Structure and Regulation of the Movement of Human Myosin VIIA*

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Background: Regulation of myosin VIIA (HM7A) has been studied in Drosophila but not in humans.
Results: The tail domain inhibits HM7A ATPase activity and translocation to filopodial tips.
Conclusion: The tail domain inhibition mechanism of HM7A is operating both in vitro and in vivo.
Significance: The results provide a clue to understand the mechanism of human Usher syndrome.

Human myosin VIIA (HM7A) is responsible for human Usher syndrome type 1B, which causes hearing and visual loss in humans. Here we studied the regulation of HM7A. The actin-activated ATPase activity of full-length HM7A (HM7AFull) was lower than that of tail-truncated HM7A (HM7ATAil). Deletion of the C-terminal 40 amino acids and mutation of the basic residues in this region (R2176A or K2179A) abolished the inhibition. Electron microscopy revealed that HM7AFull is a monomer in which the tail domain bends back toward the head-neck domain to form a compact structure. This compact structure is extended at high ionic strength or in the presence of Ca2+. Although myosin VIIA has five isoleucine-glutamine (IQ) motifs, the neck length seems to be shorter than the expected length of five bound calmodulins. Supporting this observation, the IQ domain bound only three calmodulins in Ca2+, and the first IQ motif failed to bind calmodulin in EGTA. These results suggest that the unique IQ domain of HM7A is important for the tail-neck interaction and, therefore, regulation. Cellular studies revealed that dimer formation of HM7A is critical for its translocation to filopodial tips and that the tail domain (HM7ATAil) markedly reduced the filopodial tip localization of the HM7ATAil dimer, suggesting that the tail-inhibition mechanism is operating in vivo. The translocation of the HM7AFull dimer was significantly less than that of the HM7ATAil dimer, and R2176A/R2179A mutation rescued the filopodial tip translocation. These results suggest that HM7A can transport its cargo molecules, such as USH1 proteins, upon release of the tail-dependent inhibition.

Myosin VIIA has received a great deal of interest since has been identified as a responsible gene of human Usher syndrome type 1B (USH1B) and two forms of non-syndromic deafness, DFNB2 and DFNA11. The motor function of myosin VIIA was first shown for rat myosin VIIA (4, 5). The N-terminal domain of the heavy chain is a conserved motor domain, followed by the neck domain containing five IQ motifs that bind light chains. The tail domain consists of a proximal segment of short predicted coiled-coil domain followed by a globular domain, which contains two large repeats, each incorporating a myosin tail homology 4 (MyTH4) domain and a band 4.1-ezrin-radixin-moesin (FERM) domain (6), which may function as a cargo-binding site. There is an SH3 domain between the two repeats that could be involved in the protein-protein interaction. Because of the presence of the coiled-coil domain, it was originally assumed that myosin VIIA is a dimer, i.e. a two-headed structure. However, recent biochemical and structural studies have revealed that Drosophila myosin VIIA is monomer (7, 8). Between the IQ domain and the short coiled-coil domain, there is a stable single α helix domain (7, 8). The stable single α helix domain was first identified for myosin X (9), and this structure may contribute to the extension of a lever arm length suitable for processive movement (9). Myosin VIIA is localized at the pericuticular necklace in sensory hair cells, where microtubules end and are rich in membrane vesicles (3). Therefore, it is thought that the pericuticular necklace may be a transit point of cargo movement between the microtubule system and the actin filament system. Myosin VIIA is also found in the two cell types of the retina: photoreceptor cells and pigmented epithelial cells (3, 10). In both cell types, it is thought that myosin VIIA serves as a transporter (11, 12). Supporting this view, it has been found that Drosophila myosin VIIA is a high duty ratio motor

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3 The abbreviations used are: USH1B, human Usher syndrome type 1B; ELC, myosin essential light chain; RLC, myosin regulatory light chain; MyTH4, myosin tail homology 4; FERM, band 4.1-ezrin-radixin-moesin; IQ, isoleucine-glutamine; FKBP, FK506-binding protein; Calmodulin; SPIDER, System for Processing Image Data from Electron microscopy and Related fields.
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that is suitable for a cargo transporting motor (13), and the tail-truncated forced dimer of Drosophila myosin VIIA moves processively on actin filaments (14). We have found recently that the tail domain functions as an intramolecular inhibitor in vitro and that it inhibits the actin-activated ATPase activity of Drosophila myosin VIIA (8). However, it has been known that the regulation mechanism of mammalian myosin is different from the invertebrate myosin of the same subfamily, such as myosin I and myosin II (15–17). An important question is whether the tail-dependent regulation mechanism is operating for human myosin VIIA, which is responsible for Usher syndrome. Here we studied the structural basis of the regulation mechanism of human myosin VIIA. Furthermore, we studied whether the regulation mechanism found in vitro is operating in vivo by monitoring the translocation of myosin VIIA in filopodia.

