Myosin VIIA was cloned from rat kidney, and the construct (M7IQ5) containing the motor domain, IQ domain, and the coiled-coil domain as well as the full-length myosin VIIA (M7full) was expressed. The M7IQ5 contained five calmodulins. Based upon native gel electrophoresis and gel filtration, it was found that M7IQ5 was single-headed, whereas M7full was two-headed, suggesting that the tail domain contributes to form the two-headed structure. M7IQ5 had Mg\(^{2+}\)-ATPase activity that was markedly activated by actin with $K_{\text{act}}$ of 33 mM and $V_{\text{max}}$ of 0.53 s\(^{-1}\) head\(^{-1}\). Myosin VIIA required an extremely high ATP concentration for ATPase activity, ATP-induced dissociation from actin, and in vitro actin-translocating activity. ADP markedly inhibited the actin-activated ATPase activity. ADP also significantly inhibited the ATP-induced dissociation of myosin VIIA from actin. Consistently, ADP decreased $K_{\text{act}}$ of the actin-activated ATPase. ADP decreased the actin gliding velocity, although ADP did not stop the actin gliding even at high concentration. These results suggest that myosin VIIA has slow ATP binding or low affinity for ATP and relatively high affinity for ADP. The directionality of myosin VIIA was determined by using the polarity-marked dual fluorescence-labeled actin filaments. It was found that myosin VIIA is a plus-directed motor.

Myosins are mechanochemical proteins with a motor domain containing an ATP and actin-binding region, a neck domain that interacts with light chains or calmodulin, and a tail domain that serves to anchor and position the motor domain so that it can properly interact with actin filaments at a specific intracellular location. Phylogenetic analysis revealed that myosin consists of at least 18 classes (1, 2). Myosin VIs are found in human (3, 4), mouse (5), porcine (6), bullfrog (8), Caenorhabditis elegans (9), Dictostelium discoideum (10), and Drosophila (11). In vertebrates, two types of genes for type VII myosin, myosin VIIA and VIIIB, are identified. Among them, the entire coding region has been determined for myosin VIIA. In mammals, myosin VIIA is expressed in a variety of organs and tissues including eye, inner ear, olfactory epithelium, brain, choroid plexus, intestine, liver, kidney, adrenal gland, testis, and lymphocytes (12–14). In the retina, myosin VIIA is found in the pigmented epithelial cells and is postulated to play a role in phagocytosis of cell debris that accumulates as a result of sloughing off of photoreceptor outer segments. Of interest is that defects of myosin VIIA cause the mouse shaker-1 phenotype and human Ushier syndrome 1B, which are characterized by deafness, lack of vestibular function, and (in humans) progressive retinal degeneration (5, 15). In humans, two forms of dominant and recessive nonsyndromic deafness, DFNB2 and DFNA11, are also caused by myosin VIIA mutations (16–18).

Amino acid sequence analysis of myosin VIIA has indicated that this myosin has a motor domain containing actin-binding and ATP-binding sites, and five IQ motifs at the neck domain. In the tail domain, a very short predicted coiled-coil region was found; therefore, it has been assumed that myosin VIIA forms a two-headed structure. Two “band 4.1 protein, ezrin, radixin, moesin” (FERM)\(^1\) domains that have been implicated in cytoskeletal protein interactions have been found in the tail region. There are two myosin tail homology 4 domains found in the tail region. Although similar domains are found in myosin IV and XV, the function of this domain is unclear. The tail domain of unconventional myosins has been implicated to serve as the anchoring site for the cellular target proteins. For myosin VIIA, several tail binding proteins have been identified. The type I regulatory subunit of protein kinase A binds to the FERM domain at the C terminus of myosin VIIA (19), although the role of protein kinase A in the function and regulation of myosin VIIA is unknown. It was shown that the FERM domain is involved in anchoring with adherens junction via a catenin-catenin complex (20). Todorov et al. reported (21) that the myosin VIIA tail has an affinity for microtubule-associated protein 2B, suggesting the interaction of myosin VIIA with the microtubule-based motility system. Quite recently, El-Amraoui et al. (22) reported that myosin VIIA binds to myosin VIIAs- and Rab27-interacting protein, associates with melanosomes via Rab27A, and plays a role in melanosome trafficking. On the other hand, little is known about the motor function of myosin VIIA at a molecular level.

Unlike the case for the well characterized conventional myosins, it becomes evident that the motor function among various members of the unconventional myosin subfamily varies uniquely from one to another, which is thought to be critical for specific physiological roles in diverse cellular motile processes. For example, recent studies have revealed that myosin V is a processive motor that can move in large steps approximating the 36-nm pseudorepeat of the actin filament (23–26). These characteristics are quite important to understand the physiological function of myosin V, since the processive nature of myosin V with large step size is suitable for the motors involved in cargo movement in cells.

