Actin-based motor properties of native myosin VIIa

Igor P. Udovichenko, Daniel Gibbs, and David S. Williams*

Departments of Pharmacology and Neurosciences, UCSD School of Medicine, La Jolla, California 92093-0983, USA
*Author for correspondence (e-mail: dswilliams@ucsd.edu)

Accepted 11 October 2001
Journal of Cell Science 115, 445-450 (2002) © The Company of Biologists Ltd

Summary

Myosin VIIa has critical roles in the inner ear and the retina. To help understand how this protein functions, native myosin VIIa was tested for mechanoenzymatic properties. Myosin VIIa was immunoprecipitated from retinal tissue and found to be associated with calmodulin in a Ca^{2+}-sensitive manner. Myosin VIIa Mg-ATPase activity was detected; in the absence of Ca^{2+} (i.e. with bound calmodulin), it was stimulated by f-actin with a K_{cat} of 4.3 s^{-1} and with 7 μM actin required for half-maximal activity. In a sliding filament motility assay, myosin VIIa moved actin filaments with a velocity of 190 nm s^{-1}. These results demonstrate that myosin VIIa is a calmodulin-binding protein and a bona fide actin-based motor.

Key words: Unconventional myosin, Myosin VIIa, MYO7A, Usher syndrome

Introduction

Mutations in the gene encoding myosin VIIa can cause deafness and blindness, known as Usher syndrome 1B (Weil et al., 1995). Myosin VIIa is found in a variety of tissues (Hasson et al., 1995; Wolfrum et al., 1998). Most importantly, it is found in the hair cells of the inner ear and in the epithelium and photoreceptor cells of the retina (Hasson et al., 1995; Liu et al., 1997). Suggestions for the function of myosin VIIa have been made as a result of its subcellular localization and observations of mutant mouse tissue (Gibson et al., 1995; Mburu et al., 1997). Proposed roles include receptor transport (Richardson et al., 1997; Richardson et al., 1999) and organization of the stereocilia (Hasson et al., 1997; Self et al., 1998) in the hair cells, melanosome transport in the retinal epithelium (Liu et al., 1998) and opsin transport in photoreceptor cells (Liu et al., 1999). A myosin VII in *Dicyostelium* has been suggested to function in the delivery of adhesion proteins to the site of adhesion during phagocytosis (Tuxworth et al., 2001).

These suggestions of roles for myosin VIIs are based on the prediction that myosins of this class are indeed mechanoenzymes. This prediction follows from their myosin-like primary structure. Myosin VIIa has a head of 730 amino acids, followed by a 130 amino-acid domain consisting of five IQ motifs, a short coiled-coil domain of ~70 amino acids and a long tail that contains domains of MyTH4 (myosin tail homology 4), SH3 and FERM (band 4.1, ezrin, radixin, moesin) homology (Hasson et al., 1995; Chen et al., 1996; Weil et al., 1996; Levy et al., 1997) (Fig. 1A). Its head has been predicted to contain binding sites for MgATP and f-actin and possess actin-based mechanoenyzmatic properties. Its IQ motifs represent potential light-chain-binding sites. Nevertheless, these properties have not been demonstrated for any myosin VII. The main purpose of the present paper was to test myosin VIIa for actin-based motility properties and to determine some basic characteristics of native myosin VIIa.

Materials and Methods

Materials and animals

Unless otherwise stated, chemicals were obtained from Fisher Scientific or Sigma-Aldrich Ltd. Enhanced chemiluminescence (ECL) reagents and Sepharose CL 4B were obtained from Amersham Pharmacia. Nitrocellulose was obtained from BioRad and Protein-A-coated microbeads were obtained from Polysciences. Two different myosin VIIa antibodies were used. One was generated against a recombinant protein, including residues 941-1071 of mouse myosin VIIa. This tail domain antibody was the same as pAb 2.2 described previously (Liu et al., 1997). The other antibody was produced by Affinity Bioreagents Inc. (catalogue number PA1-936) against a peptide corresponding to amino acids 16-31 of mouse, human and porcine myosin VIIa. A monoclonal antibody against calmodulin was obtained from Sigma-Aldrich (catalogue number C3545, clone number 6D4). Secondary antibodies conjugated to horseradish peroxidase (HRP) were obtained from Chemicon. Purified calmodulin was obtained from Sigma-Aldrich. Rabbit skeletal muscle myosin II was purified as described (Pollard, 1982). Rabbit skeletal muscle actin was kindly provided by Tom Pollard's laboratory (Salk Institute, La Jolla).

Our *Myo7a*^{4626SB} mouse colony was established by rederivation of mice kindly sent to us by Karen Steel (Nottingham, UK). They originated from ENU-induced mutations on a BALBc background. They were back-crossed repeatedly to the BS strain used at Oak Ridge, and then back-crossed to the BALBc strain in our vivarium. Mice were bred from homozygous×heterozygous parents and maintained on a 12-hour light/12-hour dark cycle, with exposure to 10-50 lux of fluorescent lighting during the light phase. They were treated according to NIH and UCSD animal care guidelines. Tissues were isolated after euthanasia by cervical dislocation.