Experimental Procedures

Materials—Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA). Pfu ultra high-fidelity DNA polymerase was purchased from Stratagene (La Jolla, CA). Oligonucleotides were synthesized by Invitrogen. The baculovirus expression system, including the vector pFastBac-HT and SF9 cells, was from Invitrogen (18, 19). The vector containing the 3×FLAG tag (MDYKDHDGDYK-DHIDIDYKDDDDK) was constructed by replacing the hexahisidine tag with the 3× FLAG tag sequence in pFastBac-HT. Actin was prepared from rabbit skeletal muscle acetone powder according to Spudich and Watt (20). Recombinant calmodulin was expressed in Escherichia coli as described previously (21). Anti-FLAG M2 affinity gel, phosphoenolpyruvate, and pyruvate kinase were obtained from Sigma. 3×FLAG peptides were synthesized by GenScript (Piscataway, NJ).

Expression and Purification of RLC and ELC—The cDNA fragments encoding nonmuscle myosin regulatory light chain (RLC), MYL12A cDNA, and nonmuscle myosin essential light chain (ELC), MYL6 cDNA were obtained from a human cDNA library. The amino acid sequences were identical with the submitted sequences, NP_006462 and NP_066299, respectively. Both cDNAs were amplified by PCR using each single set of primers containing restriction sites and subcloned into the pET-30a vector (Novagen, Darmstadt, Germany) at the NdeI/HindIII site in the modified pFastBacHT baculovirus transfer vector (Life Technologies) containing a 3×FLAG sequence at the 5′ end or GFP-C1 vector (Clontech, Mountain View, CA). HM7A ΔTail cDNA was amplified by PCR using a single set of primers, including restriction sites, and subcloned into the 3×FLAG pFastBacHT vector or GFP-C1 vector at the EcoRI/KpnI sites. The HM7A ΔTail cDNA mutant (R2176A/K2179A) was created by site-directed mutagenesis (22). To make the HM7A ΔTail (coil to GCN4) construct (HM7A ΔTail/LZ), HM7A ΔTail was mutated by inverse PCR methods (23) using a single set of primers containing 5′ phosphorylated ends (gagaacactttcacttgctcagtggctcatcaggcctctgtagctgctgcgc and gagaaagtatccttggaatagtgctgcaactgctagagggggccgc). DNA fragments having a various number of IQ motifs of myosin VIIA (IQ1, 738–773 amino acids; IQ1-2, 738–796 amino acids; IQ1-3, 738–819 amino acids; IQ1-4, 738–833 amino acids; and IQ1-5, 738–931 amino acids) were amplified by PCR and subcloned into a modified PET30a vector (EMD Millipore, Billerica, MA) containing a GST sequence upstream of the multiconing site. Each PCR-amplified fragment was subcloned in-frame with the C-terminal His tag into PET30a vector.

Expression and Purification of Human Myosin VIIA Proteins—HM7A proteins were produced by the baculovirus expression system according to the protocol of the manufacturer (24). To express HM7A proteins, SF9 cells were coinfected with two viruses expressing HM7A proteins and Xenopus calmodulin (25) using methods described previously (26). The infected cells were cultured for 3 days at 28 °C, and the cell pellets were suspended in lysis buffer (50 mM HEPES-KOH (pH 7.5), 300 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 2 mM MgATP, 50 μg/ml Calmodulin, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 μg/ml trypsin inhibitor, and 1 mM DTT). After gentle homogenization with a Wheaton Potter-Elvehjem-type tissue grinder (5 strokes) using a Wheaton overhead stirrer, the cell suspension was centrifuged at 100,000 × g for 30 min. The supernatant was incubated with anti-FLAG M2 affinity gel and rotated gently for 1 h at 4 °C. After extensive washing with lysis buffer and wash buffer (30 mM HEPES-KOH (pH 7.5), 200 mM KCl, 0.1 mM EGTA, 2 mM MgCl₂, and 1 mM DTT), the recombinant proteins were eluted with elution buffer containing 30 mM HEPES-KOH (pH 7.5), 200 mM KCl, 0.1 mM EGTA, 2 mM
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Expression and Purification of Human Myosin VIIA Constructs—We produced various human myosin VIIA (HM7A) constructs to study the regulation mechanism (Fig. 1A). Each 3× Flag-tagged construct was expressed in Sf9 cells using a baculovirus expression system. For the expression of constructs having an IQ domain, we coexpressed calmodulin with the heavy chain of human myosin VIIA. The isolated

MgCl₂, 1 mMDTT, 0.1 mg/ml 3× FLAG peptide, and 10% trehalose. The purified proteins were snap-frozen in liquid nitrogen and stored at −80 °C. Protein concentration was determined by the densitometry of SDS-PAGE using BSA as a standard and analyzed with National Institutes of Health ImageJ software.

