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Myosin X transports Mena/VASP to the tip of filopodia☆

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Abstract

Myosin X (M10) is a two-headed actin based motor expressed in a variety of cell types, that is thought to play a role in cargo movement in mammalian cells, but its cellular function is unknown. Here we found that M10 binds to Mena/VASP, which facilitates actin polymerization by competing with actin capping proteins. Immunocytochemistry revealed that endogenous M10 co-localized with Mena/VASP at the tip of filopodia. Consistently, both EGFP-M10 and RFP-VASP were found at the tip of filopodia. The result raises a hypothesis that M10 transports Mena/VASP towards the tip of filopodia. Supporting this idea, the amount of VASP at the tip of filopodia was proportional to that of M10. Furthermore, we directly visualized the movement of M10 and VASP in living HeLa cells under fluorescence microscope. EGFP-M10 and RFP-VASP move together from the root to the tip of the filopodia. Interestingly, the amount of M10 at the tip of filopodia was linearly related to the length of filopodia, consistent with the actin filament extending function of VASP. These results show that M10 is a specific motor carrying Mena/VASP from the root to the tip of the filopodia where extension of actin filament takes place.

Keywords: Myosin; Myosin X; VASP; Mena; Transport; Filopodia

Myosins are actin-based motor proteins that convert the chemical energy of ATP to mechanical work in cells. It is now clear that myosin constitutes a diverse superfamily that plays a critical role in diverse cellular motile events [1,2]. Among them, M10 is expressed ubiquitously in various mammalian tissues [3,4]. Based upon the deduced amino acid sequence, it is predicted that M10 consists of a motor domain, three IQ motifs that function as light chain binding sites, coiled-coil domain, and a tail domain [4]. Because of the presence of a coiled-coil domain, it has been thought that M10 is a two-headed myosin. The tail domain is quite different from conventional myosin and it is thought that M10 does not form thick filaments. There are three PH domains, one MyTH4 (myosin tail homology 4) domain, and one FERM domain in each heavy chain. While the actual function of these domains is not known, it is thought that the tail domain functions as the anchoring site for the M10 binding partners. This is because the tail domain of myosin is diverse among the superfamily and it is expected that the binding of the specific docking partner at the tail domain does not interfere the motor activity. For other myosin superfamily members, it has been shown that various myosin specific docking partners bind the tail domain of myosin [5,6].

By immunocytochemistry, it was found that M10 is present at the edge of lamellipodia, membrane ruffles, and the tip of filopodial actin bundles in cultured cells, suggesting that M10 plays a role in regions where actin is in dynamic reorganization [4]. However, the physiological function of M10 is largely unknown. Biochemical study has shown that M10 is an actin based motor molecule that moves on actin filaments towards plus end [7]. It has also suggested that the duty ratio of M10 is larger than conventional myosin [7], implying that M10 may function as a cargo carrier to transport target molecules to particular destination in cells. One of the most critical issues is what the targeting molecules of M10 are and how the transportation of such targeting

☆ *Abbreviations:* M10, myosin X; EGFP, enhanced green fluorescent protein; DsRed, *Discosoma* sp. red fluorescent protein; VASP, vasodilator-stimulated phosphoprotein; Mena, mammalian enabled.

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molecules by M10 influences the function of cells. In the present study, we found that M10 co-localizes with Mena/VASP at the tip of filopodia. Mena/VASP are actin binding proteins that localize to actin stress fiber, filopodia, and the lamellipodial leading edge [8]. Recently, it was reported that Ena/VASP antagonizes the ability of capping proteins to inhibit actin polymerization at barbed ends in vitro [8]. Therefore, it is anticipated that the distribution of Ena/VASP at specific cellular location may regulate the elongation of actin filaments that is correlated with the change in cytoskeletal structure and cell shape. As described below, we found that M10 moves VASP from the root to the tip of filopodia. The result suggests that M10 carries VASP along actin filaments to its barbed end where actin polymerization takes place.