Recently, myosin VI was also demonstrated to be a processive motor with a large step size (27, 28). A unique feature of

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\(1\) The abbreviations used are: FERM, band 4.1 protein, ezrin, radixin, moesin; M7full, full-length myosin VIIA; DTT, dithiothreitol; NTA, nitritroacetic acid.
myosin VI is that it moves backward on the actin filament (29). It was originally proposed that the myosin VI unique large insertion between the neck and converter domains is responsible for the reverse directionality of myosin VI (29). This view was based upon the hypothesis that the orientation of the motor domain of myosin VI against actin filament is the same direction as other myosins but the attachment of the position of the "lever arm" (a long a-helical region following the motor domain) (30) is different so that the same movement of the catalytic core would rotate the lever arm in the opposite direction on actin. Since the insertion is located between the converter domain (a compact subdomain thought to amplify the conformational change of the motor domain) (31, 32) and light chain binding helix, it was hypothesized that the 53-amino acid unique insertion of myosin VI is critical in determining the reverse directionality of myosin VI. However, a recent study revealed that the myosin VI unique insertion is not important to the reverse in directionality (33). Instead the motor core domain is responsible for the change in the directionality of myosin movement (33). This finding has raised the possibility that other classes of myosin may be capable of moving to the minus-end of actin filaments. Actually, it was found quite recently that myosin IX is another myosin that shows reverse directionality (34).

There is no doubt that myosin VIIA plays a role in various cellular motile processes. How myosin VII functions in cellular motile events is unknown. This is at least partly due to a lack of biochemical information on myosin VIIA. Furthermore, little is known about the motor characteristic of myosin VIIA at the molecular level. The present study was initiated to clarify the motor function of myosin VIIA at a molecular level.

EXPERIMENTAL PROCEDURES

Materials—Escherichia coli strains Y1090, XL1-Blue, and DH10BAC were purchased from Clontech (Palo Alto, CA), Stratagene (San Diego, CA), and Invitrogen, respectively. Vector plasmid pBluescript II SK(--), pCR-TOPO2.1, and pFastBac1 were purchased from Stratagene, Invitrogen, and Invitrogen, respectively. For screening of cDNA for rat myosin VIIA, oligo(dt) and random primed rat, Sprague-Dawley, kidney 5'-stretch plus λ gt11 cDNA library was purchased from Clontech. Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA). Rabbit fast skeletal muscle actin was purified according to Spudich and Watt (36). Recombinant calmodulin from Xenopus oocyte (37) was expressed in E. coli as described (38). Myosin II was prepared from rabbit skeletal muscle according to Perry (39). Skeletal muscle HMM was prepared from skeletal myosin by α-chymotrypsin digestion (40), and produced HMM was purified by Sephacryl S-300HR (Amersham Biosciences) gel filtration. Hexahistidine tag-fused smooth muscle myosin II HMM or S1 was co-expressed with two light chains in S9 cell and purified with nickel-nitrilotriacetic acid-agarose (Qiagen, Hilden, Germany).

Preparation of Anti-myosin VIIA Antibodies—A peptide, GAETRKRSPTLS, corresponding to residues 600–612 of rat myosin VIIA was synthesized with an N-terminal Cys residue and conjugated to keyhole limpet hemocyanin (Genemed Synthesis, Inc., South San Francisco, CA). Antibodies (MVIIC1000) were prepared by injecting two rabbits with keyhole limpet hemocyanin-coupled peptide. The myosin VIIA antibody affinity column was prepared from the purified MVIIC1000 antibody and formyl-cellulofine (Seikagaku Kogyo, Tokyo, Japan) according to the manufacturer’s protocol.

cDNA Cloning and Sequencing—Total RNA of rat adult male was prepared from kidney by using an RNeasy minikit (Qiagen). Poly(A)+ RNA was isolated using an oligotex mRNA minikit (Qiagen). Random oligonucleotides were used to synthesize cDNA from the poly(A)+ RNA. Sense (5'-GAACTTCACTGGAACAGCCTTC-3') and antisense (5'-GCTATGGCCCTGCAAGGTGATC-3') primers were synthesized according to the nucleotide sequence of mouse myosin VIIA (45), and the myosin VIIA cDNA of 1,185 bp, which corresponds to residues 445–567 of mouse myosin VIIA, was amplified. The amplified cDNA was random labeled with 32P by using the Megaprime labeling kit (Amersham Biosciences) and used as a probe to screen rat kidney λgt11 cDNA library. Plaque hybridization was carried out at 65 °C in Church buffer (41). The cDNA inserts encoding rat myosin VIIA were obtained by the EcoRI digestion of the cloned phage DNA and then subcloned into pBluescript II SK(--). The nucleotide sequence was analyzed with the PerkinElmer terminator ready reaction mix using a model 377 DNA sequencer (Applied Biosystems, Foster City, CA).