To express GST fusion proteins having various number of IQ motifs of myosin VIIA, recombinant plasmids (PET30a-GST-IQ motifs) were transfected into E. coli BL21 (DE3) and grown up in a 2YT medium. The cells were grown to an A₆₀₀ of 0.5, and protein expression was induced with 0.3 mM isopropyl 1-thio-β-D-galactopyranoside for 4 h at 25 °C. Pellets were lysed with sonication in lysis buffer (0.5 M KCl, 50 mM HEPES-KOH (pH 7.5), 1% Triton X-100, 5 mM β-mercaptoethanol, 5 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, and 1 μg/ml trypsin inhibitor). After centrifugation at 100,000 × g for 20 min, the supernatant was mixed with nickel-nitriolotriacetic acid-agarose (Qiagen, Hilden, Germany) and rotated gently for 1 h at 4 °C. After extensive washing with lysis buffer plus 25 mM imidazole, the protein was eluted with elution buffer containing 0.3 M KCl, 10 mM HEPES-KOH (pH 7.5), 5 mM β-mercaptoethanol, 300 mM imidazole, and 10% sucrose. The purified proteins were snap-frozen in liquid nitrogen and stored at −80 °C.

Steady-state ATPase Assay—The steady-state ATPase activity was measured in buffer containing 20 μM actin, 30 mM HEPES-KOH (pH 7.5), 50 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.2 mg/ml calmodulin, and 1 mM EGTA or 0.1 mM CaCl₂ in the presence of an ATPase regeneration system (20 units/ml pyruvate kinase and 2 mM phosphoenolpyruvate) at 25 °C (27). The final concentration of HM7A proteins was 0.05 μM, and total volume was 100 μL. The reaction solution was preincubated at 25 °C for 10 min before adding 1 mM ATP to start the reaction. The ATPase activity was calculated from the released Pi (mole) per mole of myosin VIIA per second (28). Data fitting and analysis were performed using Prizm.

Cell Culture and Transfection—HeLa cells (ATCC) were cultured with DMEM containing 10% FBS. The cells were kept at 37 °C and 5% CO₂. Transient transfections were performed with GeneJet transfection reagent (Aigilent Technologies, Santa Clara, CA) according to the instructions of the manufacturer. Plasmid DNA was purified using Qiagen Mini- or Maxi-prep columns. The cells were observed about 16 h after transfection.

Confocal Microscopy—Fluorescence images were viewed with a Leica DM IRB laser-scanning confocal microscope controlled by Leica TCS SP II systems (Leica Microsystems, Buffalo Grove, IL) equipped with a Plan-Apochromat ×601.40 numerical aperture oil immersion objective (Leica Microsystems). The images were processed using Photoshop software (Adobe, San Jose, CA).

Immunofluorescence Staining—Cells cultured on glass cover-slips were fixed in fix buffer (4% formaldehyde, 2 mM MgCl₂, and 1 mM EGTA in PBS) for 20 min at room temperature, washed twice with PBS, and permeabilized with 0.25% Triton X-100 in PBS for 10 min at room temperature. Cells were washed twice with PBS and blocked with 5% BSA in PBS for 1 h at room temperature. The cells were incubated at 4 °C overnight with the indicated primary antibodies and anti-HA tag antibodies (C29F4; 1:1000, Cell Signaling Technology, Danvers, MA) and then for 1 h with fluorescent secondary antibodies (Alexa Fluor 647-conjugated antibody, 1: 400, Invitrogen). For actin staining, Alexa Fluor 568 phalloidin (1:600, Invitrogen) was used.

Electron Microscopy and Single-particle Image Processing—For negative staining, HM7AFull (300 nm) was mixed with 6 μM of calmodulin (ratio of 1:20 to enhance calmodulin binding) and then 10-fold diluted with low (high) salt buffer containing 50 mM Na acetate (300 mM NaCl), 1 mM EGTA (1.1 mM CaCl₂), 2 mM MgCl₂, 10 mM MOPS, and 200 μM ATP (pH 7.5). After dilution, 5 μL of the final mixture was applied to a carbon-coated grid that had been glow-discharged (Harrick Plasma, Ithaca, NY) for 3 min in air, and the grid was immediately (~5 s) negatively stained using 1% uranyl acetate (29). Grids were examined in a Tecnai G2 Spirit Twin Transmission Electron Microscopy (FEI) operated at 120 kV, and images were recorded at a magnification of 65,000 (0.37 nm/pixel). Single-particle image processing was carried out using SPIDER (System for Processing Image Data from Electron microscopy and Related fields; Health Research Inc., Rensselaer, NY) according to procedures described previously (30). Averaged images were produced by alignment and classification of windowed particles (120 × 120 pixels, 701 total particles) from micrographs. Representative averages showing the clearest view of the structures were selected from 25 total class averages. UCSF Chimera was used for visualization and analysis of PDB structures (31). Atomic models fitting to average images were taken from the motor and FERM-MyTH4 domains, PDB codes 1QV1 (deleted lever domain with the plant region from scallop myosin S1 (32)) and 3PVL (deleted SH3 domain from original atomic structure (33)), respectively.