Materials and methods

Plasmid construction. Bovine M10 cDNA fragments were kindly provided by Dr. D.P. Corey (Harvard University). M10CC construct containing the entire motor domain, neck domain, and coiled-coil domain was prepared as described [7]. An *Xba*I site was created at the 3′ side of the stop codon and an *Xba*I fragment (residues 3238–6400) encoding the tail domain of M10 was inserted into M10CC/pFastbac1 after an *Xba*I site at nucleotide 2517 was eliminated to create pFastM10 containing entire coding region of M10. An *Nhe*I/*Xba*I fragment was excised from an M10CC clone and subcloned into pEGFP-C1 (Clontech). An *Xba*I fragment encoding the tail domain was then ligated into pEGFP-C1/M10CC to produce pEGFP/M10WT (EGFP-M10). An *Xba*I fragment excised from pFastBac-M10 was inserted into the pFastBacHTb vector to obtain pFastBac-M10 tail.

A cDNA library was generated by reverse transcription (Superscript II reverse transcriptase; Gibco-BRL) using human lung total RNA (Clontech). VASP cDNA was amplified from the cDNA library by PCR using *Pfu* polymerase with sense primer 5′-ATGAGC GAGACGGTCATCTGTTCCAGC-3′ and antisense primer 5′-TCA GGGAGAACCCCGCTTCCTCAGCTC-3′. A unique *Xho*I site and a unique *Eco*RI were created at the 5′ side of the initiation codon and at the 3′ side of the stop codon, respectively. The amplified cDNA was digested with *Xho*I/*Eco*RI, and ligated into the pDsRed2-C1 mammalian expression vectors (Clontech), to obtain pDsRed2-VASP (dsRed-VASP). A VASP cDNA digested with *Xho*I/*Sal*I was cloned to *Sal*I site of the pET30a *Escherichia coli* expression vector (Novagen).

Expression and purification. Expression and purification of recombinant M10 tail were carried out as described [9]. VASP was produced as a recombinant protein with an amino-terminal His-tag in *E. coli* BL21 (DE3). Purification step of recombinant VASP was same as M10 tail.

Antibodies. Anti-Mena monoclonal antibody was purchased from BD Transduction Laboratories. Anti-VASP monoclonal antibody was purchased from Alexis biochemicals. The anti-M10 antibody was raised against a synthetic peptide CDDDAFKDSPNPSEHGHSDQRTS (corresponding to amino acids 1000–1021 of M10), injected to a rabbit, and purified according to published methods [10]. All secondary antibodies were purchased from Jackson ImmunoResearch, except for the horseradish peroxidase-conjugated secondary antibodies for Western blotting (Bio-Rad).

Cell culture and transfection. HeLa cells (American Type Culture Collection) were cultured with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin (Life Technologies). Transient transfections were done with a Fugene-6 (Roche Biochemicals) according to the manufacturer's instructions.

Immunoprecipitation. Cells were lysed in ATP containing lysis buffer (20 mM Hepes, pH 7.4, 50 mM NaCl, 2 mM $MgCl_2$, 0.2 mM EGTA, 1 mM ATP, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml pepstatin A, and 1 μg/ml trypsin inhibitor). The samples were centrifuged at 10,000*g* for 30 min at 4 °C and the supernatants were incubated with antibodies conjugated to Affi-Prep Protein A (Bio-Rad) 4 h at 4 °C. The mixture was centrifuged (30*g*) in the presence of ATP to precipitate the protein A beads. The precipitates were washed five times in ice-cold lysis buffer. The precipitates were subjected to immunoblot analysis.

In vitro binding assay. His6-VASP (400 nM) was incubated for 1 h on ice with 400 nM His6-M10 tail in 250 μl of binding buffer (20 mM Hepes, pH 7.4, 50 mM NaCl, 2 mM $MgCl_2$, 0.2 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride). The mixture was then incubated with shaking for an additional 1 h at 4 °C with antibodies conjugated to Affi-Prep Protein A (Bio-Rad). After extensive washing with the binding buffer, proteins bound to the beads were subjected to immunoblot analysis.

Western blot analysis. The samples were subjected to 4–10% gradient SDS–PAGE, and the proteins were transferred to a nitrocellulose membrane (Bio-Rad). The membrane was incubated with primary antibody, followed by horseradish peroxidase-conjugated secondary antibody. The immunoreactive bands were detected with enhanced chemiluminescence (Amersham Biosciences).