Production of MTIQ5 Construct—Of the cloned rat myosin VIIA cDNA, L1 clone (encoding residues 414–1,062) and L21 clone (encoding residues 1–581) were used to construct MTIQ5 expression vector. The Cla I site was introduced at nucleotide position 1,537 by site-directed mutagenesis without changing the amino acid. The L1 clone in pBluescript II SK(--), digested with ClaI and SacI, and the excised fragment was ligated into the L21 clone in pBluescript II SK(--), digested with the same enzymes. A sense primer, MVII HAm1, containing a BamHI site upstream of the initiation codon and an antisense primer, MVIIH948–MYCHIS–, containing c-myc and hexahistidine, and EcoRI sequences downstream of residue 948 were made and used for PCR amplification of myosin VIIA cDNA (2,895 bp) using L1/L22 as a template. This fragment was subcloned into pCR-TOPO2.1. The myosin VIIA cDNA fragment was obtained with BamHI/EcoRI digestion and subcloned into pFastBac1 baculovirus transfer vector (Invitrogen). The full myosin VIIA expression vector containing 2,177 amino acid residues was also constructed using the cDNA clones of myosin VIIA (L21, L1, L7, and L9 clones encoding residues 1–582, 414–1,040, 985–1,856, and 1,754–2,177, respectively). Hexahistidine tag was fused at the C terminus of myosin VIIA. After the sequence was confirmed, bacmid DNA was prepared by manufacturer’s protocol to produce the recombinant virus expressing MTIQ5.

Preparation of Myosin VIIA Proteins—To express MTIQ5, 200 ml of S9 cells (approximately 1 × 109) were co-infected with two separate viruses expressing the MTIQ5 heavy chain and calmodulin, respectively. The cells were cultured at 28 °C in 175-cm2 flasks and harvested after 72 h. Cells were lysed by sonication in 100 ml of lysis buffer (20 mM KPi, pH 8.0, 0.6 mM KCl, 1 mM glutamic acid, 5 mM MgCl2, 5 mM ATP, 5 mM 2-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, 0.01 mg/ml leupeptin, 0.002 mg/ml pepstatin A, and 320 µg/ml calmodulin). After centrifugation at 100,000 g for 20 min, the supernatant was mixed with 0.2 ml of Ni2+-NTA agarose (Qiagen) in a 50-ml conical tube on a rotating wheel for 30 min at 4 °C. The resin suspension was then loaded on a column (1 × 10 cm) and washed with a 10-fold volume of buffer containing 20 mM imidazole, pH 7.5, 0.6 mM KCl, 0.1 mM EGTA, and 5 mM 2-mercaptoethanol. MTIQ5 was eluted...
with buffer containing 200 mM imidazole, pH 7.5, 0.6 M KCl, 0.1 mM EGTA, and 5 mM 2-mercaptoethanol. Fractions containing M7IQ5 were pooled and dialyzed against 20 mM imidazole, pH 7.5, 0.15 M KCl, and 5 mM 2-mercaptoethanol. The purified M7IQ5 was stored on ice and used within 2 days. Typically, 0.5 mg of isolated M7IQ5 was obtained.

The full-length myosin VIIA (M7full) was also expressed in Sf9 cells and purified as described above except for using immunoaffinity chromatography instead of Ni^2+ -NTA-agarose chromatography. M7full was expressed and purified as described under “Experimental Procedures.” The purified protein was analyzed by native gel electrophoresis followed by Western blot using anti-hexahistidine antibodies as probes. HMM, smooth muscle myosin II HMM; S1, smooth muscle myosin II S1; M7IQ5, truncated myosin VIIA. E, native gel electrophoresis of M7full. M7full, full-length myosin VIIA. F, estimation of the molecular mass by gel filtration. Sephacryl S-300HR gel filtration chromatography was performed as described under “Experimental Procedures.” Inset, immunoblotting of the fractions probed by the anti-hexahistidine antibodies.
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co-precipitated with F-actin and then dissociated from F-actin in the presence of ATP as described above. Dissociated M7full was applied to a MVIIL600-formyl cellulofine column (0.8 × 1.5 cm) and was washed with 5 volumes of buffer B (20 mM imidazole, pH 7.5, 0.6 mM KCl, 1 mM EGTA, and 5 mM 2-mercaptoethanol). Bound full myosin VIIA was eluted by buffer B containing 0.2 mg/ml antigen peptide. Fractions containing M7full were pooled and dialyzed against 20 mM imidazole, pH 7.5, 0.15 mM KCl, and 5 mM 2-mercaptoethanol.

**Gel Electrophoresis and Immunoblot Analysis—**SDS-polyacrylamide gel electrophoresis was carried out on a 5–20% polyacrylamide gel using the discontinuous buffer system of Laemmli (42). Molecular mass markers used were smooth muscle myosin heavy chain (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), myosin regulatory light chain (20 kDa), and α-lactalbumin (14.2 kDa). Gels were stained with Coomassie Brilliant Blue R-250. To estimate bound calmodulin with M7IQ5, the gel was stained with GelCode Blue Stain Reagent (Pierce, Rockford, IL) by the manufacturer’s protocol, and densitometric analysis was performed using the NIH Image version 1.62 software as described (43).

Native gel electrophoresis was performed as previously described (44). The gel consisted of 4.75% acrylamide, 0.25% bisacrylamide, 40 mM sodium pyrophosphate, pH 8.8, 2 mM ATP, 2 mM MgCl2, 1 mM EGTA, 1 mM cysteine, and 10% glycerol. Electrophoresis was performed for 16 h at 4 °C with a constant current of 20 mA.