GST Pulldown Assay and Data Analysis—The purified 5 μM GST or GST fusion proteins having various numbers of IQ motifs of myosin VIIA were incubated with 200 μM calmodulin, ELC, or RLC in 200 μl of buffer containing 30 mM HEPES-KOH (pH 7.5), 200 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.1% Triton X-100, and 1 mM EGTA or 0.1 mM CaCl₂. These mixtures were incubated for 1 h on ice and mixed with 20 μl of GST glutathione-Sepharose 4B (GE Healthcare). After incubation for 1 h at 4 °C on a rotator, the beads were washed twice with 400 μl of the same buffer and spun down, and then the supernatant was removed completely using gel loading tips. The beads were resuspended in SDS sample loading buffer at 100 °C for 5 min, and the samples were loaded on 12% SDS-polyacrylamide gels. Proteins were visualized by Coomassie Brilliant Blue R staining. Protein amounts were quantified by comparing band density using ImageJ (National Institutes of Health).

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Electron microscopy and single-particle image processing—For negative staining, HM7AFull (300 nm) was mixed with 6 μM of calmodulin (ratio of 1:20 to enhance calmodulin binding) and then 10-fold diluted with low (high) salt buffer containing 50 mM Na acetate (300 mM NaCl), 1 mM EGTA (1.1 mM CaCl₂), 2 mM MgCl₂, 10 mM MOPS, and 200 μM ATP (pH 7.5). After dilution, 5 μL of the final mixture was applied to a carbon-coated grid that had been glow-discharged (Harrick Plasma, Ithaca, NY) for 3 min in air, and the grid was immediately (~5 s) negatively stained using 1% uranyl acetate (29). Grids were examined in a Tecnai G2 Spirit Twin Transmission Electron Microscopy (FEI) operated at 120 kV, and images were recorded at a magnification of 65,000 (0.37 nm/pixel). Single-particle image processing was carried out using SPIDER (System for Processing Image Data from Electron microscopy and Related fields; Health Research Inc., Rensselaer, NY) according to procedures described previously (30). Averaged images were produced by alignment and classification of windowed particles (120 × 120 pixels, 701 total particles) from micrographs. Representative averages showing the clearest view of the structures were selected from 25 total class averages. UCSF Chimera was used for visualization and analysis of PDB structures (31). Atomic models fitting to average images were taken from the motor and FERM-MyTH4 domains, PDB codes 1QV1 (deleted lever domain with the plant region from scallop myosin S1 (32)) and 3PVL (deleted SH3 domain from original atomic structure (33)), respectively.

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Expression and Purification of Human Myosin VIIA

Constructs—We produced various human myosin VIIA (HM7A) constructs to study the regulation mechanism (Fig. 1A). Each 3× Flag-tagged construct was expressed in Sf9 cells using a baculovirus expression system. For the expression of constructs having an IQ domain, we coexpressed calmodulin with the heavy chain of human myosin VIIA. The isolated
HM7A heavy chain containing the IQ domain was copurified with calmodulin light chain (Fig. 1B). The apparent molecular mass of each constructs determined from its mobility in SDS-PAGE was 250 and 120 kDa for HM7AFull and HM7A/H9004 Tail, respectively, which is consistent with the calculated molecular masses of these constructs (Fig. 1B).

The Tail Domain Inhibits the Actin-activated ATPase Activity of Human Myosin VIIA—We found that the actin-activated ATPase activity of HM7AFull was significantly lower than that of HM7A/H9004 Tail (Fig. 2A). The actin concentration dependence of the ATPase activity showed that the actin-activated ATPase activity of HM7AFull in EGTA was significantly lower than that in the presence of Ca²⁺/H11001. On the other hand, this Ca²⁺/H11001 dependence was not observed for HM7A/H9004 Tail (Fig. 2, B and C). The result suggests that the tail domain functions as an intramolecular inhibitor of human myosin VIIA. Although Ca²⁺/H11001 reverses the tail-induced inhibition, the ATPase activity of HM7AFull in Ca²⁺ (Vₘₐₓ of 0.15 ± 0.02 s⁻¹) was less than that of HM7A/H9004 Tail (Vₘₐₓ of 0.26 ± 0.03 s⁻¹ in Ca²⁺ and 0.32 ± 0.04 s⁻¹ in EGTA). This result suggests that the effect of Ca²⁺, presumably because of the binding to calmodulin light chain, does not completely abolish the tail-dependent inhibition.

Basic Residues in the C-terminal Region of the Tail Are Involved in Inhibition—As shown in Fig. 2D, deletion of the C-terminal 40 residues significantly increased the ATPase activity of HM7AFull to activity similar to HM7A/H9004 Tail. It has been shown previously that a cluster of the basic residues Arg-2140/Lys-2143 is involved in the regulation of Drosophila myosin VIIA (7). Therefore, we examined whether Arg-2176/Lys-2179 of human myosin VIIA, which are within the C-terminal 40 residues, are involved in the tail-dependent inhibition of human myosin VIIA. R2176A/K2179A mutation significantly increased the ATPase activity of HM7AFull to a level similar to the activity of HM7A/H9004 Tail and HM7AFull/−40. The result suggests that these basic residues are critical for the tail-dependent inhibition of HM7AFull (Fig. 2D). This property is similar to myosin VA, in which the basic residues in the globular tail domain interact with an acidic residue in the motor domain, therefore inhibiting motor activity (34).