Immunofluorescence. Cells were fixed with fix buffer (4% formaldehyde, 2 mM $MgCl_2$, and 1 mM EGTA in PBS) at room temperature for 20 min, washed twice with PBS, and permeabilized with 0.5% Triton X-100 in PBS at room temperature for 10 min. Cells were washed twice with PBS and blocked with 5% BSA (Gibco-BRL) in PBS for 30 min. Cells were incubated with primary antibodies for 1 h, washed three times with PBS, and then incubated with anti-rabbit or mouse fluorescein isothiocyanate (FITC)-conjugated antibody for an hour. For actin staining, cells were incubated with 5 U/ml Rhodamine phalloidin (Sigma) for 20 min at room temperature. The samples were mounted in 3% 1,4-diazabicyclo[2.2.2]octane (Sigma), 90% glycerol in PBS. Images were viewed and analyzed as described previously [11]. Differential interference contrast (DIC) and fluorescence images were viewed with a Leica DM IRB laser scanning confocal microscope controlled by Leica TCS SP II systems (Leica Microsystems).

Determination of the amount of M10 and VASP at the tip of filopodia, and the length of filopodia. To quantify the amount of M10 and VASP at the tip of filopodia (see Fig. 4), HeLa cells were plated onto poly-L-lysine (Sigma) coated glass coverslips for 16 h, and fixed and permeabilized same as above. The cells were double-stained with anti-M10 antibody, detected by FITC-conjugated anti-rabbit secondary antibody, and anti-VASP mononal antibody, detected by indodicarbocyanine (Cy5)-conjugated anti-mouse secondary antibody. The tips of filopodia stained by FITC and Cy5 were outlined manually, and the total fluorescence signals of both FITC (M10) and Cy5 (VASP) in each tip of filopodia acquired from the digital confocal microscope were obtained, respectively.

Filopodia were defined as thin phalloidin-stained protrusions that extended from the cell margin. Filopodia and cell margins were traced and the lengths of filopodia was measured with Leica confocal software. The intensity of the fluorescence signal of M10 at the tip measured as described above was plotted against the length of each filopodia simultaneously.

Statistics. The ratio of fluorescent intensity of M10 to the fluorescent intensity of VASP, and the length of filopodia to the fluorescent intensity of M10 were scatter-plotted and analyzed by linear regression analysis using PRISM 3.0 (GraphPad software).

Video microscopy. Cells were imaged using the same system of immunofluorescence to control illumination shutters and camera exposure (Leica Microsystems). Time-lapse images were obtained by

sequential epifluorescent and phase illumination. The intervals were 10 s and exposure times were 100–300 ms, depending on the time-lapse interval and level of fluorescence. Cells were imaged over periods of 3–10 min at room temperature (25–30 °C).

Results

Binding of M10 to Mena and VASP

We performed immunoprecipitation of total cell lysates with anti-M10 antibodies. The immunoprecipitated sample was analyzed by SDS–PAGE followed by Western blot. As shown in Fig. 1A, Mena was co-immunoprecipitated with M10. The result suggests that M10 and Mena are in the same protein complex and interact each other either directly or indirectly. To examine whether VASP directly bind to M10, M10 tail domain (M10 tail) and VASP were expressed and purified. The purified proteins were subjected to pull down assay using anti-VASP antibodies. As shown in Fig. 1B, M10 tail was co-immunoprecipitated with VASP. The M10/VASP mixture was also immunoprecipitated with anti-M10 antibodies, and it was found that VASP was co-immunoprecipitated with M10 tail (not shown). These results indicate that VASP directly binds to the tail domain of M10.

M10 co-localizes with Mena and VASP in mammalian cells

Next, we compared the subcellular localization of M10 and Mena/VASP by immunocytochemical analysis using HeLa cells. As shown in Fig. 2A, indirect immunofluorescent studies revealed that anti-M10 antibody specifically detected concentrated M10 at tips of filopodia, and this is consistent with earlier results [12]. And VASP localized at actin stress fibers, lamellipodial leading edges, focal adhesion sites, and tips of filopodia (Fig. 2B). These subcellular localizations of VASP correspond with the reported data [8,13–15]. These results suggested that endogenous M10 and endogenous VASP co-localized at the tips of filopodia (Fig. 2C). To support these results, we detected that EGFP-M10 exclusively localized at the tip of filopodia (Fig. 2D), and dsRed-VASP also localized at the tip of filipodia (Fig. 2E). We found that endogenous Mena was also localized at the tips of filopodia where M10 was localized (Figs. 2H and I). The results are consistent with the fact that M10 directly binds to VASP in vitro, and further suggests that M10 interacts with Mena/VASP in the cell environment. A critical question is whether M10 functions as a motor carrying Mena/VASP to the specific location in cells.