**ATPase Assay**—The ATPase activity was measured in 20 mM imidazole, pH 7.0, 50 mM KCl, 2 mM MgCl2, 1 mM EGTA, 1 mM cysteine, and 10% glycerol. Electrophoresis was performed for 0.46 s at 25 °C. Assays were initiated by adding 0.2 mM ATP was used to assay the ATPase activity of M7IQ5. Other assay conditions are as described in Fig. 3, except without F-actin, at 25 °C. All assays were initiated by adding γ32P-ATP (Amersham Biosciences) to the reaction mixture. The liberated 32P was measured as described previously (46) to determine ATPase activity. The actin-activated ATPase activity was also assayed

![Fig. 3. ATP dependence of the actin-activated Mg2+-ATPase activity of M7IQ5.](image)

**Table I**

| M7IQ5 | ATPase activity (V/sec.head) |
|-------|-----------------------------|
| **KATP** | **Vmax** | **KADP** | **Kactin** | **Vmax** |
| Without actin | 154 | 0.04 | 9.1 | 32.7 |
| With actin | 231 | 0.48 | 7.3 | 6.9 |

* Values were obtained from the ADP assay.

**ATPase activity was measured in the presence of 0.3 mM ADP without an ATP regeneration system.**

**ATPase activity was measured in 20 mM imidazole, pH 7.0, 50 mM KCl, 2 mM MgCl2, 1 mM EGTA with or without F-actin, at 25 °C. All assays were initiated by adding γ32P-ATP (Amersham Biosciences) to the reaction mixture. The liberated 32P was measured as described previously (46) to determine ATPase activity. The actin-activated ATPase activity was also assayed

For immunoblotting analysis, samples were electroblotted to polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) after separation with SDS-polyacrylamide gel or native gel electrophoresis. Anti-myosin VIIA polyclonal, anti-calmodulin monoclonal (Upstate Biotechnology, Inc., Lake Placid, NY), or anti-hexahistidine polyclonal (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies were used as primary antibodies followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibodies as described previously (45). Signal detection was done with Supersignal West Femto Maximum Sensitivity Substrate (Pierce).

**Gel Filtration Chromatography—**M7IQ5 was dialyzed against a solution containing 20 mM Tris-HCl, pH 7.5, 0.15 mM KCl, 1 mM EGTA, 5 mM MgCl2, 5 mM ATP, and 5 mM 2-mercaptoethanol and applied to a Sephacryl S-300HR column (1.0 × 46 cm). The protein was eluted with a dialyzed buffer at a flow rate of 0.16 ml/min. Fractions were analyzed with immunoblotting with anti-hexahistidine polyclonal antibody as described above. The following molecular mass standards were also applied to the column: tyroglobulin, immunoglobulin, ovalbumin, myoglobin, and vitamin B12.
Actin concentration dependence of the Mg2+-ATPase activity of M7IQ5 was measured as a function of actin concentration in 1 mM ATP in the presence of the ATP regeneration system (inset). Solid lines, calculated based upon the equation $V = V_{\text{max}}/[\text{actin}] / (K_{\text{actin}} + [\text{actin}])$. According to the analysis, $V_{\text{max}}$, $K_{\text{actin}}$, and $K_{\text{ADP}}$ were obtained for 0.53 s⁻¹ head⁻¹ and 32.7 μM in the absence of ADP. In the presence of 0.3 mM ADP, $V_{\text{max}}$, and $K_{\text{actin}}$ were reduced to 0.081 s⁻¹ head⁻¹ and 17.5 μM, respectively.

RESULTS

Cloning and Sequencing of cDNAs Encoding Rat Myosin VIIA—From $5 \times 10^5$ recombinant phage plaques, 22 positive clones were obtained. Among them, four clones covered the entire open reading frame of rat myosin VIIA as shown in Fig. 1. A nucleotide sequence of 6,807 bp with an open reading frame of 6,531 bp at positions 272–6,805 was obtained. No polyadenylation signal or poly(A) tail were found in the 3'-untranslated region. From this nucleotide sequence, a 2,177-amino acid sequence was deduced with the entire open reading frame of rat myosin VIIA as shown in Fig. 3.

Actin Co-sedimentation Assay—The binding of calmodulin to M7IQ5 heavy chain was determined by an actin co-sedimentation assay. M7IQ5 was incubated in buffer containing 20 mM imidazole, pH 7.5, 50 mM KCl, 20 mM MgCl₂, 1 mM EGTA, F-actin, 1 mM DTT, 18 μg/ml catalase, 0.1 mg/ml glucose oxidase, 3.0 mg/ml glucose, 0.5% methylcellulose, and various concentrations of Mg²⁺-ATP with or without an ATP regeneration system (20 units/ml pyruvate kinase and 3 mM phosphoenol pyruvate). Actin filament velocity was calculated from the movement distance and the elapsed time in successive snapshots. Student’s t-test was used for statistical comparison of mean values. A value of $p < 0.01$ was considered to be significant.

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in the presence of 20 units/ml pyruvate kinase and 3 mM phosphoenol pyruvate for determining ATP concentration dependence of the activity. The liberated pyruvate was determined as described previously (47).