The Inhibited State of Human Myosin VIIA Forms a Single-headed Folded Structure under Low Ionic Strength—We carried out an electron microscope analysis of HM7AFull molecules. Images of negatively stained molecules under high ionic strength exhibit an extended appearance of the monomer, and such molecules have two distinct domains from the head to the tail/neck with a total length of 57–64 nm (Fig. 3A). Under low ionic strength, similar appearances of extended monomers were observed in the presence of 0.1 mM free Ca²⁺/H11001, whereas the molecules were seen as a folded structure in the absence of free Ca²⁺ (Fig. 3, B and C). Compared with the extended monomers, the length of the folded molecules was shorter (29–32 nm), demonstrating that the head and tail/neck domains were close...
together. Averaged images confirmed the observation of folded molecules (Fig. 3D). This structure resembled the inhibited conformation of single-headed Drosophila myosin VIIa (8). The folded structure of HM7AFull showed the densities of the folding back tail with the head/neck, supported by a two-dimensional fitting of atomic models, assembled motor domain (PDB code 1QVI (32)) with the tip of the tail domain (FERM-MyTH4 domain, PDB code 3PVL (33)) to the averaged image (Fig. 3E). This demonstrated that HM7AFull forms a single-headed folded structure induced by the intramolecular interaction. Taken together with biochemical results (Fig. 2), the low actin-activated ATPase activities of HM7AFull were due to the interaction between the head and tail/neck, whereas, in the absence of this interaction, the molecules became extended monomers (both in high ionic strength and in low ionic strength with the presence of Ca\(^{2+}\)), causing activation of the actin-activated ATPase activities. This observation indicated that the folded structure represents an inhibited form of HM7AFull, and intramolecular interaction within the structure is deemed to have a critical role in promoting the inhibition state.

The Five IQ Motifs of Myosin VIIA Bind Only Three Calmodulins—As shown in Fig. 4, although myosin VIIA has five IQ motifs in the neck domain, the distance between the head and tail seems to be much shorter than the neck domain of myosin V, which has six IQ motifs (7, 35–37). This raises the question of whether the five IQ motifs of myosin VIIA bind five calmodulin light chains. To ask this question, we determined the numbers of bound CaM to full-length human myosin VIIa (Fig. 4, A and B). HM7AFull was incubated with CaM and subjected to a pulldown assay using Anti-FLAG M2 affinity gel under both EGTA and Ca\(^{2+}\) conditions, and we analyzed the number of bound CaM to HM7AFull. The results showed that HM7AFull bound 2.1 ± 0.45 CaM in EGTA and 2.8 ± 0.37 CaM in Ca\(^{2+}\) (Fig. 4, A and B).

To further identify the CaM binding IQ motifs, we produced constructs having various numbers of myosin VIIA IQ motifs and examined them for the binding of calmodulin molecules. The first IQ motif bound calmodulin only in the presence of
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**FIGURE 4.** Calmodulin light chain binding at the IQ domain of myosin VIIA. A, SDS-PAGE of HM7AFull in EGTA and pCa4. CalM was incubated with HM7AFull-immobilized anti-FLAG resin or anti-FLAG resin alone and subjected to a pull-down assay in the presence or absence of Ca\(^{2+}\). Proteins that were pulled down were subjected to SDS-PAGE, followed by Coomassie Brilliant Blue staining. B, molar ratio of CaM to HM7AFull. The concentrations of HM7AFull and CaM were determined by densitometry using a known concentration of CaM to make a standard curve. Error bars represent mean ± S.D. from three independent experiments. C, purified GST or GST-fused constructs having various number of IQ motifs of myosin VIIA were incubated with CaM in the presence or absence of Ca\(^{2+}\), and binding was examined using a GST pulldown assay. Proteins that were pulled down were subjected to SDS-PAGE and Coomassie Brilliant Blue staining. Shown is a representative image of SDS-PAGE. D, molar ratio of calmodulin to GST-HM7AIQ domains. The concentrations of GST-HM7AIQ domains and CaM were determined by densitometry. The experiment was performed three times, and error bars represent mean ± S.D.

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Ca\(^{2+}\) but not EGTA (Fig. 4, C and D). The second IQ motif bound calmodulin under both EGTA and Ca\(^{2+}\) conditions, whereas the third IQ motif did not bind CaM. In EGTA, the fifth IQ motif, but not the fourth IQ motif, bound calmodulin, whereas, in Ca\(^{2+}\), the fourth IQ domain, but not the fifth IQ domain, bound calmodulin. A total of three calmodulin molecules bound to the five IQ motifs of myosin VIIA in Ca\(^{2+}\), whereas two calmodulin molecules bound in EGTA (Fig. 4, A and B). The results are consistent with the structural observations (Fig. 3) and show that myosin VIIA has a much shorter neck domain than myosin V.