Immunoprecipitation of myosin VIIa

Fresh bovine eyes were obtained from a local slaughterhouse and the retinas were dissected and homogenized in 25 mM Tris-Cl, pH 7.5, 10 mM EDTA, 10 mM EGTA, 5 mM ATP, 2 mM dithiothretiol (DTT) and 10 μg/ml each of leupeptin, pepstatin and aprotinin using a Potter mechanical homogenizer. The cytosolic fraction was obtained by centrifugation at 100,000 ***g*** for one hour at 4°C (TL100, Beckman).

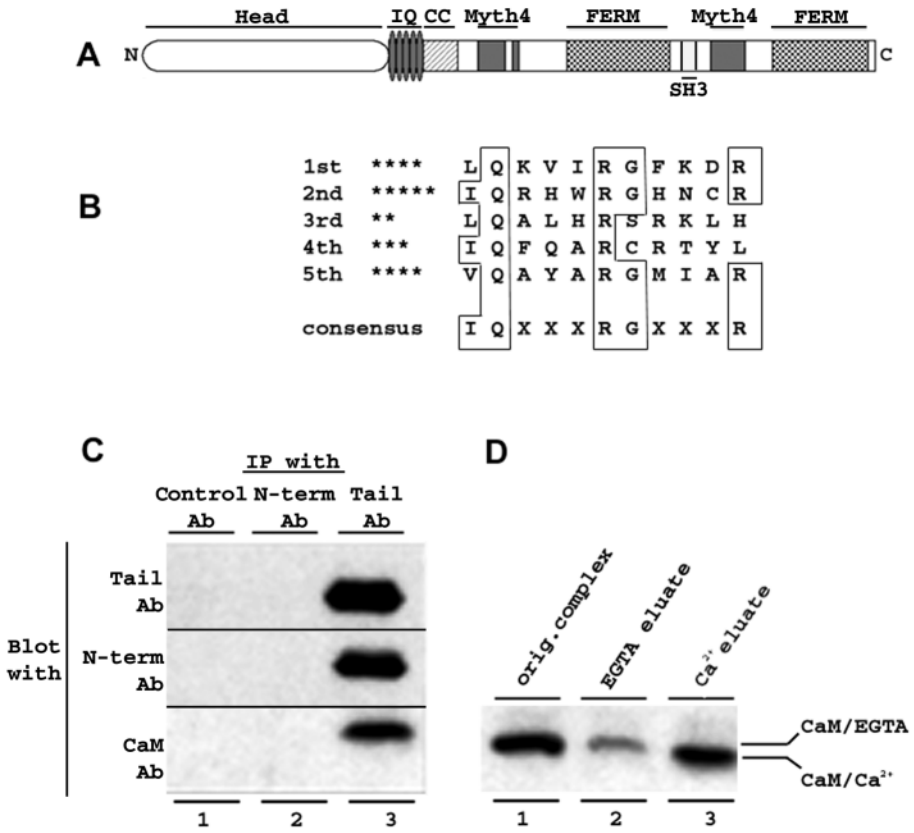

Fig. 1. Calmodulin binding to myosin VIIa. (A) Diagram of the primary structure of native myosin VIIa, indicating the motor domain (head), the five IQ motifs (and potentially bound light chains (grey ellipses)), the coiled-coil domain (CC) and the tail, which includes the Myth4 and FERM domains (Chen et al., 1996; Weil et al., 1996). (B) Sequences of the five IQ motifs of human myosin VIIa (Hasson et al., 1995) compared with the IQ consensus motif. The number of asterisks indicates the extent of similarity of each motif to the consensus motif. Mouse myosin VIIa differs from the human at the following amino acids: N to H in IQ2; first Q to E and T to A in IQ4 (Mburu et al., 1997). (C) Immunoprecipitation of myosin VIIa and calmodulin from bovine retina with myosin VIIa antibodies. Preimmune serum (control Ab) and the N-terminal myosin VIIa antibody fail to immunoprecipitate myosin VIIa or calmodulin (lanes 1 and 2), whereas the tail myosin VIIa antibody immunoprecipitates both. (D) Release of calmodulin from immunoprecipitate. The beads containing the immunprecipitate (lane 1) were washed with buffer containing 1 mM EGTA (lane 2) or 10 μM Ca^{2+} (lane 3). More calmodulin is released in the presence of Ca^{2+}, and this calmodulin characteristically has a slightly higher mobility in SDS-PAGE.

This fraction was then incubated overnight at 4°C with the myosin VIIa tail antibody. Before use, this antibody was affinity purified with the fusion protein, which contained amino acids 941-1071 of mouse myosin VIIa, coupled to a Sepharose CL 4B column. The reaction mix was then incubated with gentle shaking for one hour at 4°C with Protein A Sepharose beads. Immunocomplexes were collected by a brief centrifugation at 10,000 ***g*** and 4°C.

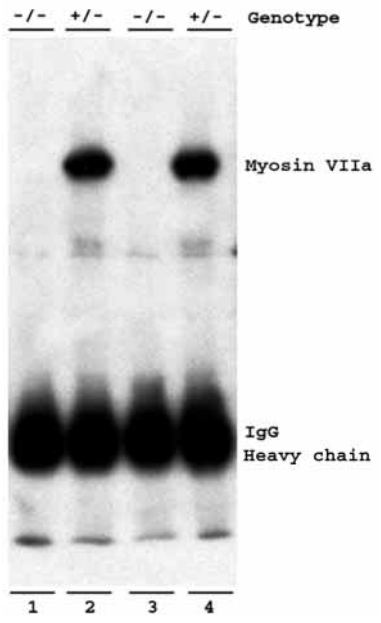

Fig. 2. Immunoprecipitation of myosin VIIa from *shaker1* mouse tissue. Western blot showing the result of immunoprecipitation of myosin VIIa from mouse testes of *shaker1*-null mutants ($^{-/-}$) and control littermate heterozygotes ($^{+/-}$).