Preparation of the Dual Labeled F-actin—F-actin (30 μM) was first labeled with a 5 μM excess of tetramethylrhodamine-5-(or -6-) maleimide (Molecular Probes, Inc., Eugene, OR) in the presence of 8 μM phalloidin, 25 mM KCl, 5 mM MgCl₂, 1 mM EGTA, and 25 mM imidazole, pH 7.5 (buffer C) in the dark for 40 min at 4°C. After the reaction was stopped by adding 10 mM DTT, the fluorescently labeled F-actin was obtained by centrifugation (250,000 × g for 10 min). The pellet was homogenized with buffer C plus 5 mM DTT and then precipitated again (250,000 × g for 10 min) to remove the residual dye. The pellet was again homogenized with buffer C with 5 mM DTT. The homogenate was diluted with the same buffer (F-actin concentration should be ~0.8 μM) and subjected to sonication to make minus end cap. The fragmentation of the fluorescently labeled F-actin was confirmed under fluorescence microscope. 0.1–1 μM G-actin was added to the fragmented fluorescently labeled F-actin (2–15 μg/ml) in 25 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 25 mM imidazole, pH 7.5, and 10 units/ml fluorescein phalloidin (Molecular Probes). The elongation of actin filament was carried out overnight in the dark at 4°C.

In Vitro Motility Assay—The motile activities were measured by in vitro motility assay. A coverslip was first coated with nitrocellulose, and then M7IQ5 was then applied to the coverslip. Actin filament motility was observed in 25 mM imidazole, pH 7.4, 25 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 18 μg/ml catalase, 0.1 mg/ml glucose oxidase, 3.0 mg/ml glucose, 0.5% methylcellulose, and various concentrations of Mg²⁺-ATP with or without an ATP regeneration system (20 units/ml pyruvate kinase and 3 mM phosphoenol pyruvate). Actin filament velocity was calculated from the movement distance and the elapsed time in successive snapshots. Student’s t-test was used for statistical comparison of mean values. A value of $p < 0.01$ was considered to be significant.

Actin Co-sedimentation Assay—The binding of calmodulin to M7IQ5 heavy chain was determined by an actin co-sedimentation assay. M7IQ5 was incubated in buffer containing 20 mM imidazole, pH 7.5, 50 mM KCl, 20 mM MgCl₂, 1 mM EGTA, and various concentrations of ATP or ADP at 25°C for 30 min. The sample was ultracentrifuged at 100,000 × g for 30 min, and the pellets were analyzed by SDS-polyacrylamide gel electrophoresis. The amounts of the co-sedimented M7IQ5 heavy chain and calmodulin were determined by densitometry as described previously (43).
[59x113]and various concentrations of ADP were used. The conditions are as described for actin.

A, dissociation of acto-M7IQ5 by ATP. Inset, dissociation of skeletal myosin II HMM from actin. The co-sedimentation assay was performed as follows. M7IQ5 or skeletal HMM (0.1 mg/ml) was incubated in buffer containing 20 mM imidazole, pH 7.5, 50 mM KCl, 20 mM F-actin, 2 mM MgCl₂, and various concentrations of Mg²⁺-ATP in the presence of the ATP regeneration system (20 units/ml pyruvate kinase and 3 mM phosphoenol pyruvate) at 25°C for 30 min. The samples were ultracentrifuged at 100,000 x g for 30 min, and the pellets were analyzed by SDS-polyacrylamide gel electrophoresis. The amount of the co-sedimented heavy chain was determined by densitometry as described previously (43).

Solid lines, calculated based upon the equation [bound M7IQ5] = [ATP] + [ADP]K_ATP/([ATP] + [ADP]K_ATP), according to the analysis, K_ATP value was obtained for 202 μM. B, effect of ADP on the ATP-induced dissociation of acto-M7IQ5. The conditions are as described for A, except 1 mM ATP and various concentrations of ADP were used. Solid lines are the calculated ones based upon the equation [bound M7IQ5] = 1 - [ATP] + K_ATP + [ADP]K_ATP/([ATP] + [ADP]K_ATP). According to the analysis, K_ATP value was obtained for 6.9 μM in the presence of EGTA.

Fig. 7. Effect of nucleotides on the dissociation of M7IQ5 from actin. A, dissociation of acto-M7IQ5 by ATP. Inset, dissociation of skeletal myosin II HMM from actin. The co-sedimentation assay was performed as follows. M7IQ5 or skeletal HMM (0.1 mg/ml) was incubated in buffer containing 20 mM imidazole, pH 7.5, 50 mM KCl, 20 mM F-actin, 2 mM MgCl₂, and various concentrations of Mg²⁺-ATP in the presence of the ATP regeneration system (20 units/ml pyruvate kinase and 3 mM phosphoenol pyruvate) at 25°C for 30 min. The samples were ultracentrifuged at 100,000 x g for 30 min, and the pellets were analyzed by SDS-polyacrylamide gel electrophoresis. The amount of the co-sedimented heavy chain was determined by densitometry as described previously (43).