**The Five IQ Motifs of Myosin VIIA Bind RLC**—It has been reported recently that IQ motifs from unconventional myosins, such as myosin 18A and myosin 15A, can also bind RLCs and ELCs (38, 39). Myosin 18A has two predicted IQ motifs, and these bind an ELC and a RLC, but it is unclear which IQ motif binds ELC or RLC (38, 39). Myosin 15A has three predicted IQ motifs. RLC binds the first IQ domain, and ELC is associated with the second IQ domain (39). This raises the question of whether the IQ motif of myosin VIIA also binds RLC and/or ELC. To answer this question, we produced non-muscle myosin RLC MYL12A and ELC MYL6 and investigated the binding to the IQ motif of myosin VIIA (Fig. 5A). The five IQ motifs of myosin VIIA bound to both RLC and ELC (Fig. 5A), but when both RLC and ELC were incubated simultaneously with the IQ motifs, only RLC bound to the IQ motifs. This result suggests that RLC binds preferentially to the myosin VIIA IQ motifs. Therefore, we further examined the binding of RLC to various IQ motifs of myosin VIIA (Fig. 5, B and C). The first and fifth IQ motifs bound RLC under both EGTA and Ca\(^{2+}\) conditions. RLC did not bind to the third IQ motif, which was similar to calmodulin. The sequence alignment of each IQ motif of myosin VIIA, myosin VA, myosin 15A, and myosin 18A showed that the amino acid sequences of the first, second, fourth, and fifth IQ motifs of myosin VIIA conform well to the typical IQ motif consensus sequence (ILV)QXGGGXX(RK), which forms an amphipathic α-helix (Fig. 5D) (40, 41). On the other hand, the third IQ motif lacks the canonical Arg/Lys at the last consensus position, which is replaced by His (Fig. 5D). Our result is consistent with this feature of the third IQ motif, and it is thought that the failure of binding of CaM/RLC to the third IQ motif is due to the lack of the Arg/Lys at the last consensus position.

We also examined the competition between CaM and RLC in the presence and absence of Ca\(^{2+}\) (Fig. 5E). When both CaM and RLC were incubated simultaneously with the IQ motifs, only CaM bound to the IQ motifs under both EGTA and Ca\(^{2+}\) conditions. This result suggests that CaM binds preferentially to the IQ motifs of myosin VIIA (Fig. 5E). Because CaM is expressed ubiquitously in a variety of cell types, RLC binding to myosin VIIA may occur only under limited conditions.

**The Tail Domain Attenuates the Translocation of Tail-truncated Human Myosin VIIA to Filopodial Tips**—An important question is whether the tail inhibition mechanism of human myosin VIIA is operating in vivo. To address this question, we studied the effect of HM7ATail in the translocation of myosin VIIA to filopodial tips. Because dimer formation is critical of myosin VIIA for the movement of this myosin on actin filaments (14, 42), we produced a GFP-tagged forced dimer con-
struct having a FK506-binding protein (FKBP), a drug-inducible homodimerization module, GFP-HM7AΔTail-FKBP to produce a stable dimer of myosin VIIA (42). GFP-HM7AΔTail-FKBP diffused cytosolically, but it showed discrete localization at the tip of filopodia upon addition of AP20187, a homodimerizer (Fig. 6A). This result suggests that GFP-HM7AΔTail moves on actin filaments in filopodia in cells upon dimer formation. It should be noted that it is possible that the dimer formation of GFP-HM7AΔTail stimulates filopodium formation at the membrane and that it remains at the tip as the filopodium grows. However, as we reported previously, the dimer formation of GFP-HM7AΔTail did not induce filopodium formation (42), unlike myosin X. It has also been shown previously that time-lapse images of dimerizer-induced translocation of GFP-HM7AΔTail-FKBP show GFP-HM7AΔTail-FKBP gradually accumulating at the tip of pre-existing filopodia (42). Therefore, it is unlikely that HM7A localizes at the filopodial tips by this mechanism.

When HA-HM7ATail was coexpressed, the localization of GFP-HM7AΔTail-FKBP at the filopodial tips was diminished significantly (Fig. 6, B and C). One potential problem is that the high level of tail overexpression may artificially inhibit the translocation of the tail-truncated human myosin VIIA. To minimize this possibility, we selected cells moderately expressing the tail domain compared with the expression of tail-truncated myosin VIIA. The result supports the notion that the
binding of the tail domain to the head/neck domain of human myosin VIIA inhibits the translocation of GFP-HM7AΔTail. To further evaluate this idea, we coexpressed HA-HM7ATail (R2176A/K2179A), which does not inhibit motor activity (Fig. 2), along with GFP-HM7A/H9004 Tail-FKBP. The tail-dependent inhibition of the translocation of GFP-HM7A/H9004 Tail-FKBP was attenuated significantly with R2176A/K2179A mutation (Fig. 6, B and C). These results indicate that the tail domain inhibits motor activity in vivo, which causes the inhibition of translocation of the HM7AΔTail dimer to filopodial tips.