M10 transports VASP from the base to the tip of filopodia

The crucial evidence to answer this question was obtained from direct imaging of M10 and Mena/VASP in living cells.

Fig. 3 shows the movement of EGFP-M10 and dsRed-VASP in HeLa cell. We found that the green spot and the red spot moved together from the root to the tip of filopodia, indicating that M10 and VASP moved together. The velocity of the movement was ~120 nm/s that is similar to the velocity reported for M10 [7]. The movement was stopped when EGFP and dsRed signals reached to the tip of filopodia. These results indicate that M10 carries VASP from the root to the tip of filopodia along with actin cables and stops moving when M10 reached the end of actin filament at the tip of filopodia. Interestingly, we found that M10 and VASP also moved back together from the tip to the root of filopodia. The velocity of the reverse movement, however, was much slower than that of the forward movement and it is anticipated that M10/VASP complex are accumulated at the tip of the filopodia. This is actually demonstrated by the localization of the fixed cells, which show the accumulation of M10 and VASP at the tip of filopodia (Fig. 1). While we do not completely understand how M10/VASP moves

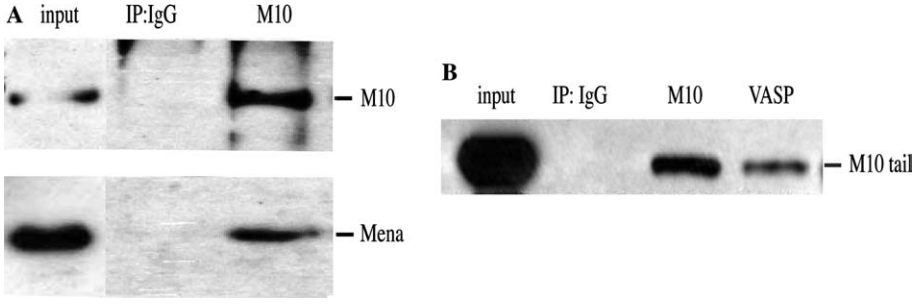

Fig. 1. Binding of M10 to Mena and VASP. (A) Co-immunoprecipiation of Mena with M10. HeLa cell lysates were subjected to immunoprecipitation with anti-M10 antibodies or control IgG. The immunoprecipitated samples were subjected to SDS–PAGE followed by Western blot with anti-M10 and anti-Mena antibody, respectively. (B) Binding of purified VASP and M10 tail. The purified M10 tail and VASP were mixed at 4 °C for 1 h, then the protein complex was immunoprecipitated with anti-M10, anti-VASP antibodies, and control IgG. The immunoprecipitated samples were subjected to Western blot analysis with anti-M10 antibody.