For the analysis of ATPase activity, mouse myosin VIIa was immunoprecipitated as above from heterozygous or homozygous mutant *Myo7a*^{4626SB} mouse testes with the following modification: Protein-A-coated 1 μm microspheres were used as the solid substrate rather than Sepharose beads. Unlike Sepharose beads, the microspheres do not contain any cavities so that all of the immunoprecipitated protein would be exposed and readily accessible to actin filaments.

Determination of amount of calmodulin bound to immunoprecipitated myosin VIIa

A 1075 amino-acid HMM (heavy meromyosin-like) fragment of human myosin VIIa (Hasson et al., 1995), with a His_6 tag at its C-terminus, was expressed using the baculogold™ (Pharmingen) baculovirus system as described previously (Liu et al., 1997). This baculovirus-expressed protein was purified to homogeneity using centrifuge fractionation and a Ni^{2+} agarose (Qiagen) column. Although use of this column results in severe inhibition of enzymatic activity (MgATPase activity was only ~0.2 s^{-1}), the pure protein could be used as a standard on western blots as it contains the epitopes recognized by both the N-terminal and tail myosin VIIa antibodies. Standard curves of immunolabel density versus amount of protein were made for the myosin VIIa and calmodulin antibodies using this pure HMM myosin VIIa and purified calmodulin (purchased from Sigma). Amounts of pure protein were determined by Coomassie blue staining and by comparison to BSA. In order to minimize differences in transfer efficiency of HMM myosin VIIa and full-length myosin VIIa, the transfer to Immobilon was performed for an extended period (overnight). After this period, there was no immunodetectable HMM myosin VIIa on a second sheet of Immobilon placed behind the first. Moreover, we found that an additional 24 hours of attempted transfer to new Immobilon yielded no immunodetectable full-length

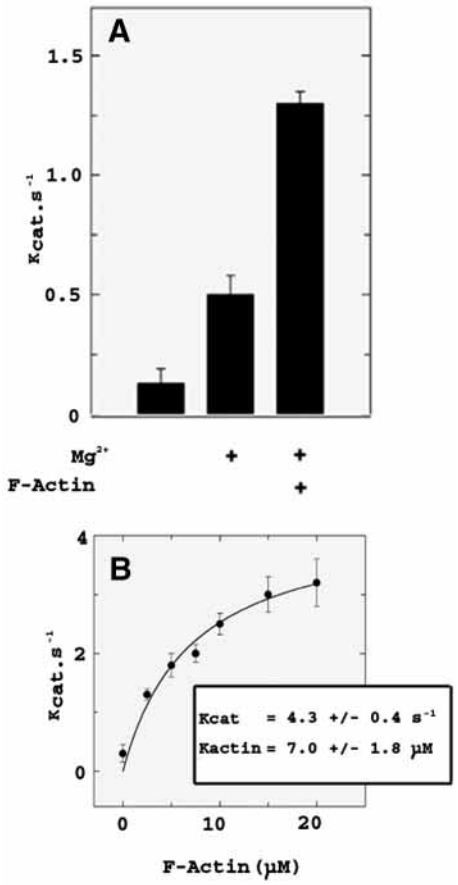

Fig. 3. ATPase activity of myosin VIIa. (A) A histogram illustrating the dependence of Mg^{2+} (2 mM) and actin filaments (2.5 μM) on myosin VIIa ATPase activity. (B) Relationship between myosin VIIa ATPase activity and the concentration of actin filaments. From these data a maximal ATPase activity (K_{cat}) of 4.3 s^{-1}, with 7.0 μM of f-actin required for half-maximal activity (K_{actin}), was calculated.

myosin VIIa. The molar ratio of myosin VIIa to calmodulin in immunoprecipitates from the cytosolic fraction of bovine retinas was determined by densitometry and reference to these standard curves. Relative densitometric units were calculated from digitized images using the NIH image v.1.62 software.

ATPase activity assay

The ATPase activity of mouse myosin VIIa (ATP/s per myosin VIIa head) was determined following immunoprecipitation from wild-type or heterozygous *Myo7a*^{4626SB} testes. It was measured at 30°C for 10 minutes in buffer A (0.5 mM ATP, 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA and 20 mM Tris-Cl, pH 7.5) with 1 mM phosphocreatine and 0.2 mg/ml creatine phosphokinase (to regenerate ATP). Actin filaments (2-20 μM) and 2 μM calmodulin were added unless otherwise specified. The final assay volume was 70 μl, containing 5.0 pmoles of myosin VIIa dimer (the amount of myosin VIIa was determined as above by comparison on western blots with purified HMM myosin VIIa). Assays using testes from homozygous-null *Myo7a*^{4626SB} littermates were performed in parallel and used to obtain background measurements of released phosphate. Reactions were stopped with an equal volume of 0.6 M perchloric acid. Released phosphate was quantified using the malachite green method (Kodama et al., 1986), when an equal volume of malachite green reagent (0.2% sodium molybdate, 0.03% malachite green oxalate, 0.05% Triton X-100, 0.7 M HCl) was added to the reaction mix. After developing for 15 minutes, the absorbance was measured at 650nm. Inorganic phosphate (KH_2PO_4) was used as a standard.

