fig. 2C). These results suggest that MBD alone is sufficient for the activation of myosin Va. It should be mentioned that the ionic strength dependence of  $\Delta$ -ABD and MBD stimulated ATPase activity is quite similar to that of the wild type, supporting that MBD contains all the binding and activating function of melanophilin to myosin Va (data not shown).

It was shown previously (23) that the interaction between myosin Va and melanophilin is regulated by a melanocyte (Mc)-specific alternative splicing in the tail domain of myosin Va. Melanophilin strongly interacts with the Mc-type myosin Va containing the Mc-specific exon-F but weakly interacts with brain-type myosin Va lacking exon-F. It has been shown that there are two separate myosin Va binding sites in MBD, *i.e.* the N terminus of MBD binds to myosin Va globular tail domain (MBD-1 site) and the C terminus of MBD binds to the exon-F region of Myosin Va (MBD-2 site) (28). To further identify the binding sites required for the activation of myosin Va, we produced two constructs containing MBD-1 and MBD-2 sites, respectively. As shown in Fig. 2, MBD-1 and MBD-2 differently stimulated the actin-activated ATPase activity of McM5. MBD-1 activated the ATPase activity of McM5 linearly correlated with the concentration of MBD-1 and could not reach saturation at  $30 \mu\text{M}$ . On the other hand, MBD-2 activated the ATPase activity with Michaelis-Menten kinetics with the dissociation constant similar to that of the entire MBD (Fig. 2 and Table I). However,  $V_{\max}$  of MBD-2-induced activation was much lower than that of whole MBD-induced activation (Table

**FIG. 2. Effect of melanophilin on the actin-activated ATPase activity of McM5.** The actin-activated ATPase activity of McM5 in the presence of various concentrations of melanophilin constructs (except MBD-1) was fit to the Michaelis-Menten equation. The experiments were conducted in buffer A containing 20 mM MOPS-KOH, pH 7.0, 0.2 M KCl, 1 mM MgCl<sub>2</sub>, 1 mM DTT, 0.25 mg/ml BSA, 12 μM CaM, 0.5 mM ATP, 2.5 mM phosphoenolpyruvate, 20 units/ml pyruvate kinase, 1 mM EGTA, in the presence of 40 μM actin, 25–50 nM myosin Va, and various concentrations of melanophilin constructs. *A*, melanophilin; *B*, Δ-ABD; *C*, MBD (open circle) and MBD-2 (closed triangle); *D*, MBD-1. For all figures in this paper, the ATPase activity on the y-axis represents the actin-activated ATPase activity.