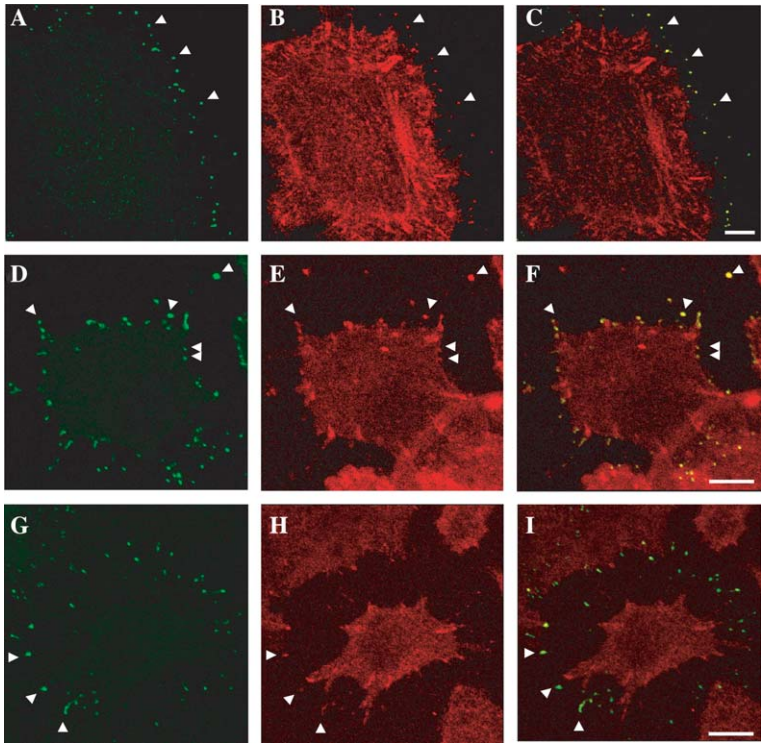

Fig. 2. M10 co-localizes with Mena and VASP in mammalian cells. HeLa cells were double-stained with anti-M10 and anti-VASP antibodies. (A) Endogenous M10 localizes at the tips of filopodia. (B) Endogenous VASP localizes at actin stress fibers, lamellipodial leading edges, focal adhesion sites, and tips of filopodia. (C) Endogenous M10 and VASP co-localize at the tips of filopodia. HeLa cells were co-transfected with EGFP-M10 and dsRed-VASP. The cells were fixed at 16 h after transfection. (D) EGFP-M10 localizes at the tip of filopodia. (E) dsRed-VASP also localizes at the tip of filopodia in addition at focal adhesion sites. (F) A merged image of (D) and (E) shows clear co-localization of M10 and VASP at the tip of filopodia. HeLa cells transfected with EGFP-M10 were fixed at 16 h after transfection and endogenous Mena was immunostained with anti-Mena antibody followed by TexasRed conjugated second antibody. (G) EGFP image; (H) TexasRed image of endogenous Mena; and (I) merged image of (G,H). Yellow color in (C,F,I) indicates the co-localization of green and red fluorescence signals (arrowheads). Scale bar, 10 μm.

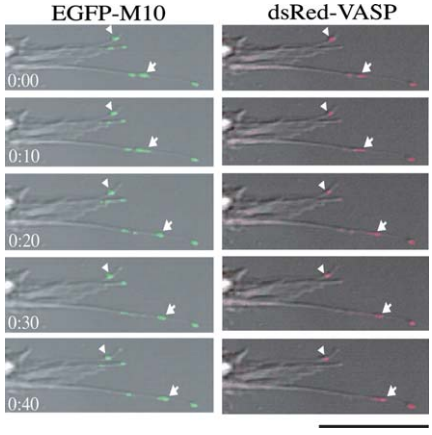

Fig. 3. M10 transports VASP from the base to the tip of filopodia. Simultaneous imaging of EGFP-M10 and dsRed-VASP in living HeLa cell is shown. HeLa cells were co-transfected with EGFP-M10 and dsRed-VASP. After 24 h, the EGFP and dsRed image was captured by video microscopy. A time-lapse series shows movements of EGFP-M10 (left) with corresponding dsRed-VASP images (right). Within the filopodia, EGFP and dsRed positive puncta move together to both anterograde (arrow) and retrograde (arrowhead) directions. The fluorescence images are superimposed with DIC images. Scale bar, 5 μm. Numerals on the pictures indicate time (minutes:seconds) from the start of observation.

backward, we think that this is due to the diffusion because: (1) M10 is a plus directed motor and actin filaments in filopodia are lined up with their plus end towards the tip of filopodia. (2) The velocity of backward movement is much slower than the forward motion. However, we cannot exclude the possibility that the reverse movement is achieved by minus directed motor(s).