Full-length Human Myosin VIIA Can Translocate to Filopodial Tips When the Tail Inhibition Is Released—A potential issue is that, although the tail domain can inhibit the motor activity of the tail-truncated construct, the tail domain of full-length human myosin VIIA may not interact with the head/neck domain, therefore inhibiting motor activity in cells. To
FIGURE 7. The full-length myosin VIIA dimer showed less filopodial localization than the tail-truncated myosin VIIA, but the R2176/R2179 mutation restored the filopodial tip localization of the full-length human myosin VIIA dimer. A, replacement of the coiled-coil region with the GCN4 sequence (HM7AFull/LZ) increased the filopodial tip localization of full-length myosin VIIA. However, GFP-HM7AFull/LZ showed significantly less filopodial tip accumulation than the mutant (R2176A/K2179A). HeLa cells were transfected with GFP-HM7AFull, GFP-HM7AFull/LZ, or the mutant (R2176A/K2179A). Scale bar = 10 μm. B, qualitative analysis of the effect of the mutation (R2176A/K2179A) of dimerized HM7AFull on filopodial tip accumulation. Filopodia with and without GFP-myosin VIIA at the tips were counted in each cell that expressed GFP-HM7AFull (n = 20), GFP-HM7AFull/LZ (n = 81), and GFP-HM7AFull/LZ (R2176A/K2179A) (n = 67). Values are mean ± S.E. GFP-HM7AFull, 0% ± 0%; GFP-HM7AFull/LZ, 19.25% ± 2.6%; GFP-HM7AFull/LZ (R2176A/K2179A), 39.68% ± 3.8%.
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address this issue, we monitored the filopodial tip localization of full-length human myosin VIIA. HM7AFull did not localize at the filopodial tips (Fig. 7A). This is because human full-length myosin VIIA is monomeric, and dimer formation is required for human myosin VIIA to move in filopodia (Fig. 6). To produce a stable myosin VIIA dimer, we introduced a GCN4 LZ motif at the short coiled-coil region. It is known that a coiled-coil domain forms a heptad repeat pattern in which the first and the fourth positions (Fig. 1C, a and d) are hydrophobic amino acids. We substituted the coiled-coil domain sequence of GFP-HM7AFull for a GCN4 sequence without changing the registration of the heptad repeat pattern (Fig. 1C) to make the GFP-HM7AFull/LZ construct. Although GFP-HM7AFull/LZ showed some filopodial tip localization, the percent of tip localization was much less than that of the GFP-HM7AΔTail dimer (GFP-HM7AΔTail-FKBP) (Fig. 7, A and B). These results support the idea that the tail domain inhibits the motor activity of the full-length myosin VIIA in vivo, therefore attenuating the translocation of human myosin VIIA to the filopodial tips. To evaluate this idea, we next asked whether the R2167A/R2169A mutation, which interferes with the tail-dependent inhibition of motor activity, abolishes the tail-induced inhibition of motor activity, resulting in the translocation of GFP-HM7AFull/LZ to filopodial tips. As shown in Fig. 7, A and B, the R2167A/R2169A mutation, which increases actin-activated ATPase activity (Fig. 2D), significantly enhanced the filopodial tip localization of GFP-HM7AFull/LZ in COS7 cells. These results further support the hypothesis that the tail inhibition mechanism is operating in full-length human myosin VIIA in vivo.

Discussion

Myosin VIIA is known as the gene responsible for Usher syndrome type 1B (1). It has also been found that myosin VIIA interacts with other USH1 gene products, such as harmonin and sans, and interactions between the USH1 proteins have been observed in vitro (43–47). Myosin VIIA deficiency results in improper localization of USH1 proteins at the stereocilia in hair cells. Myosin VIIA is also found in retinal epithelial cells, and it is thought that myosin VIIA is involved in the tethering of melanosomes at the root of actin bundles (48). These findings suggest that human myosin VIIA functions as a transporter of other USH1 proteins to their proper destination in hair cells and/or force producer for tethering the target proteins.

One of the most important unanswered questions is how human myosin VIIA function is regulated. We and others have reported previously that the tail domain of Drosophila myosin VIIA inhibits actin-activated ATPase activity (7, 8). However, it is important to study the regulatory mechanism of human myosin VIIA because it is known that the regulation mechanism of mammalian myosin is different from the invertebrate counterparts of the same subfamily, such as myosin I and myosin II (15–17).

This study shows that human myosin VIIA is a monomeric myosin and that its motor activity is inhibited by the tail domain under physiological ionic conditions. Structural analysis using electron microscopy revealed that the tail domain folds back to the head/neck domain of human myosin VIIA under inhibited conditions, whereas the molecules show an extended conformation at high ionic strength, suggesting that the ionic interaction between the tail and the head/neck stabilizes the folded conformation. These findings suggest that the tail inhibition mechanism of motor activity regulation is operating for human myosin VIIA.