Solid lines, calculated based upon the equation [bound M7IQ5] = [ATP] + [ADP]K_ATP/([ATP] + [ADP]K_ATP), according to the analysis, K_ATP value was obtained for 202 μM. B, effect of ADP on the ATP-induced dissociation of acto-M7IQ5. The conditions are as described for A, except 1 mM ATP and various concentrations of ADP were used. Solid lines are the calculated ones based upon the equation [bound M7IQ5] = 1 - [ATP] + K_ATP + [ADP]K_ATP/([ATP] + [ADP]K_ATP). According to the analysis, K_ATP value was obtained for 6.9 μM in the presence of EGTA.

Fig. 8. Actin-translocating activity of M7IQ5 as a function of ATP concentration. The actin-translocating activity was measured in 25 mM imidazole, pH 7.4, 25 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 18 μg/ml catalase, 0.1 mg/ml glucose oxidase, 3.0 mg/ml glucose, 0.5% methylcellulose, and various ATP concentrations at 25°C. The experiments were done in the presence of an ATP regeneration system (20 units/ml pyruvate kinase and 3 mM phosphoenol pyruvate). The bars represent the S.D. with 14–26 actin filaments observed for the motility assay. A and B, plots of M7IQ5 and rabbit skeletal HMM, respectively.

were positioned at 745–857, suggesting the binding of five light chains. The region of residues 862–933 was predicted by the program COILS to form the coiled-coil structure (48). The tail region, residues 1,322–1,492 and 1,934–2,095, was found to contain two FERM domains. At the upstream of each FERM domain, two myosin tail homology 4 domains were positioned at 1,148–1,250 and 1,752–1,858, respectively. An Src homology 3 domain was also found at residues 1,565–1,634.

Expression and Purification of Mammalian Myosin VIIA—A rat myosin VIIA construct was produced and expressed in Sf9 insect cells. The construct (M7IQ5) contains the entire coiled-coil domain in addition to the complete head and neck domains with a C-terminal hexahistidine tag to aid purification (Fig. 2A). The histidine tagging at the C-terminal end of the molecule has been performed with conventional (49) as well as unconventional myosin (50), and no influence on motor function has been observed. The cells were co-infected with an appropriate ratio of myosin VIIA-expressing virus and calmodulin-expressing virus, since it was reported that calmodulin copurified with myosin VIIA heavy chain (21), suggesting that calmodulin plays a role as the light chain subunits. It should be noted that functional myosin VIIA was only obtained with co-infection of a calmodulin-expressing virus, in contrast to myosin V, in which functional protein can be obtained without calmodulin co-infection (40). The purification of M7IQ5 involved basically two steps: F-actin co-precipitation followed by ATP-induced dissociation from F-actin with Ni²⁺-NTA-agarose
affinity chromatography using the hexahistidine tag (see “Experimental Procedures”). The former step selects the functionally active molecules, and the second step eliminates the endogenous Sf9 cell myosin and F-actin. Fig. 2B shows SDSPolyacrylamide gel electrophoresis of the purified myosin VIIA. The purified M7IQ5 construct was composed of a high molecular mass band and a low molecular mass band, free of the 200-kDa Sf9 conventional myosin and actin. The high molecular mass band (110 kDa) was consistent with the calculated molecular mass of M7IQ5 and was recognized by anti-rat myosin VIIA (MVII600) antibodies (Fig. 2C) and anti-His6 antibodies (Santa Cruz Biotechnology) (not shown), indicating that the high mass band is the expressed myosin VIIA heavy chains. The small subunits showed a mobility shift with a change in Ca2+ that is characteristic of calmodulin, suggesting that the small subunits are indeed calmodulin (not shown). The identification of the small subunit was also confirmed using anti-calmodulin antibodies (Fig. 2C). The stoichiometries of calmodulin versus myosin VIIA heavy chain were determined by densitometry to be 4.9 versus 1.0, consistent with the five IQ motifs in the M7IQ5 construct. Fig. 2D shows the native gel electrophoresis of M7IQ5. The mobility of M7IQ5 was similar to that of myosin II S1 but much larger than that of myosin II HMM. Based upon the calculated molecular mass of heavy chain plus five calmodulin light chains (194,717 Da), the results suggest that M7IQ5 is single-headed, although it contains an entire short coiled-coil region of myosin VIIA. This was also confirmed with gel filtration chromatography using Sephacryl S-300HR. The estimated molecular mass of 206 kDa was obtained according to the elution positions of the molecular mass standard proteins (Fig. 2F). On the other hand, the mobility of the full-length myosin VIIA was much lower than M7IQ5 and similar to myosin II HMM (Fig. 2E). To further confirm the two-headed structure of the full-length myosin VIIA, we performed gel filtration chromatography. The elution position of M7full was similar to that of myosin II HMM, suggesting the two-headed structure of M7full (Fig. 2F). These results indicate that the full-length myosin VIIA is a dimer. The results obtained here suggest that the coiled-coil domain is not sufficient to form the two-headed structure of myosin VIIA and that its C-terminal domain contributes to form the stable doubled-headed structure.