Transportation of VASP by M10 to the tip of filopodia affects the filopodia length

The above results indicate that M10 is responsible for transportation of VASP to the tip of filopodia. To further ascertain this idea, we studied the amount of endogenous M10 and VASP at the tip of filopodia among various length of filopodia. As shown in Figs. 4A and B, longer filopodia tips (arrows) had greater amounts of M10 and VASP than shorter filopodia (arrowheads). To analyze the data more quantitatively, we determined the amount of proteins that localized at the tip of filopodia using the digital confocal microscope system. If M10 transports VASP, it is expected

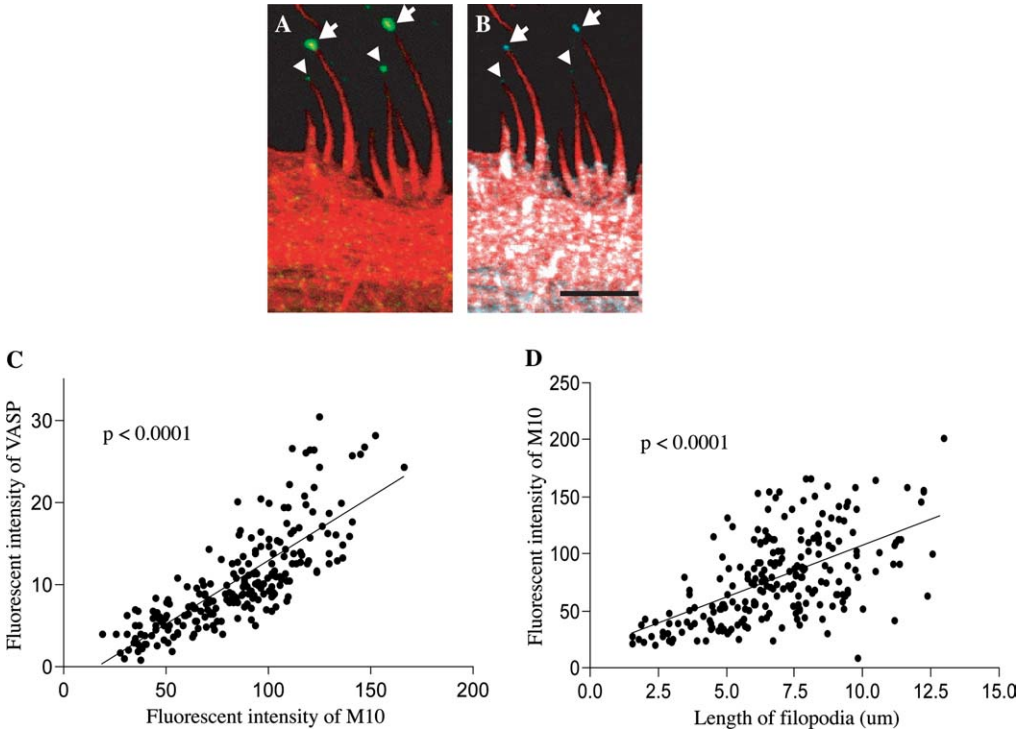

Fig. 4. Correlation between the length of filopodia and the concentration of VASP and M10 at the tip of filopodia. (A,B) HeLa cells were double-stained with anti-M10 and anti-VASP antibodies. Longer filopodia tips (arrows) had larger amounts of M10 (A: FITC) and VASP (B: Cy5) than shorter filopodia (arrowheads). Red: rhodamine phalloidin, Scale bar, 5 μm. (C) The amount of VASP at the tip of filopodia was increased in proportion to the amount of M10. The amounts of VASP and M10 were determined by the fluorescence intensity of the labelled antibodies interact with VASP and M10, respectively. The fluorescent intensities at the tips of filopodia were quantified by using digital confocal microscope (21 cells, 224 filopodia). (D) The amount of M10 at the tip of filopodia showed linear relationship to the length of filopodia. The fluorescent intensities of the labeled antibodies recognizing M10 and length of filopodia were determined as described in "Materials and methods" (20 cells, 224 filopodia).

that the amount of VASP is correlated to the amount of M10 localized at the tip of filopodia. As shown in Fig. 4C, the fluorescent intensity of VASP was increased in proportion to the fluorescent intensity of M10, supporting the idea that M10 is responsible for VASP transportation to the tip of filopodia. Important finding was that the amount of M10 was significantly higher at the longer filopodia than the shorter filopodia. Fig. 4D shows the amount of M10 plotted against the filopodia length. The result clearly indicates that the amount of M10 at the tip of filopodia was linearly increased with the length of filopodia. These results indicate that M10 is critical to the transportation of VASP at the tip of filopodia, where VASP plays a role in extending filopodia.