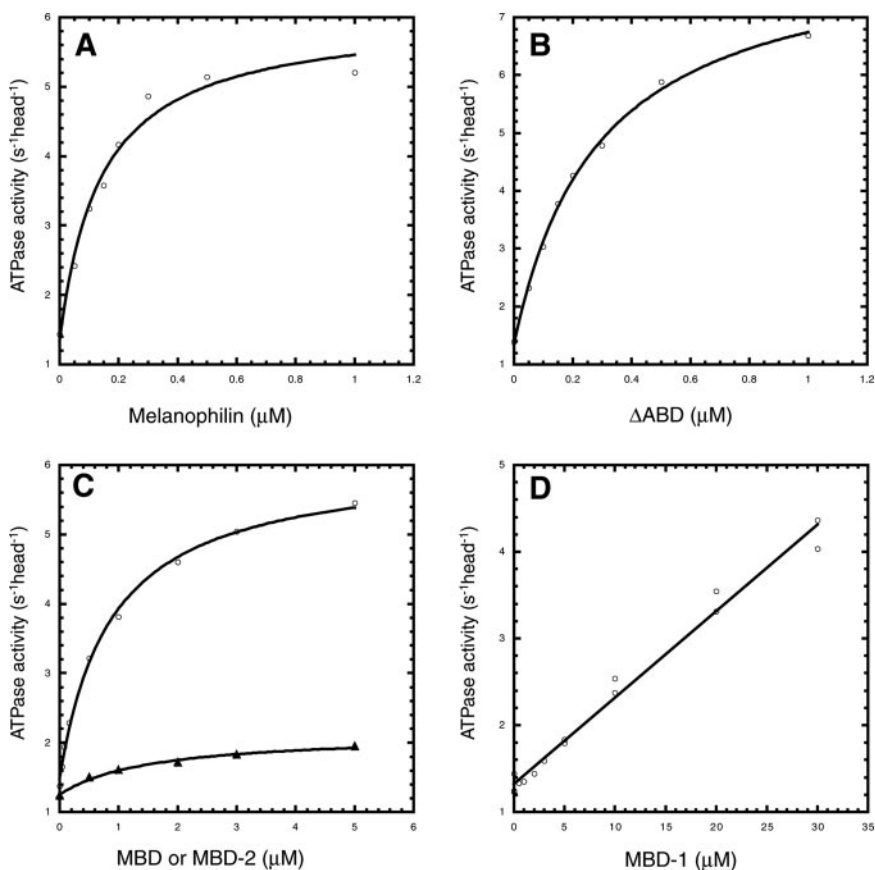


TABLE I

$K_m$  and  $V_{max}$  of Melanophilin constructs-stimulated actin-activated ATPase activity of McM5

Assay conditions were as described in the legend to Fig. 2. The activity of McM5 in the absence of melanophilin constructs, which is  $1.25 \pm 0.14 \text{ s}^{-1}\text{head}^{-1}$ , was subtracted. Curves are the least squares fits of the data points based on the equation,  $V = (V_{max} \times [M]) / (K_m + [M])$ , where  $[M]$  is the concentration of melanophilin constructs. All data in the table are the average of two independent assays.

	$V_{max}$ $\text{s}^{-1}\text{head}^{-1}$	$K_m$ $\mu\text{M}$
Melanophilin	$5.39 \pm 0.25$	$0.15 \pm 0.01$
Δ-ABD	$6.20 \pm 1.05$	$0.28 \pm 0.01$
MBD	$4.26 \pm 0.33$	$1.07 \pm 0.19$
MBD-1 <sup>a</sup>		
MBD-2	$0.81 \pm 0.08$	$2.24 \pm 0.70$

<sup>a</sup> The actin-activated ATPase activity of McM5 increased linearly with the increase of MBD-1 up to 30 μM.

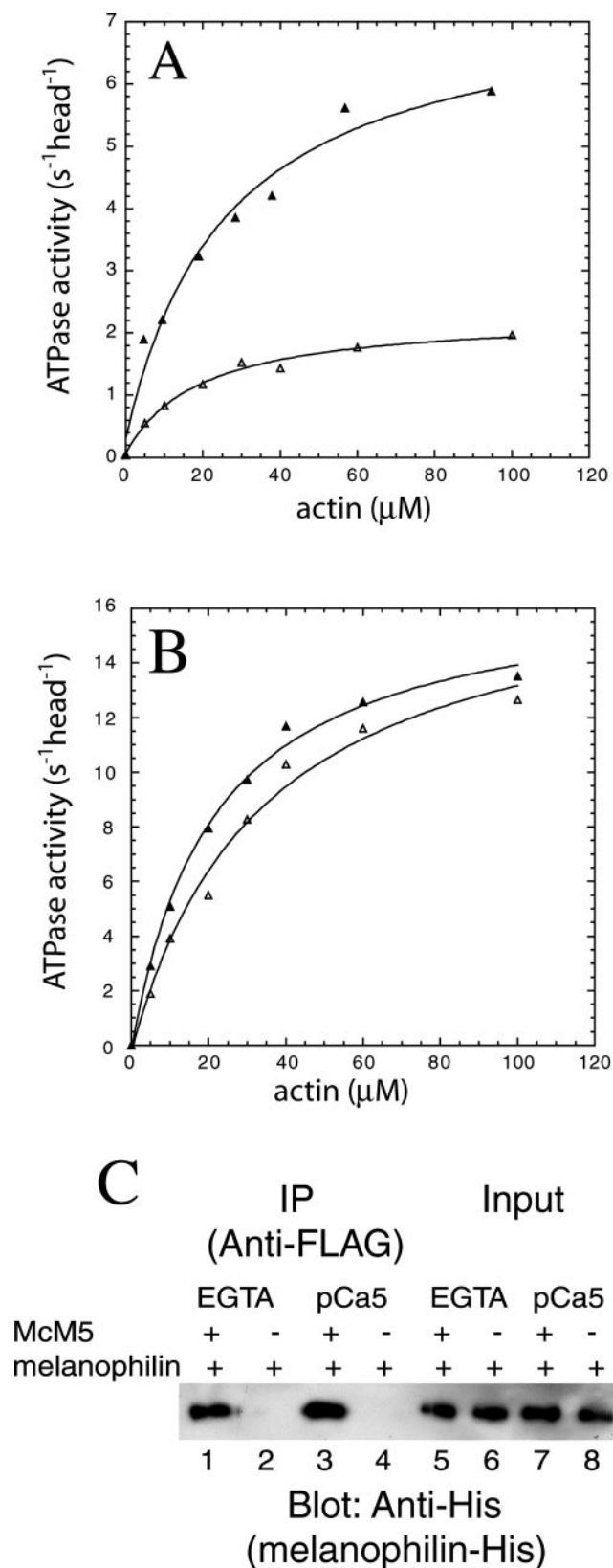
D). These results suggest that the MBD-2 site interacting with the exon-F of myosin Va plays a predominant role in the binding between myosin Va and melanophilin. On the other hand, the interaction between myosin Va and MBD-1 site is critical to the activation of the ATPase activity. Fig. 1A summarized the effects of truncation of melanophilin on the stimulation of actin-activated ATPase of McM5. McM5 activity was markedly stimulated by low concentration (1 μM) of full-length melanophilin and truncated melanophilin having both MBD-1 and MBD-2 domains, moderately stimulated by MBD-1 and MBD-2, but not stimulated by ABD.