In vitro motility assay

The movements of f-actin filaments labeled with rhodamine-phalloidin (Molecular Probes) were observed on coverslips coated with nitrocellulose, following the procedure of Kron and Spudich (Kron and Spudich, 1986). Coverslips were coated and assembled into an in vitro motility chamber. In the case of myosin II (rabbit skeletal muscle), the purified protein (0.2 mg/ml) was immobilized directly onto the nitrocellulose-coated coverslips. Myosin VIIa from retinas of *Myo7a*^{4626SB} mice was immobilized indirectly using the affinity-purified myosin VIIa tail antibody by a method similar to that described for myosin IXb (Post et al., 1998). Each time the experiment was performed with retinas from control ($^{+/+}$ or $^{+/-}$) *Myo7a*^{4626SB} mice, it was also performed in a separate chamber with retinas from homozygous mutant ($^{-/-}$) *Myo7a*^{4626SB} mice.

The myosin VIIa antibody was incubated on coated coverslips for three hours (0.2 μg in 50 μl per 1 cm^2 area of coverslip). The retinal cytosolic fraction (100,000 ***g*** for one hour) was then incubated with the antibody-coated coverslips (two retinas per coverslip) overnight at 4°C in buffer H (100 mM NaCl, 20 mM potassium phosphate, 5 mM EDTA, 5 mM EGTA, 0.05% Tween 20, 5 mM DTT, 1 mM ATP, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 10 μg/ml pepstatin, pH 7.5). The chamber was then washed with buffer H and perfused with buffer A (50 mM KCl, 30 mM Tris-Cl, 4 mM $MgCl_2$, 1 mM EGTA, 5 mM DTT, pH 7.5) before applying rhodamine-phalloidin-f-actin (20 nM in buffer A) for three minutes. F-actin was obtained by the polymerization of 2 μM G-actin in the presence of 2 μM rhodamine-phalloidin in Buffer P (50 mM KCl, 10 mM Tris-Cl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 100 μM ATP, pH 7.5) for one hour at 4°C.

All motility assays were performed at room temperature in buffer M (50 mM KCl, 30 mM Tris-Cl, 4 mM $MgCl_2$, 1 mM ATP, 25 mM DTT, 1 mM EGTA, 0.7% methyl cellulose (Sigma-Aldrich, M-0512) and 2 μM calmodulin, pH 7.5). Glucose oxidase (0.2 mg/ml), catalase (0.04 mg/ml) and glucose (4.5 mg/ml) were also included in buffer M as oxygen scavengers to inhibit photobleaching, and phosphocreatine (1 mM) and creatine phosphokinase (0.2 mg/ml) were included to regenerate ATP. The movement of actin filaments was observed using an Axiophot epifluorescence microscope (Zeiss, Germany) with an Orca 1 C4742-95 digital camera (Hamamatsu, Japan). Image acquisition and post acquisition analysis were performed using the Openlab v.2.2.5 software package (Improvision) running on a Macintosh G4 desktop computer (Apple).

Data and statistical analyses

Paired Student *t*-tests were performed to determine the probability (*p*) of no significant difference.

Results

Association of calmodulin with native myosin VIIa

Of the five IQ motifs in each myosin VIIa subunit, only one (the second) fits the consensus sequence perfectly. The others can approximate an IQ motif to varying degrees (Fig. 1B). The best-known candidate for binding IQ motifs of other unconventional myosins is calmodulin (Wolenski, 1995). To test if calmodulin bound to retinal myosin VIIa, myosin VIIa was immunoprecipitated from supernatants of bovine retina using antibodies against the N-terminus or against the first part of the tail (amino acids 941-1,071). The N-terminal antibody failed to immunoprecipitate myosin VIIa, suggesting that this

