Myosin VIIA is specifically associated with calmodulin and microtubule-associated protein-2B (MAP-2B)

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Myosin VIIA is a motor molecule with a conserved head domain and tail region unique to myosin VIIA, which probably defines its unique function *in vivo*. In an attempt to further characterize myosin VIIA function we set out to identify molecule(s) that specifically associate with it. We demonstrate that 17 and 55 kDa proteins from mouse kidney and cochlea co-purify with myosin VIIA on affinity columns carrying immobilized anti-myosin VIIA antibody. N-terminal sequencing and immunoblotting analysis identified the 17 kDa protein as calmodulin, whereas MS and immunoblotting analysis identified the 55 kDa protein as microtubule-associated protein-2B (MAP-2B). Myosin VIIA can also be co-immunoprecipitated from kidney homogenate using anti-

calmodulin or anti-MAP2 (recognizing isoforms 2A and 2B) antibodies, confirming the strong association between calmodulin and myosin VIIA and between MAP-2B and myosin VIIA. Myosin VIIA binds to calmodulin with an apparent K_d of 10^{-9} M. Scatchard analysis of the binding of myosin VIIA to MAP-2B provided evidence for two binding sites, with K_d values of 10^{-10} and 10^{-9} M, which have been mapped to medial and C-terminal tail domains of myosin VIIA. The characterization of the interaction of calmodulin and MAP-2B with myosin VIIA provides new insights into the function of myosin VIIA.

Key words: hearing, retina, Usher syndrome.

INTRODUCTION

The large superfamily of myosins contains at least 15 structurally distinct classes [1,2] and includes the myosin II family of skeletal-muscle myosins. The classes are defined on the basis of sequence similarities in the head or motor domain. The myosin motor domain binds actin in an ATP-dependent manner with ATP hydrolysis generating mechanical force. The tail domain is believed to direct the interaction of myosins with specific proteins or other cargo. The structure of the tail domain is unique for each myosin class and is clearly an important determinant governing the specific cargo functions of any particular myosin. Both biochemical and genetic studies have hinted at potential cellular functions for a number of myosin classes, including membrane trafficking, cell movements and signal transduction [2]. However, for most myosin classes the specific cellular functions are unknown and the myosin cargo remains unidentified.

Between the head and tail regions of myosins lies the neck region that contains a variable number of 'IQ' light-chain-binding motifs. To date, all myosins whose primary structure has been determined have contained at least one IQ motif. A number of myosin classes, including myosin I, myosin III and myosin V [3–6] are known to bind calmodulin at the light-chain-binding motifs. As well as calcium-mediated regulation of myosin activity, the binding of calmodulin to myosins could enable its sequestering in specific cellular compartments. However, for many myosins the nature of light-chain binding has not been explored.

The myosin VII class has been implicated in deafness through a number of genetic studies. The myosin VIIA gene was shown initially to underlie the mouse deafness mutations, *shaker1* [7], as well as to encode mutations leading to the deaf-blind syndrome,

Usher type 1b, in the human population [8]. Subsequently, myosin VIIA mutations were shown to be responsible for both recessive and dominant forms of non-syndromic deafness in the human population [9–11]. Myosin VIIA is expressed in both inner and outer hair cells of the inner ear neuroepithelium, the organ of Corti, that is responsible for sound transduction [12]. In mammalian hair cells, myosin VIIA is distributed along the hair-cell stereocilia as well as throughout the body of the hair cell [12]. The protein is also expressed in the retinal pigmented epithelium and the photoreceptor cells of the eye [13,14], as well as a number of other tissues [7,15]. Studies of myosin VIIA mutants in the mouse have suggested that myosin VIIA plays a role in organizing the array of hair-cell stereocilia, the site of mechanotransduction, at the apical hair-cell surface [16]. In photoreceptor cells, myosin VIIA may also play a role in opsin traffic through the connecting cilium from the inner to outer segments [17]. Although there are substantial clues about the role of myosin VIIA in these critical cell types, we know nothing of the cargo or light-chain interactions of myosin VIIA.