To further assess the roles of MBD-1 and MBD-2, we examined the effects of MBD-1 and MBD-2 on the stimulation of McM5 ATPase activity by wild type melanophilin. As shown in Fig. 5, the ATPase activity of McM5 in the presence of melanophilin (0.1 μM) was further increased with MBD-1, but the activity decreased with MBD-2. It is thought that MBD-2 com-

peted with the full-length melanophilin and decreased the activation, because MBD-2 itself did not have a strong activating function. These results support the view that MBD-1 is essential for the activation and MBD-2 is critical for the high affinity of melanophilin to McM5. To determine if the presence of exon-F in McM5 is required for the activation by melanophilin, we produced McM5ΔF, an exon-F-deleted melanocyte-type myosin Va. Similar to McM5, the ATPase activity of McM5ΔF in pCa5 is about six times that in EGTA (see Fig. 7) indicating that McM5ΔF is well regulated by Ca<sup>2+</sup>, and exon-F is not involved in the Ca<sup>2+</sup>-dependent regulation of myosin Va. On the other hand, full-length melanophilin (0.5 μM), and MBD (5 μM) only slightly stimulated the ATPase activity of McM5ΔF, indicating that the presence of exon-F is critical for full activation of myosin Va by melanophilin.

Because myosin Va, melanophilin, and Rab27a could form a ternary protein complex, it is interesting to know if Rab27a plays a role in the stimulation of myosin Va by melanophilin. To test this possibility, we measured the actin-activated ATPase of McM5 in the presence of melanophilin with or without Rab27a. As shown in Fig. 6, neither GTP-bound nor GDP-bound of Rab27a changes the stimulation of McM5 ATPase activity by melanophilin. Similarly, Rab27a containing Q78L mutation (Rab27a-Q78L), which should drive Rab27a toward to GTP-bound state, has no effect on the stimulation of McM5 by melanophilin. It should be mentioned that Rab27a-Q78L coimmunoprecipitated with McM5 under the ATPase assay condition in the presence of melanophilin, but not in its absence (not shown), that was consistent with previous reports (7, 19).

*Effects of Phosphorylation at the Globular Tail Domain on the Regulation of Myosin Va*—Quite recently, it was found that organelle transport by myosin Va is down-regulated by the phosphorylation (possibly by CaMKII) in the C-terminal tail domain of mouse myosin Va (Ser-1650) (30). Phosphorylation of Ser-1650 resulted in the release of the motor from the or-



**FIG. 3. Actin dependence of McM5 ATPase activity in the presence or absence of melanophilin.** The actin-activated ATPase activity of McM5 in the absence (open triangle) and presence (closed triangle) of  $0.5 \mu\text{M}$  of melanophilin are fit to the Michaelis-Menten equation. Assay conditions are as described in Fig. 2. A, EGTA; B, pCa5; C, binding of melanophilin to McM5 in EGTA and pCa5 conditions. His-tagged melanophilin coimmunoprecipitated with FLAG-His-tagged McM5 was detected by mouse monoclonal antibody recognizing His tag. The lanes in the right panel (input) indicate the total melanophilin-His (1/13 volume of the reaction mixture) used for immunoprecipitation.

ganelle. However, it is unknown whether the tail domain phosphorylation regulates the motor activity of myosin Va.