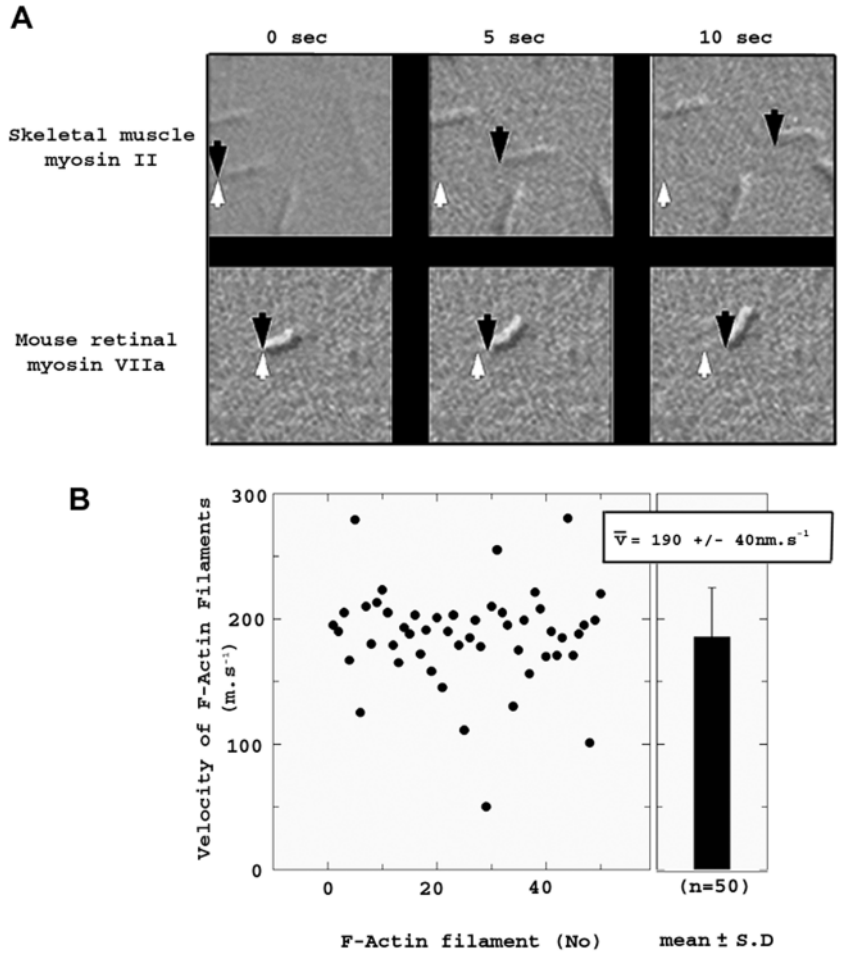

Fig. 4. In vitro motility of myosin VIIa. (A) Series of images illustrating movement of actin filaments by rabbit skeletal muscle myosin II (upper) and by mouse retinal myosin VIIa (lower). The white arrow indicates the starting point. The black arrow indicates the trailing end of a single actin filament. (B) Plot of the measured velocities of 50 individual actin filaments moved by mouse retinal myosin VIIa.

part of the protein might not be exposed in the native protein. However, myosin VIIa was immunoprecipitated by the tail antibody, together with a protein that reacted with antibodies against calmodulin (Fig. 1C). This anticalmodulin-reactive protein comigrated with purified calmodulin in 15% SDS-PAGE (data not shown), supporting the notion that it is indeed calmodulin. The calmodulin was more readily eluted from the immunoprecipitated complex by buffer containing 10 μM Ca^{2+} than by buffer without Ca^{2+} (Fig. 1D), indicating that the affinity of myosin VIIa for calmodulin decreases in the presence of Ca^{2+}. Using a purified, truncated myosin VIIa from a baculovirus-expressed construct (Liu et al., 1997) and calmodulin as standards on western blots, we determined that an average of 3.2 mols of calmodulin was bound to each myosin VIIa dimer after the immunoprecipitation process in the absence of Ca^{2+}. It is likely that some calmodulin was lost during the immunoprecipitation process. By extending the incubations by an hour, we measured an average of 2.4 mols per myosin VIIa dimer. The ratio of 3.2 indicates that a minimum of two of the five IQ motifs on myosin VIIa bind to calmodulin.

MgATPase activity of native myosin VIIa

Owing to the scarcity of myosin VIIa in tissues, myosin VIIa was not purified to homogeneity. Instead, myosin VIIa was immunoprecipitated, and background activity from possible contaminants was determined by simultaneously performing experiments with normal and myosin-VIIa-null tissues. Immunoprecipitation was effected using the myosin VIIa tail antibody conjugated via protein A to solid polystyrene microspheres. Tissues were from *shaker1* mice of the 4626 allele, which possesses a mutation that appears to be null; mutant homozygotes lack even a truncated product (Liu et al., 1999). We attempted to use retinal tissue, but could not obtain sufficient quantities of myosin VIIa for the assays. Instead, we used testes from mutant ($^{-/-}$) and unaffected ($^{+/-}$) mice (Fig. 2). Myosin VIIa MgATPase activity was determined by subtracting that measured in $^{-/-}$ samples from that in $^{+/-}$ samples; the inorganic phosphate measured in the $^{-/-}$ samples represented the background from all sources and was typically less than 20% of the total measured in the $^{+/-}$ samples.

An ATPase activity of ~0.5 s^{-1} was measured in the absence of actin. Addition of 2.5 μM f-actin increased the activity nearly threefold (Fig. 3A). The relationship between actin concentration and activity in the presence of 5 mM Mg^{2+} and the absence of Ca^{2+} at 30°C is illustrated in Fig. 3B. A maximal ATPase activity (K_{cat}) of 4.3 s^{-1}, with 7.0 μM of f-actin required for half-maximal activity (K_{actin}), was calculated.

In vitro motility of myosin VIIa

To test whether myosin VIIa could move along actin filaments, we performed an in vitro sliding actin filament assay by adhering mouse retinal myosin VIIa to coverslips via antibodies. When the tissue source was from wild-type or heterozygous *Myo7a*^{4626SB} mouse retinas, rhodamine-phalloidin-labeled actin filaments were observed moving over the myosin VIIa at a velocity of 190±40 nm s^{-1}. These velocities were five or six times slower than that measured for skeletal muscle myosin II under the same conditions (Fig. 4). No movement of any actin filaments was observed when the experiment was performed under identical conditions with retinas from homozygous mutant *Myo7a*^{4626SB} mice.