The myosin VIIA gene in mouse and human encodes a protein of 2215 amino acids with a predicted molecular mass of 254 kDa [18,19]. Mouse myosin VIIA is 96% identical at the amino acid level to human myosin VIIA [19]. The myosin VIIA neck region contains five IQ motifs whereas the tail contains a number of domains, including a region of identity with myosin IV and a region sharing identity with the Band 4.1 family of membrane-associated proteins [18]. Intriguingly, these regions of shared identity also demonstrate very significant sequence similarity to the tail domain of a calmodulin-binding kinesin protein found in plants [19]. In order to identify myosin VIIA-interacting molecules that might regulate activity or modulate physical proximity to other cell substrates, we have employed affinity chromatography to identify molecules that associate with myosin

Abbreviations used: ECL, enhanced chemiluminescence; MAP-2B, microtubule-associated protein-2B.

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VIIA. We report here that a number of specific proteins interact with myosin VIIA, two of which have been identified as calmodulin and microtubule-associated protein-2B (MAP-2B).

EXPERIMENTAL

Materials

The enhanced chemiluminescence (ECL) detection kit, streptavidin-horseradish peroxidase conjugate and Na^{125}I (0.1 mCi) were purchased from Amersham Pharmacia Biotech. PinPoint™ XaI T-vector and SoftLink™ Soft Release Avidin Resin were from Promega. Affi-Gel Hz, protein assay, Trans-Blot® transfer medium and Sequi-Blot™ PVDF membrane were obtained from Bio-Rad (Hemel Hempstead, Herts., U.K.), and Centriplus® concentrator (Amicon) was from Millipore. Protein A-peroxidase conjugate, Protein A-Sepharose and all chemicals were purchased from Sigma-Aldrich.

Antibodies

A fragment corresponding to the last 17 amino acids of the C-terminus of the myosin VIIA tail (SQMLTAMSKQRNSRSGR) was synthesized with an additional Cys residue at its C-terminus and coupled to keyhole-limpet haemocyanin. Rabbits were primed with 500 µg of the protein together with Freund's adjuvant and received three additional boosts (300 µg each) at 4 week intervals. The antibodies to the myosin VIIA tail were affinity-purified on a column with the immobilized synthetic peptide. The purified polyclonal antibody against myosin VIIA tail was used for the affinity purification and Western-blotting analysis as described below. An antibody (anti-55p) against the 55 kDa myosin-associated protein (55p) was raised by injection of the gel fragments containing the protein into rabbits. The gel was first fragmented by passing it repeatedly through a syringe. Anti-calmodulin monoclonal antibody (mouse ascites fluid) and anti-MAP2 monoclonal antibody (recognizing isoforms 2A and 2B; mouse ascites fluid) were obtained from Sigma-Aldrich.

Affinity purification of myosin VIIA-associated proteins

Mouse kidney or cochlea were homogenized in PBS containing 0.5 mM PMSF, 0.5 mM EGTA and 1 mM dithiothreitol. The homogenate was subjected to low-speed centrifugation at 3000 g for 15 min at 4 °C. The supernatant was concentrated using a Centriplus® concentrator containing a membrane filter with a molecular-mass cut-off of 10000 Da. Any precipitate was removed by centrifugation at 3000 g for 20 min at 4 °C, and the supernatant was applied overnight to an affinity column with anti-myosin VIIA polyclonal antibody coupled to Affi-Gel Hz. The coupling was done according to the manufacturer's instructions and yielded 5 mg of anti-myosin VIIA antibody/ml of gel. The column was washed with PBS until no further protein was eluted, and the retained proteins were eluted with 100 mM glycine/HCl, pH 2.5. After neutralization with 0.5 ml of 1 M Tris/HCl, pH 8.0, the immunoreactivity of the individual fractions was determined by Western blotting using anti-myosin VIIA antibody. The positive fractions were combined, and the volume was reduced by ultrafiltration in a Centriplus® concentrator against water. The protein sample was subjected to electrophoretic and immunoblotting analysis. The bands of co-purified material eluted from the anti-myosin VIIA affinity column were analysed subsequently by N-terminal microsequencing and MS analysis.