We produced two McM5 mutations, S1650A and S1650E, because it has been shown that the S1650E mutation has the same effect as phosphorylation of Ser-1650 in inducing the release of myosin Va from the organelle (30). We examined whether the actin-activated ATPase activity of myosin Va is affected by the mutation of Ser-1650. As shown in Fig. 7, we found no significant change in the actin-activated ATPase activity of McM5 by mutation of Ser-1650 to Glu or Ala. McM5-S1650A and -S1650E have low activity in EGTA, and high activity in pCa5, respectively, which is similar to wild-type McM5. Furthermore, MBD, MBD-1, and MBD-2 stimulated the ATPase activity of McM5-S1650A and -S1650E in an extent similar to that of wild-type McM5, indicating that phosphorylation of Ser-1650 does not regulate the activation of McM5 by melanophilin.

#### DISCUSSION

Although the motor head domain is highly conserved for myosin superfamily members, the tail domain is quite diverse and it is thought that the unique tail domain serves as a targeting site for myosin isoform specific partners. Melanophilin has been identified as a melanocyte type myosin Va binding partner (7, 19–22). As myosin Va functions as a cargo transporting motor, it is thought that melanophilin is a key protein for myosin Va-dependent vesicular trafficking. In the present study, we found that melanophilin directly activates the actin-activated ATPase activity of melanocyte-type myosin Va (McM5). Melanophilin markedly stimulated the activity of McM5, but only marginally activated that of McM5 lacking exon-F. Consistently, it was found that melanophilin binds strongly to McM5, but weakly to McM5 lacking exon-F (23, 28). Therefore, the activation of the ATPase activity is due to the direct binding between myosin Va and melanophilin. It should be mentioned that no significant ATPase activity was found in the purified melanophilin, and the increase of ATPase activity is not due to the contamination of ATPase from the purified melanophilin. This is the first demonstration that the binding of the cargo molecule directly activates myosin motor activity.

It was reported previously that the actin-binding domain of melanophilin is required for melanosome distribution in melanocytes (29). However, the present results indicate that the actin-binding domain of melanophilin does not play a role in the activation of myosin Va activity because  $\Delta\text{ABD}$ , which lacks the actin-binding domain, activated the actin-activated ATPase activity of myosin Va with similar  $V_{\text{max}}$  and  $K_m$  to those of the wild type melanophilin. The function of the actin-binding domain of melanophilin is obscure, and it is unlikely that this domain tightly associates actin, because this would prevent the movement of myosin Va on actin filaments. The actin binding ability of melanophilin may contribute to increase the localized concentration of both myosin Va and melanophilin at actin filaments thus facilitating the interaction between them.

It was shown previously that melanophilin binds to exon-F of myosin Va that is unique to the melanocyte type myosin Va (23). On the other hand, it has been known that missense mutation in the globular tail domain of myosin Va causes *dilute* coat color phenotype in mice (31), suggesting a role of the globular tail domain for myosin Va function. Quite recently, it was reported that the globular domain also contributes to the binding of myosin Va to melanophilin (28). The globular tail and exon-F of myosin Va bind to melanophilin at the N-terminal (MBD-1, amino acids 137–321) and C-terminal side (MBD-2, amino acids 241–405) of the MBD of melanophilin, respectively. In the present study, we found that MBD-2/

TABLE II

 $K_{actin}$  and  $V_{max}$  of actin-activated ATPase activity of McM5 in the absence and presence of melanophilin

Assay conditions were as described in the legend to Fig.3. All data are the average of three independent assays.

	EGTA condition		PCa5 condition	
	$V_{max}$ $s^{-1}head^{-1}$	$K_{actin}$ $\mu M$	$V_{max}$ $s^{-1}head^{-1}$	$K_{actin}$ $\mu M$
Control	$2.44 \pm 0.29$	$23.9 \pm 6.3$	$20.5 \pm 1.3$	$53.2 \pm 16.4$
+ 0.5 $\mu M$ melanophilin	$6.60 \pm 0.80$	$22.3 \pm 5.5$	$19.5 \pm 1.3$	$28.3 \pm 5.4$