Discussion

Myosin VIIa plays critical roles in the inner ear and retina as illustrated by the Usher 1B syndrome, which results from mutations in the *myosin VIIa* gene. Patients with this disorder are born profoundly deaf, have balance problems and then in

the second decade of life begin to lose their vision owing to progressive photoreceptor degeneration. Myosin VIIa is also found in other tissues (Hasson et al., 1995; Wolfrum et al., 1998), although there is no conclusive evidence that Usher 1B patients suffer from disorders that are not related to vision, hearing and balance. To understand the function of myosin VIIa, it is imperative to determine its biochemical capabilities. In the present study, we have shown that myosin VIIa shares basic properties found in previously characterized unconventional myosins: namely, it binds to calmodulin and has actin-based motility.

Calmodulin binding

Calmodulin was coimmunoprecipitated with myosin VIIa from retinal supernatants. During the course of the present study, the binding of calmodulin to kidney and cochlear myosin VIIa was reported from studies using immunoaffinity chromatography and immunoprecipitation (Todorov et al., 2001). This report concluded that the binding was not sensitive to Ca^{2+} and that there was only one binding site for calmodulin on each myosin VIIa heavy chain. By contrast, our study shows that Ca^{2+} causes the elution of calmodulin from myosin VIIa, indicating Ca^{2+} sensitivity. In addition, we found an average of 3.2 mols of calmodulin bound to each dimer of myosin VIIa in the absence of Ca^{2+}, indicating more than one binding site on each myosin VIIa heavy chain. As noted in the Results, our measurement of bound calmodulin is probably an underestimate, given that the immunoprecipitation process included a long incubation of the proteins diluted in buffer. Nevertheless, it is plausible that all five IQ motifs might not normally bind to calmodulin; myosin VIIa might have other light chains. Two other unconventional myosins, myosin Va (Cheney et al., 1993; Wang et al., 2000) and myosin X (Rogers and Strehler, 2001) bind to other light chains besides calmodulin. A calmodulin-like protein (CLP) binds to the third IQ motif of myosin X. Although CLP migrates slightly faster than calmodulin in SDS-PAGE (Yaswen et al., 1992), the sequence of this protein is 85% identical to that of calmodulin (Koller and Strehler, 1988; Rhyner et al., 1992). However, CLP appears unlikely to be a light chain of myosin VIIa. First, CLP has a restricted tissue distribution that does not overlap with myosin VIIa (Yaswen et al., 1992; Hasson et al., 1995; Wolfrum et al., 1998). Second, the third IQ motif of myosin X (FQKQLRGQIAR), which binds to CLP, bears little resemblance to any of the myosin VIIa IQ motifs (Fig. 1B). Yet, it is intriguing to consider whether, in addition to calmodulin, myosin VIIa binds to a different calmodulin-like protein, which we did not detect in our study. As illustrated in Fig. 1B, the IQ motifs of myosin VIIa differ from each other (including in their conformity to an IQ consensus sequence), a feature that might be related to binding different light chains. The sequences of these different motifs do appear to be important. For example, in the fourth motif – the second most divergent – a missense mutation (Ala to Thr) has been linked to Usher syndrome 1B in a number of patients (Adato et al., 1997).

Actin-based motility

Functional myosins are characterized by actin-activated Mg^{2+}-ATPase activity, which is used to power movement along actin filaments. Myosin VIIa has been predicted to be an actin-based motor on the basis of the similarity of its first 730 amino acids to the motor domains of conventional myosins (class II) and representative unconventional myosins from classes I, V, VI, and IX. Myosins from these classes possess actin-based motility. However, to date, the vast majority of myosins, including all myosins in class VII, have not. Demonstration of actin-based motility is required not only to confirm their classification as myosins but to help understand their cellular function. The present study provides the first documentation of actin-based motility of myosin VIIa.

ATPase activity attributable to myosin VIIa increased eightfold from ~0.5 s^{-1} to over 4 s^{-1} with saturating f-actin. Interestingly, the concentration of f-actin required for half-maximal activity was 7 μM at 30°C, which is relatively low when compared with class I myosins (Pollard et al., 1991; Zhu et al., 1998). However, this value is higher than the 1 to 2 μM, which was reported for a native myosin Va in a similar salt concentration but at 37°C (Nascimento et al., 1996). Myosin Va is the most extensively studied two-headed unconventional myosin. Its high affinity for f-actin is important in its processive movement along an actin filament (Mehta et al., 1999). It would be interesting to determine whether myosin VIIa, with its somewhat higher K_{actin}, moves processively.

Our comparison with skeletal muscle myosin II showed that myosin VIIa moved along actin filaments five to six times more slowly (190 nm s^{-1} compared with 1 μm s^{-1}). However, the velocity of myosin VIIa compares more favorably to velocities reported by others for other unconventional myosins. Although comparisons of velocities measured by different experimenters should be made with caution, it is interesting to compare maximal values obtained with other myosins in the same Kron and Spudich sliding filament assay that we used. Native myosin Va has been reported to move actin filaments at 400 nm s^{-1} (Cheney et al., 1993). Velocities of baculovirus-expressed recombinant forms, with a truncated tail, are 250 to 350 nm s^{-1} (Homma et al., 2000; Wang et al., 2000). The velocity of baculovirus-expressed recombinant myosin Iβ from bovine adrenal gland is also in this range (300-500 nm s^{-1}) (Zhu et al., 1996). But, a number of others form a distinctly slower group. Brush-border myosin I has been reported to move at less than 50 nm s^{-1} (Collins et al., 1990), myosin VI (which moves in the opposite direction) at 58 nm s^{-1} (Wells et al., 1999) and myosin IXb at 15 nm s^{-1} (Post et al., 1998). Thus, myosin VIIa appears to belong to the faster group of unconventional myosins.