It is known that there are five IQ motifs in the neck domain of myosin VIIA. However, electron microscopy revealed that the neck length is much shorter than that of myosin VA, which has six IQ motifs (7, 35–37). This raises the issue that calmodulin light chains do not bind all of the five IQ motifs of myosin VIIA. We determined the stoichiometry of bound calmodulin with constructs having various numbers of IQ motifs of myosin VIIA and found that only three and two calmodulin molecules bind to the IQ motifs of myosin VIIA in Ca2+/EGTA, respectively. Calmodulin binds to the first IQ motif in the presence of Ca2+ but not in its absence, and this result suggests that the dissociation of calmodulin from this site in the absence of Ca2+ induces flexibility at this site to facilitate the formation of a folded conformation of myosin VIIA. In other words, the binding of calmodulin at this site induces a rigid neck structure to promote the formation of an extended conformation.

Evidence has accumulated that unconventional myosin can also bind to RLC and ELC. We found that the IQ domain of myosin VIIA binds preferentially to RLC rather than ELC. Ca2+ (0.1 mM) did not affect the binding because the EF-hand pocket of RLC is a Ca2+/Mg2+ site (49) and because, under physiological conditions containing millimolar concentrations of Mg2+, the site is occupied by Mg2+. Moreover, our result shows that CaM binds preferentially to the IQ motifs of myosin VIIA. This result implies that myosin VIIA molecules with bound RLC may only exist in a specific cell environment, and further study is required to determine the physiological significance of RLC binding to myosin VIIA molecules.

A critical unanswered question is whether the tail inhibition mechanism is operating in vivo. On the basis of the findings of this study, we concluded that human myosin VIIA motor activity is inhibited by the tail domain in vivo. The tail-truncated HM7A translocated to the filopodial tips upon dimer formation, and the overexpression of the tail domain significantly diminished the tip translocation of the tail-truncated HM7A dimer. Moreover, this tail-dependent inhibition of filopodial tip translocation of the tail-truncated HM7A dimer was diminished by R2176A/K2179A mutation of the tail, which abolishes the tail-dependent inhibition of motor activity. These findings support our conclusion. An important point is that this tail-dependent inhibition mechanism is operating for full-length myosin VIIA. We found that the translocation of full-length human myosin VIIA to filopodial tips is significantly less than that of tail-deleted myosin VIIA, even with the GCN4 motif, which forms a stable dimer. These results suggest that dimer formation is not sufficient for translocation because of tail inhibition of the motor activity. Supporting this view, the R2176A/K2179A mutation in the tail domain of HM7AFull promoted the translocation of the HM7AFull dimer to filopodial tips. These results further support the abovementioned conclusion.

Human myosin VIIA is known as one of the USH1 genes, and mutation of myosin VIIA results in defects of stereocilia in inner ear hair cells (1). Interactions between USH1 proteins
have been observed in vitro (43–45), and all USH1 proteins are localized to the tip of the hair bundle (50, 51). These findings have led to the idea that human myosin VIIA may function as a transporter of the USH1 protein complex. Our results show that wild-type full-length human myosin VIIA is a monomer, that its motor activity is inhibited by the tail, and that it translocates to filopodial tips when tail inhibition is released. Therefore, it is likely that human myosin VIIA transports cargo molecules along with its translocation.

An important question is how myosin VIIA becomes activated and produces a dimer. It has been shown previously that melanophilin, a cargo molecule of myosin VA, activates the actin-activated ATPase activity of mammalian myosin VA (52). It has also been shown that phospholipid binding activates the motor activity of myosin X (53). Because USH1 proteins can interact with myosin VIIA (44), it is plausible that USH1 proteins may activate the motor activity of human myosin VIIA, therefore transporting the USH1 complex to stereocilia. Whether human myosin VIIA forms a dimer in inner ear hair cells is another concern. It has been suggested that myosin VI may form a dimer when it binds to its binding partner molecule optineurin and Dab2 (54, 55). It has also been suggested that myosin VI dimer formation can be induced upon actin binding (56). We have reported recently that full-length myosin X is a monomer but that it forms a dimer upon binding to phospholipids (53). Therefore, it is plausible that human myosin VIIA may form a dimer when it associates with its binding partner molecules, such as USH1 proteins. Supporting this notion, we have found previously that wild-type full-length human myosin VIIA translocates to filopodial tips when it associates with the MyRip-Rab27a complex in ARPE19 cells, presumably because of the dimer formation (42). It is also plausible that MyRip-Rab27a binding may activate the motor activity of myosin VIIA, further study is required to clarify the regulatory mechanism of cargo-dependent activation of myosin VIIA. Alternatively, a cluster of monomeric human myosin VIIA may be able to transport the USH1 complex. Further study is required to understand the regulation of myosin VIIA-based transportation of its cargo molecules, such as the USH1 protein complex.

Author Contributions—T. S. performed biochemical and cell biological experiments and wrote the manuscript. H. S. J. and D. J. Y. performed electron microscopy analysis. M. I. performed molecular cloning. O. S. and M. D. Y. helped with protein purification. R. I. performed molecular cloning. M. I. supervised the project, designed the experiments, and wrote the manuscript.

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