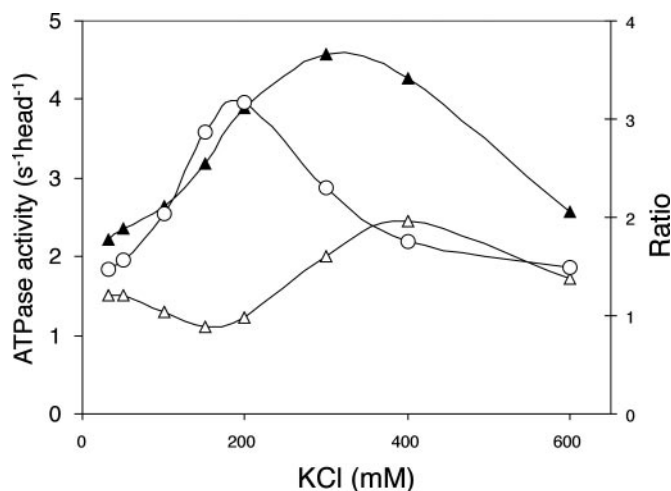


FIG. 4. Ionic strength dependence of stimulation of actin-activated McM5 ATPase activity by melanophilin. The actin-activated ATPase activity of McM5 was measured in various concentrations of KCl. The assay was conducted as described in Fig. 2 except that various KCl concentrations were used. *Open triangle*, in the absence of melanophilin; *closed triangle*, in the presence of 0.5  $\mu M$  melanophilin; *open circle*, the ratio of ATPase activity in the presence and absence of melanophilin.

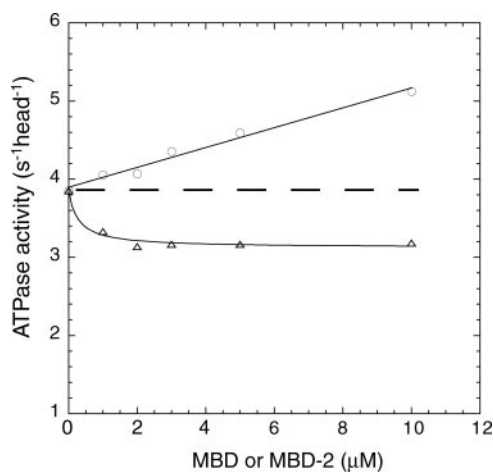


FIG. 5. Effects of MBD-1 and MBD-2 on the melanophilin-stimulated actin-activated ATPase activity of McM5. The assay conditions are as described in Fig. 2. Except that 0.1  $\mu M$  melanophilin and the indicated concentration of MBD-1 (*open circle*) or MBD-2 (*open triangle*) were added to the assay solution. The *broken line* represents the activity of McM5 with 0.1  $\mu M$  melanophilin only.

exon-F interaction has higher affinity than MBD-1/myosin Va tail interaction and plays a predominant role in the binding between the two proteins. However, the MBD-2 activated the ATPase much less than the entire MBD domain. On the other hand, MBD-1 highly activated the ATPase activity of myosin Va, although the concentration required for the activation was much higher than whole MBD. The results suggest that the two domains have distinct roles in the activation of myosin Va, *i.e.* MBD-1 is responsible for the activation, whereas MBD-2 is mainly responsible to the high affinity binding to myosin Va,