In conclusion, the present study demonstrates that myosin VIIa possesses actin-based motility. Basic parameters of that motility have been quantified. They provoke a number of questions that should be addressed in further studies. For example, the Ca^{2+} sensitivity of calmodulin binding suggests that motility might be regulated by Ca^{2+}. And, as noted above, the measured K_{actin} of myosin VIIa justifies testing it for processivity. Because of the difficulty in obtaining sufficient quantities of native myosin VIIa for biochemical analyses, a sensible approach to these further studies would be first to develop an expression system (e.g. using baculovirus) that generates suitable quantities of myosin VIIa with the same basic properties as we report here for native myosin VIIa.

We thank Tama Hasson for ongoing helpful discussions, Laurent Blanchoin (Tom Pollard Laboratory) for providing us with actin, and Karen Steel for *Myo7a*^{4626SB} mice. The research was supported by NIH grant EY07042 and a grant from the Foundation Fighting Blindness.

References

Adato, A., Weil, D., Kalinski, H., Pel-Or, Y., Ayadi, H., Petit, C., Korostishevsky, M. and Bonne-Tamir, B. (1997). Mutation profile of all 49 exons of the human myosin VIIA gene, and haplotype analysis, in Usher 1B families from diverse origins. *Am. J. Hum. Genet.* **61**, 813-821.

Chen, Z. Y., Hasson, T., Kelley, P. M., Schwender, B. J., Schwartz, M. F., Ramakrishnan, M., Kimberling, W. J., Mooseker, M. S. and Corey, D. P. (1996). Molecular cloning and domain structure of human myosin-VIIa, the gene product defective in Usher syndrome 1B. *Genomics* **36**, 440-448.

Cheney, R. E., O'Shea, M. K., Heuser, J. E., Coelho, M. V., Wolenski, J. S., Espreafico, E. M., Forschner, P., Larson, R. E. and Mooseker, M. S. (1993). Brain myosin-V is a two-headed unconventional myosin with motor activity. *Cell* **75**, 13-23.

Collins, K., Sellers, J. R. and Matsudaira, P. (1990). Calmodulin dissociation regulates brush border myosin I (110-kD-calmodulin) mechanochemical activity in vitro. *J. Cell Biol.* **110**, 1137-1147.

Gibson, F., Walsh, J., Mburu, P., Varela, A., Brown, K. A., Antonio, M., Beisel, K. W., Steel, K. P. and Brown, S. D. M. (1995). A type VII myosin encoded by mouse deafness gene shaker-1. *Nature* **374**, 62-64.

Hasson, T., Gillespie, P. G., Garcia, J. A., MacDonald, R. B., Zhao, Y., Yee, A. G., Mooseker, M. S. and Corey, D. P. (1997). Unconventional myosins in inner-ear sensory epithelia. *J. Cell Biol.* **137**, 1287-1307.

Hasson, T., Heintzelman, M. B., Santos-Sacchi, J., Corey, D. P. and Mooseker, M. S. (1995). Expression in cochlea and retina of myosin VIIa, the gene product defective in Usher syndrome type 1B. *Proc. Natl. Acad. Sci. USA* **92**, 9815-9819.

Homma, K., Saito, J., Ikebe, R. and Ikebe, M. (2000). Ca(2+)-dependent regulation of the motor activity of myosin V. *J. Biol. Chem.* **275**, 34766-34771.

Kodama, T., Fukui, K. and Kometani, K. (1986). The initial phosphate burst in ATP hydrolysis by myosin and subfragment- 1 as studied by a modified malachite green method for determination of inorganic phosphate. *J. Biochem. (Tokyo)* **99**, 1465-1472.

Koller, M. and Strehler, E. E. (1988). Characterization of an intronless human calmodulin-like pseudogene. *FEBS Lett.* **239**, 121-128.

Kron, S. J. and Spudich, J. A. (1986). Fluorescent actin filaments move on myosin fixed to a glass surface. *Proc. Natl. Acad. Sci. USA* **83**, 6272-6276.

Levy, G., Levi Acobas, F., Blanchard, S., Gerber, S., Larget Piet, D., Chenal, V., Liu, X. Z., Newton, V., Steel, K. P., Brown, S. D. et al. (1997). Myosin VIIA gene: heterogeneity of the mutations responsible for Usher syndrome type IB. *Hum. Mol. Genet.* **6**, 111-116.

Liu, X., Vansant, G., Udovichenko, I. P., Wolfrum, U. and Williams, D. S. (1997). Myosin VIIa, the product of the Usher 1B syndrome gene, is concentrated in the connecting cilia of photoreceptor cells. *Cell Motil. Cytoskel.* **37**, 240-252.

Liu, X. R., Ondek, B. and Williams, D. S. (1998). Mutant myosin VIIa causes defective melanosome distribution in the RPE of shaker-1 mice. *Nat. Genet.* **19**, 117-118.

Liu, X. R., Udovichenko, I. P., Brown, S. D. M., Steel, K. P. and Williams, D. S. (1999). Myosin VIIa participates in opsin transport through the photoreceptor cilium. *J. Neurosci.* **19**, 6267-6274.