Producing tail fragments of mouse myosin VIIA in *Escherichia coli*

Five fragments encoding different regions (amino acid positions 1026–1241, 1268–1586, 1585–1649, 1733–1867 and 1893–2155) of the mouse myosin VIIA tail domain were subcloned into the PinPoint™ XaI T-vector and overexpressed in *E. coli* according to the supplier's instructions. The PinPoint vector carries a segment encoding a peptide that becomes biotinylated in *E. coli* and subsequently functions as a purification tag. The biotinylated fusion proteins produced with this system were affinity-purified using the SoftLink™ Soft Release Avidin Resin, according to the manufacturer's instructions (Promega). The identity of the fusion proteins was confirmed by Western-blot analysis with streptavidin-horseradish peroxidase conjugate and ECL.

PAGE and Western-blotting analysis

Gels were prepared according to the method of Laemmli [20] and consisted of a 5 % (w/v) stacking gel and either SDS/PAGE (15 or 8 % gel, w/v) or 10 % (w/v) non-denaturing PAGE resolving gels. Gels were either stained with Coomassie Brilliant Blue R-250 or transferred to nitrocellulose membranes that had been blocked with 5 % (w/v) dried milk powder in 0.15 % (v/v) Tween 20 in PBS at 4 °C overnight. The membranes were washed for 15 min and twice for 5 min in Tween/PBS and incubated further in Tween/PBS containing 1.5 % (w/v) dried milk powder and 10 µg/ml of the purified anti-myosin VIIA antibody for 1 h at room temperature. After being washed three times as above, the filters were incubated for 1 h with Protein A-peroxidase conjugate at a 1:5000 dilution, followed by one 15 min wash and four 5 min washes with 0.5 % (v/v) Tween 20 in PBS. The immunoreactive bands were visualized using the ECL system. For blotting with biotinylated fusion peptides, gels were transferred to nitrocellulose membranes as above and blocked overnight. The washed membranes were incubated further for 2 h at room temperature with Tween/PBS containing 1.5 % dried milk powder and 50 µg/ml of purified fusion protein. After being washed three times as above, the filters were incubated for 1 h with streptavidin-horseradish peroxidase conjugate at a 1:1000 dilution, followed by one 15 min wash and four 5 min washes with 0.1 % Tween-20 in PBS. Bands were detected with an ECL system.

Immunocytochemistry

Wild-type mice were killed at 3 days old by cervical dislocation and the heads bisected and immersed in 4 % paraformaldehyde (pH 7.2). Heads were dehydrated through increasing concentrations of alcohol, cleared in Safeclear and embedded in paraffin wax. Sections of 10 mm were cut through the inner ear. Sections were deparaffinized for 15 min in xylene and put through two changes of 100 % ethanol. Sections were then immersed in 0.3 % hydrogen peroxide in methanol for 30 min. After washing in PBS, sections were placed in a 0.2 % solution of Triton X-100 in PBS for 30 min. Sections were washed in PBS and blocked in 5 % dried milk powder in PBS for 5 min. Antibodies were applied to the slides in a 1.5 % solution of dried milk powder. The myosin VIIA antibody was used at a concentration of 20 µg/ml. Slides were left overnight at 4 °C in a hydrated chamber. Slides were washed in PBS and biotinylated goat anti-rabbit secondary antibody was applied in PBS for 30 min. Slides were washed again in PBS and the avidin-biotin complex (Vector Laboratories) was applied for 30 min. After further washing in PBS slides were diaminobenzidine stained until brown cells were apparent. The reaction was quenched in PBS, and sections were counterstained with haematoxylin and dehydrated through

an alcohol series. Slides were mounted with DPX (10 g of distrene/80 ml of dibutyl phthalate/35 ml of xylene), had coverslips added and were photographed.

N-terminal sequence and MS analysis

The proteins required for sequencing were separated on 10% (w/v) gels, transferred to Sequi-Blot™ PVDF membrane and stained with Coomassie Brilliant Blue R-250. Proteins were 'on-membrane' digested with trypsin, and the generated peptides were separated on HPLC and subjected to N-terminal sequence analysis using the Edman procedure by the Protein Structure Core Facility, University of Nebraska Medical Center, Omaha, NE, U.S.A.

The bands required for MS analysis were excised or electroeluted from the gel and digested with trypsin prior to analysis by matrix-assisted laser-desorption ionization (MALDI) MS. Subsequently, MALDI fingerprints were checked against current protein sequence databases including SwissProt, NCBItr and Genpept. All MS analysis was carried out by Genomic Solutions, Huntingdon, Cambs., U.K.