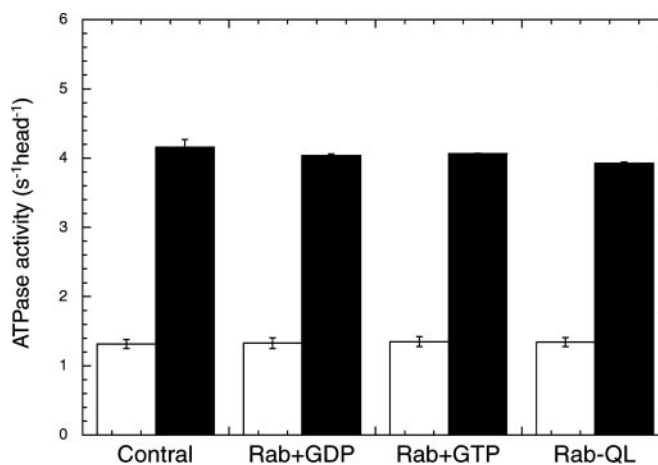


FIG. 6. Effects of Rab27a on the stimulation of actin-activated ATPase activity of McM5 by melanophilin. GTP-bound Rab27a was prepared by incubating 50  $\mu M$  Rab27a with 100  $\mu M$  GTP $\gamma$ S in the presence of 1 mM EDTA at 25  $^{\circ}C$  for 30 min, then adding  $MgCl_2$  to 5 mM. GTP-Rab27a was stored on ice and used within 2 h. A similar procedure was used to prepare GDP-bound Rab27a except using GDP instead of GTP $\gamma$ S. The ATPase activity assay was conducted in a solution containing 20 mM MOPS-KOH, pH 7.0, 0.2 M KCl, 1 mM  $MgCl_2$ , 1 mM DTT, 0.25 mg/ml BSA, 12  $\mu M$  CaM, 0.5 mM ATP, 2.5 mM phosphoenolpyruvate, 20 units/ml pyruvate kinase, 1 mM EGTA, 40  $\mu M$  actin, 40–50 nM myosin Va, with (*closed bar*) or without (*open bar*) 0.5  $\mu M$  melanophilin, and 2.5  $\mu M$  Rab27a or Rab27a-Q78L. All data are the average of two independent assays.

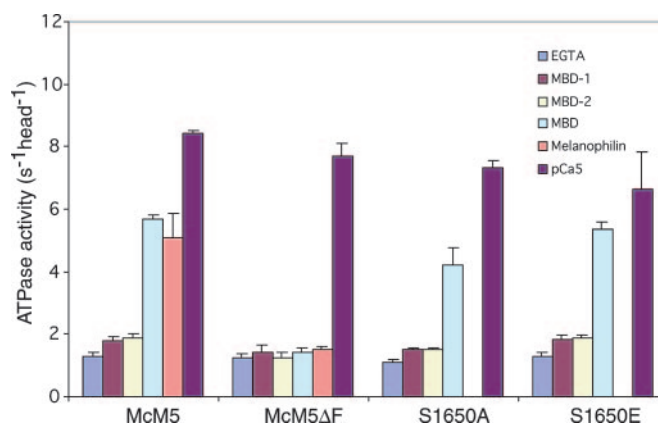


FIG. 7. Effects of melanophilin on the actin-activated ATPase activity of various McM5 constructs. The actin-activated ATPase activity of McM5 and its variants was measured in the presence of various melanophilin constructs. Assay conditions are the same as Fig. 2, except that 40  $\mu M$  actin was used. Melanophilin (0.5  $\mu M$ ),  $\Delta$ -ABD (1  $\mu M$ ), MBD (5  $\mu M$ ), MBD-1 (5  $\mu M$ ), or MBD-2 (5  $\mu M$ ) was included in the indicated assay. All data are the average of at least three independent assays.

but not essential for the activation (Fig. 2). Consistent with this view, MBD at 5  $\mu M$  activated the ATPase activity of McM5 lacking exon-F much less than the activation of the wild type McM5 that is due to the lack of higher affinity interaction between the exon-F and MBD-2 domain. To support this notion, we found that the addition of MBD-2 attenuated the activation of McM5 ATPase activity by intermediate concentra-

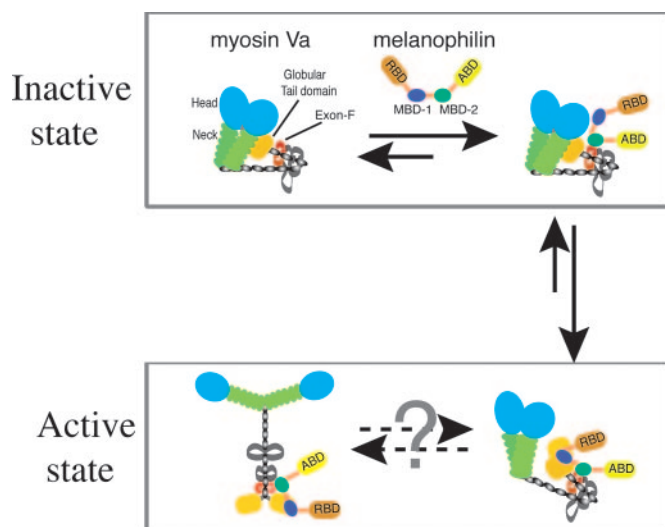


FIG. 8. **Schematic model of the activation of myosin Va ATPase activity by melanophilin.** ABD and RBD represent actin-binding domain and Rab27a-binding domain of melanophilin, respectively. Melanophilin binds to the inhibited form of myosin Va at exon-F with MBD-2 domain. The bound melanophilin then interacts with the tail domain of myosin Va with MBD-1 domain. This weakens the interaction between the tail and head-neck of myosin Va.

tion of wild type melanophilin, whereas MBD-1 rather increased the activity. This is because MBD-2 competes with the wild type melanophilin for myosin Va binding, but MBD-2 bound myosin Va is less activated than that with wild type melanophilin. MBD, which has both MBD-1 and MBD-2 sites, has much higher affinity to myosin Va than MBD-1, suggesting that MBD-1 and MBD-2 function synergistically in binding to myosin Va. The present results are consistent with previous studies that amino acids 367–400 of melanophilin comprise a minimal determinant of the myosin Va-binding domain (22).