Mburu, P., Liu, X. Z., Walsh, J., Saw, D., Jamie, M., Cope, T. V., Gibson, F., Kendrick-Jones, J., Steel, K. P. and Brown, S. D. M. (1997). Mutation analysis of the mouse myosin VIIA deafness gene. *Genes Funct.* **1**, 191-203.

Mehta, A. D., Rock, R. S., Rief, M., Spudich, J. A., Mooseker, M. S. and Cheney, R. E. (1999). Myosin-V is a processive actin-based motor. *Nature* **400**, 590-593.

Nascimento, A. A. C., Cheney, R. E., Tauhata, S. B. F., Larson, R. E. and Mooseker, M. S. (1996). Enzymatic characterization and functional domain mapping of brain myosin-V. *J. Biol. Chem.* **271**, 17561-17569.

Pollard, T. D. (1982). Myosin purification and characterization. *Methods Cell Biol.* **24**, 333-371.

Pollard, T. D., Doberstein, S. K. and Zot, H. G. (1991). Myosin-I. *Annu. Rev. Physiol.* **53**, 653-681.

Post, P. L., Bokoch, G. M. and Mooseker, M. S. (1998). Human myosin-IXb is a mechanochemically active motor and a GAP for rho. *J. Cell Sci.* **111**, 941-950.

Rhyner, J. A., Koller, M., Durussel-Gerber, I., Cox, J. A. and Strehler, E. E. (1992). Characterization of the human calmodulin-like protein expressed in *Escherichia coli*. *Biochemistry* **31**, 12826-12832.

Richardson, G. P., Forge, A., Kros, C. J., Fleming, J., Brown, S. D. M. and Steel, K. P. (1997). Myosin VIIA is required for aminoglycoside accumulation in cochlear hair cells. *J. Neurosci.* **17**, 9506-9519.

Richardson, G. P., Forge, A., Kros, C. J., Marcotti, W., Becker, D., Williams, D. S., Thorpe, J., Fleming, J., Brown, S. D. and Steel, K. P. (1999). A missense mutation in myosin VIIA prevents aminoglycoside accumulation in early postnatal cochlear hair cells. *Ann. N. Y. Acad. Sci.* **884**, 110-124.

Rogers, M. S. and Strehler, E. E. (2001). The tumor-sensitive calmodulin-like protein is a specific light chain of human unconventional myosin X. *J. Biol. Chem.* **276**, 12182-12189.

Self, T., Mahony, M., Fleming, J., Walsh, J., Brown, S. D. M. and Steel, K. P. (1998). Shaker-1 mutations reveal roles for myosin VIIA in both development and function of cochlear hair cells. *Development* **125**, 557-566.

Todorov, P. T., Hardisty, R. E. and Brown, S. D. (2001). Myosin VIIA is specifically associated with calmodulin and microtubule- associated protein-2B (MAP-2B). *Biochem. J.* **354**, 267-274.

Tuxworth, R. I., Weber, I., Wessels, D., Addicks, G. C., Soll, D. R., Gerisch, G. and Titus, M. A. (2001). A role for myosin VII in dynamic cell adhesion. *Curr. Biol.* **11**, 318-329.

Wang, F., Chen, L., Arcucci, O., Harvey, E. V., Bowers, B., Xu, Y., Hammer, J. A. and Sellers, J. R. (2000). Effect of ADP and ionic strength on the kinetic and motile properties of recombinant mouse myosin V. *J. Biol. Chem.* **275**, 4329-4335.

Weil, D., Blanchard, S., Kaplan, J., Guilford, P., Gibson, F., Walsh, J., Mburu, P., Varela, A., Levilliers, J., Weston, M. D. et al. (1995). Defective myosin VIIA gene responsible for Usher syndrome type 1B. *Nature* **374**, 60-61.

Weil, D., Levy, G., Sahly, I., Levi-Acobas, F., Blanchard, S., El-Amraoui, A., Crozet, F., Philippe, H., Abitbol, M. and Petit, C. (1996). Human myosin VIIA responsible for the Usher 1B syndrome: a predicted membrane-associated motor protein expressed in developing sensory epithelia. *Proc. Natl. Acad. Sci. USA* **93**, 3232-3237.

Wells, A. L., Lin, A. W., Chen, L. Q., Safer, D., Cain, S. M., Hasson, T., Carragher, B. I., Milligan, R. A. and Sweeney, H. L. (1999). Myosin VI is an actin-based motor that moves backwards. *Nature* **401**, 505-508.

Wolenski, J. S. (1995). Regulation of calmodulin-binding myosins. *Trends Cell Biol.* **5**, 310-316.

Wolfrum, U., Liu, X. R., Schmitt, A., Udovichenko, I. P. and Williams, D. S. (1998). Myosin VIIa as a common component of cilia and microvilli. *Cell Motil. Cytoskel.* **40**, 261-271.

Yaswen, P., Smoll, A., Hosoda, J., Parry, G. and Stampfer, M. R. (1992). Protein product of a human intronless calmodulin-like gene shows tissue-specific expression and reduced abundance in transformed cells. *Cell Growth Differ.* **3**, 335-345.

Zhu, T., Beckingham, K. and Ikebe, M. (1998). High affinity Ca^{2+} binding sites of calmodulin are critical for the regulation of myosin I beta motor function. *J. Biol. Chem.* **273**, 20481-20486.

Zhu, T., Sata, M. and Ikebe, M. (1996). Functional expression of mammalian myosin I beta: analysis of its motor activity. *Biochemistry* **35**, 513-522.