**Actin-activated ATPase Activity**—Fig. 3 shows the myosin VIIA ATPase activity as a function of ATP concentration in the presence of an ATP regeneration system. In contrast to the conventional myosin, myosin VIIA ATPase activity in the absence of actin required extremely high ATP concentrations to saturate the activity. A K\text{ATP} of 154 μM was obtained (Table I). A high K\text{ATP} value (231 μM) was also obtained in the presence of actin (Table I) (i.e. actin-activated ATPase). The results suggest either extremely slow ATP binding or weak affinity of myosin VIIA for ATP. While measuring the ATPase activity of M7IQ5, we found that the rate of Pi liberation significantly decreased with time (Fig. 4). This time-dependent inhibition of the Pi liberation was abolished when the ATP regeneration system was included in the reaction mixture (Fig. 4). In contrast, the time-dependent inhibition of the ATPase activity was not observed for skeletal HMM (Fig. 4, inset). A similar observation has been reported for myosin V. Based upon the equation, d[ADP]/dt = V_{max}[ATP]/(K_{ATP} \cdot 1 + [ADP]/K_{ADP}) + [ATP]) and K_{ATP} = 231 μM, K_{ATP} was estimated from the initial phase of the fitting curve to be 7.3 μM (Table I). To further confirm this notion, the effect of ADP on the actin-activated ATPase activity of M7IQ5 was examined (Fig. 5). ADP markedly inhibited the actin-activated ATPase activity of M7IQ5 but not skeletal HMM. Based upon the equation, V = V_{max}[ATP]/K_{ATP}(1 + [ADP]/K_{ADP}) + [ATP], K_{ADP} was calculated to be 9.1 μM (Table I). The result was consistent with Fig. 4.

**Binding of Myosin VIIA to Actin**—The dissociation of M7IQ5 from actin was examined as a function of ATP concentration. Myosin VIIA was mixed with actin in the presence of Mg2+-ATP, and the fraction of myosin VIIA dissociated from actin was determined by sedimentation analysis (Fig. 7A). The dissociation of myosin VIIA from actin required much higher ATP concentrations than that required for conventional myosin II, and the complete dissociation required 1 mM ATP. Assuming that ATP binding to myosin VIIA dissociates myosin VIIA from actin, the dissociation constant (K_{ATP}) of 202 μM was obtained from the ATP dependence of actomyosin VIIA dissociation (Table I). These results suggest the weak ATP binding of myosin VIIA. On the other hand, a significant fraction of myosin VIIA in the presence of 1 mM ATP becomes co-precipitated with actin in the presence of ADP (Fig. 7B). In contrast, the addition of ADP did not affect the binding of skeletal HMM to actin in the presence of ATP. K_{ADP} values were estimated to be 6.9 μM based upon the equation, [bound heavy chain] = 1 – [ATP]/(ATP) + K_{ATP} + [ADP]/K_{ADP} (Table I). This result is consistent with those of Figs. 5 and 6, suggesting that a signif-
significant fraction of M7IQ5 forms myosin plus ADP complex even in the presence of ATP. These results imply that, in the presence of ATP and ADP, a significant fraction of myosin VII may be present in a myosin plus ADP form, a strong actin binding form.

The Motility Activity of Myosin VIIA—The motility activity of myosin VIIA was measured as a function of ATP concentration (Fig. 8). Consistent with the high ATP requirement of M7IQ5 for the actin-activated ATPase activity, a high ATP concentration was also required for the actin-translocating activity of myosin VIIA. The velocity was saturated at 4 mM ATP with a maximum velocity of 0.16 ± 0.02 μm. This value is consistent with that obtained for the myosin VIIA prepared from tissue (53). This extremely high ATP requirement for the actin-translocating activity of myosin VIIA was not observed for myosin II. On the other hand, ADP significantly inhibited the motility activity of myosin VIIA (Fig. 9A). The inhibition by ADP was saturated at 2 mM ADP, and an approximate 50% inhibition of the motility velocity was observed. The addition of higher concentrations of ADP did not further inhibit the motility activity. In contrast, ADP did not inhibit the actin-translocating velocity of conventional myosin (skeletal muscle HMM) under the conditions used.

Direction of the Movement—Quite recently, it was shown that the mammalian class VI myosin is a minus end-directed motor, in contrast to other known myosins (25). It was originally hypothesized that myosin VI’s unique large insertion between the motor domain and the neck domain was responsible for the reverse directionality of motility (25). However, this view has been questioned recently (26), opening up the possible presence of other minus end-directed motors within the myosin superfamily.

To determine the direction of movement of the myosin VIIA, we utilized F-actin filaments in the in vitro motility assay that were labeled throughout with fluorescein and labeled with a rhodamine cap at the filament’s pointed end (see “Experimental Procedures”). The dual fluorescence-labeled F-actin filaments were visualized under the fluorescence microscope moving on coverslips coated with M7IQ5. As shown in Fig. 10A, myosin VIIA moved the dual fluorescence-labeled F-actin with the pointed end at the front of the movement. This means that myosin VIIA moves toward the barbed end of F-actin, as is known for the conventional myosins. Fig. 10B shows a histogram of the velocities of polarity-marked actin filaments on myosin VIIA-coated cover slips. Whereas some variation of the sliding velocity was observed, all actin filaments moved in the same direction. The results show that myosin VIIA is a (+)-directed motor.