Of interest is how the binding of melanophilin at the tail of myosin Va activates acto-myosin Va ATPase activity. It was shown recently by others and us that the actin-activated ATPase activity of myosin Va is regulated via change in the conformation of myosin Va (13–15). We found that myosin Va forms a folded conformation in which the globular tail domain bent back to the head-neck region of the molecule at low ionic strength in  $\text{Ca}^{2+}$ -free condition, and this conformation is abolished either at high ionic strength or binding of  $\text{Ca}^{2+}$  at the CaM light chain at the neck (13). Therefore, one possible scenario for the activation of myosin Va activity by melanophilin is that the binding of melanophilin to the inhibited form of myosin Va at the tail domain of myosin Va weakens the interaction between the tail and head-neck of myosin Va, thus shifting the conformational equilibrium of myosin Va toward the active conformation (Fig. 8). We attempted to see a large change in the sedimentation velocity of myosin Va in the presence of melanophilin, but we failed to observe a large change. Therefore, the binding of melanophilin may not unfold the folded structure of myosin Va, although the binding attenuates the interaction between the tail and the head-neck thus releasing the inhibition. Alternatively, another unknown mechanism is operating for the melanophilin-dependent activation of myosin Va. Further studies are required to understand how melanophilin binding at the globular tail domain activates the ATPase activity.

There are at least three alternative splicing isoforms of murine myosin Va (32). The alternative splicing is located in the distal region of the  $\alpha$ -helical coiled-coil region. Melanocyte-type has exon-D and -F, but no exon-B. Brain-type has exon-B, but no exon-D and -F. The third isoform, existing in skin, spleen,

thymus, and other organs, has exon-F, but no exon-B and -D. Therefore, it is plausible that myosin Va-targeting molecules in brain and other organs bind to the sequence encoded by exon-B as an anchoring site in addition to the globular tail.

Of interest is the regulation of the interaction between myosin Va and melanophilin. It was shown previously (30) that phosphorylation of myosin Va by  $\text{Ca}^{2+}$ /CaM-dependent kinase II at Ser-1650 abolishes the recruitment of myosin Va to melanosome, suggesting that the phosphorylation is a critical regulatory process for cargo binding of myosin Va. In the present study, we examined the effect of S1650E mutation that mimics the phosphorylation of myosin Va for the recruitment of myosin Va to melanosome on the melanophilin-induced enhancement of the actin-activated ATPase activity. Fukuda *et al.* (28) recently found that the binding of melanophilin to the truncated myosin Va tail domain, including exon-F and globular tail domain, is not affected by S1650E mutation. However, the binding of the tail domain to melanophilin is rather weak and the sequence encoded by exon-F plays a predominant role in the binding between the two proteins. Because our study suggests that the role of the interaction between the tail domain and melanophilin is the functional activation of the ATPase, we anticipated that Ser-1650 phosphorylation reduces the melanophilin-induced activation of the ATPase activity. Interestingly, we found no significant effect of S1650E mutation on the melanophilin-induced activation of myosin Va ATPase activity. The present result is consistent with the earlier study of Fukuda and Kuroda (28) and shows that Ser-1650 phosphorylation neither affects the binding of melanophilin to myosin Va nor changes the melanophilin-induced activation of the ATPase activity of myosin Va. At present, we cannot explain the discrepancy from the earlier results of Karcher *et al.* (30). There might be other myosin Va isoforms or myosin Va binding partners in *Xenopus* oocyte that are involved in the phosphorylation-dependent regulation of myosin Va in *Xenopus* oocyte.

In summary, the present finding raises an idea that myosin motors are switched on upon binding to the cargo molecules, thus avoiding the waste of ATP consumption. Further studies with other cargo molecules and myosin motors are required to find whether the binding of cargo molecules regulates the partner motor protein function.

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