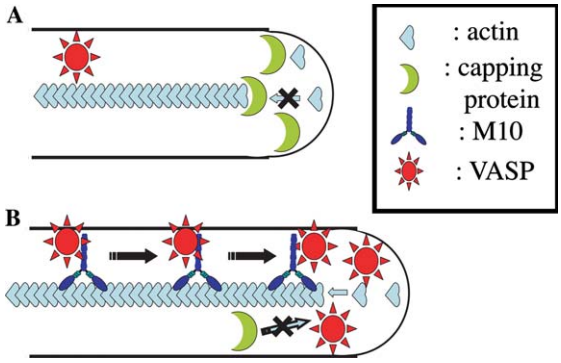

Fig. 5. Model of M10 function in filopodia elongation. (A) Actin monomers are incorporated at the plus end of actin filaments and at the tip of the filopodia. Once capping proteins bind to the plus end of actin filaments, the elongation of actin bundles is blocked. (B) M10 transports a sufficient amount of VASP to the tip of the filopodia. Transported VASP promotes actin filament elongation by interacting with the plus ends, shielding them from the capping protein.

Discussion

Present results clearly show that M10 is a motor that transports VASP to the tip of filopodia. It was reported recently that overexpression of M10 not only increases the number of filopodia, but also elongates the length of filopodia [12]. It has been known that Mena/VASP compete with actin capping proteins at the plus end [8,16,17]. Based upon the present findings and the previously reported results, we propose the model explaining the function of M10 (Fig. 5). Mena/VASP binds to the tail domain of M10 at the base of filopodia. M10 moves along actin filaments in filopodia and

transports the bound Mena/VASP to the tip of filopodia. Mena/VASP is accumulated at the tip of filopodia and competes with the capping proteins. This causes the elongation of actin filaments and filopodia. It has been shown that M10 localizes at the region where actin is in dynamic reorganization [4]. The present study suggests that M10 helps extending actin filament in various cellular domains such as filopodia, membrane raffles, and neuronal growth cone by delivering Mena/VASP. These transported Mena/VASP promote the elongation of actin filaments by interacting with the plus ends, shielding from capping proteins. This is also supported by the finding that the length of filopodia is linearly related to the amount of VASP and M10 at the tip of filopodia. Consistent with this view, it was reported that overexpression of M10 causes elongation of filopodia [12]. We also observed that there are many filpodia that we cannot detect VASP and M10 at the tip. These filopodia were much shorter than those having VASP and M10 at the tip. Therefore, we think that VASP/M10 system is critical for the elongation of filopodia rather than the initiation of them. Supporting this view, it was reported recently that RacV12 could induce lamellipodia and filopodia even in the Ena/VASP family deficient cells [14,18].

Present study revealed that M10 moves on actin filaments in filopodia with carrying Mena/VASP. While we also observed the backward movement of M10 and VASP from the tip to the root of filopodia, the rate of the movement was much slower than the forward movement. It is expected that the difference in the rate between the forward and backward movement results in the accumulation of M10 and VASP at the tip of the filopodia. Consistent with this notion, both endogenous M10 and EGFP-tagged M10 localized at the tip of filopodia. They moved back together from the tip to the root of filopodia presumably by diffusion. This suggests that M10/VASP interaction is not abandoned at the tip of filopodia and M10 delivers VASP as M10/VASP complex rather performs shuttle delivery of VASP.

It is known that myosin superfamily members play a role in vesicle movement in cells. Myosin Va moves melanosomes via binding to melanophilin/Rb27a complex [6]. At present, it is unknown whether M10/VASP or M10/Mena complex is associated to vesicle movement. However, it is likely that multiple molecules of M10 move together because the fluorescence signals of EGFP moving in filopodia were much larger than single EGFP fluorescence. Also it is reasonable to assume that the multiple molecules are involved in the long distance movement of M10/VASP or M10/Mena, since the M10 is not a processive motor [7]. It is plausible that M10 forms a larger protein complex to transport the cargos efficiently in cells. Molecular identity of such a complex requires further investigation.

In summary, we found that M10 forms a complex with Mena/VASP and transports them from the root to the tip of filopodia where elongation of actin takes place. The present finding raises a concept that specific biological motor protein (M10) delivers the specific cell regulatory proteins (Mena/VASP) to the defined cellular region where the regulatory protein function is required.

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