DISCUSSION

A number of unconventional myosins have been found during the last decade, and it has been anticipated that these newly found myosins play a key role in diverse cellular motile
These results suggest the relatively strong binding of myosin ATPase activity of myosin VIIA. Consistently, the actin-activated ADP significantly inhibited the actin-activated ATP binding or a weak affinity of myosin VIIA for ATP. A very significant fraction of myosin VIIA and the ATP-induced dissociation of myosin VIIA from actin requires an extremely high ATP concentration for its motility function. The present study has determined the motor properties of myosin VIIA. In order to prepare mammalian myosin VIIA, we decided to express recombinant myosin VIIA rather than to purify it from tissues for several reasons. First, it is known that various types of myosins are present in the same tissue and it would be difficult to completely eliminate the contamination of other myosins. This problem can be overcome by overexpressing myosin VIIA in Sf9 cells and purifying with a histidine tag. Also, large quantities of the protein can be made and prepared in a short period of time. This is critical for preventing the denaturation and degradation of the protein during preparation.

We produced a M7IQ5 construct that contains the entire head domain plus coiled-coil domain. It has been thought that myosin VIIA is a double-headed myosin because of the presence of the coiled-coil domain (12). However, the length of the coiled-coil domain is relatively short, and there is no conclusive evidence for the two-headed structure of myosin VIIA. Our results clearly demonstrated that M7IQ5, having an entire coiled-coil domain of myosin VIIA, is single-headed. However, quite interestingly, the full-length myosin VIIA formed a two-headed structure. The result suggests that the relatively short coiled-coil domain of myosin VIIA is not sufficient to stabilize the two-headed structure, and the tail domains of myosin VIIA in the two heavy chains interact with each other, which contributes to form a stable two-headed structure of myosin VIIA.

Myosin VIIA showed maximum actin-translocating velocity of 0.16 ± 0.02 μm/s at 25°C. This value agrees with that of mouse myosin VIIA (0.19 μm/s) as recently reported by Udovichenko et al. (53). However, to our surprise, myosin VIIA requires an extremely high ATP concentration for its motility activity. Consistently, a high ATP concentration was needed for the saturation of the actin-activated ATPase activity of myosin VIIA and the ATP-induced dissociation of myosin VIIA from actin (Table I). These results suggest either an extremely low ATP binding or a weak affinity of myosin VIIA for ATP. A very slow rate of ATP binding has been reported for myosin (54). On the other hand, ADP significantly inhibited the actin-activated ATPase activity of myosin VIIA. Consistently, the actin-translocating velocity of myosin VIIA was also inhibited by ADP. These results suggest the relatively strong binding of myosin VIIA to ADP. Of particular interest is the observation that ADP markedly reduced K_{cat}, thus increasing the apparent affinity for actin during the ATPase cycle (Table I). Consistently, the addition of ADP markedly increased the binding of myosin VIIA to actin filaments in the presence of ATP. Even in the presence of 1 mM ATP, the addition of ADP enhanced the binding of myosin VIIA, with nearly 100% of myosin VIIA binding to actin filaments. These results suggest that a significant fraction of myosin VIIA during the ATPase cycle is in a strong binding state in the presence of ADP, presumably in a myosin VIIA/ADP form.

If this is true, then it is plausible that myosin VIIA plays a role in connecting actin cytoskeleton and cellular components to maintain stress. Myosin VIIA is associated with cross-links between adjacent stereocilia (55), suggesting a role in maintaining their structural integrity. The present results are consistent with this earlier finding and provide a molecular basis for this notion. The weak affinity for actin in the presence of ATP suggests the low duty ratio of myosin VIIA. However, the tight binding of ADP usually suggests slow ADP dissociation. If so, the myosin would tend to give high duty ratio. In the presence of ADP, myosin movement is inhibited, but the inhibition required a relatively high ADP concentration. A likely scenario would be that the ADP affinity is not very high, but because ATP binding is weak, relatively low ADP concentration decreases the ATPase activity.

In the presence of sufficiently high ADP concentration, myosin movement is inhibited by the presence of myosin-ADP complex. If this is the case, myosin VII may function to maintain tension in cells where a significant fraction binds ADP. Tension maintenance in cells has also been proposed for myr1 (54).

Directionality of myosin movement is another important issue to understand the physiological relevance of each myosin motor. The present results clearly indicate that myosin VIIA is a (+)-ended motor, unlike myosin VI. Quite recently, it was assumed that Dictyostelium myosin VII would be a plus end-directed motor, because it may function in the assembly and disassembly of adhesion proteins at the plasma membrane, and the actin at areas of membrane extension is arranged with the plus ends outmost (55, 56). The present results agree with this assumption. Whereas myosin VI and VIIA are present in the sensory hair cells in the inner ear and the mutation of these two classes of myosins causes auditory dysfunction, the present results strongly suggest that the function of these two types of myosins in the sensory hair cells must be distinct from each other.

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