Immunoprecipitation

Antibodies (10 µl, 10 mg/ml; anti-calmodulin ascites fluid, anti-MAP2 ascites fluid and anti-55p) were coupled separately to 200 µl of hydrated Protein A-Sepharose. The mixtures were incubated for 2 h at 4 °C with agitation. The antibody-bead complexes were pelleted by centrifugation and washed in PBS containing 0.05% (v/v) glutaraldehyde for 5 min to enhance coupling. The mixtures were washed extensively in PBS followed by PBS containing 2% (w/v) glycine, and washed again in PBS prior to incubation with a mouse kidney homogenate. The kidney homogenate (prepared as described above) was incubated with each antibody-Protein A-Sepharose complex overnight at 4 °C on a rocking platform. The immunoprecipitate complexes were pelleted by centrifugation and the pellets washed three times in PBS. After the final wash the pellets were dissolved in sample buffer and subjected to electrophoretic and immunoblotting analysis. As a control, immunoprecipitation was performed without addition of primary antibody to the Protein A-Sepharose. All reaction mixtures of PBS contained 0.5 mM PMSF, 1 mM EGTA and 1 mM dithiothreitol. Immunoprecipitation with all three antibodies was performed in the absence or presence of Ca²⁺ (in the latter case the reaction mixture contained an additional 1 mM CaCl₂).

Binding studies

Affinity-purified myosin VIIA was separated from other proteins on a SDS/polyacrylamide (10%) gel. Then the bands were electroeluted from the gel, dialysed against water, and the volume was reduced to 200 µl by ultrafiltration in a Centriplus® concentrator containing a membrane filter with a molecular-mass cut-off of 10000 Da. The purified myosin VIIA was iodinated using Na¹²⁵I (0.1 mCi, specific radioactivity 17.4 mCi/µg) and iodobeads.

Mouse kidney homogenate was affinity-purified on a column with anti-myosin VIIA antibody and 10 µg of the eluted protein was loaded into each well of a SDS/polyacrylamide (15%) gel. Samples were electrophoresed and transferred electrophoretically to a nitrocellulose filter that had been preblocked overnight in blocking buffer (5% dried milk powder in PBS containing 0.1% Tween-20). The 17 kDa (calmodulin) and 55 kDa (MAP-2B) bands were excised separately from each lane and incubated with increasing concentrations of ¹²⁵I-myosin VIIA (5, 10, 20, 30, 40,

50, 60, 70, 100 and 200 nM) in blocking buffer for 2 h at room temperature. Blots were washed three times with PBS containing 0.1% Tween-20, air-dried and processed for autoradiography. The pieces were counted directly in a gamma counter. A second piece of equal area above the band was cut to subtract as non-specific binding. The apparent dissociation constants were determined from Scatchard plots of binding of myosin VIIA to calmodulin and MAP-2B.

RESULTS

Purification of myosin VIIA-associated proteins

Antisera to the tail region of myosin VIIA were generated using a synthetic peptide corresponding to the C-terminal 17 amino acids of the molecule (see the Experimental section). Myosin VIIA is known to be expressed in the lung, kidney and testis, as well as in the inner ear [7,15]. The purified anti-myosin polyclonal antibodies were capable of detecting a single band of the expected molecular mass of around 240 kDa from mouse lung, kidney and testis (Figure 1A, lanes 1–3). Furthermore, this antibody was used to affinity-purify myosin VIIA from mouse lung, kidney and testis. When these preparations were immunoblotted with the same antibody the expected band at 240 kDa was detected (Figure 1A, lanes 4–6). As an additional control (see Figure 1A, lane 7) we showed that the immunoreactive band was absent from testis homogenates from a mutant mouse, *Myo7a*^{sh14626SB}, which carries a stop mutation in the motor domain [19] and expresses less than 1% of the wild-type levels of myosin VIIA [15]. Also, the purified anti-myosin VIIA antibody was used for staining on mouse cochlear sections. The expected expression pattern of myosin VIIA was observed. The antibody labelled exclusively the hair cells within the organ of Corti (Figure 1B) as well as hair cells within the sacculus (Figure 1C). In hair cells within the organ of Corti, staining is marked at the apical hair-cell surface in the region of the cuticular plate and stereocilia hair bundles (Figure 1B and see [12]). These results confirm the specificity of the anti-myosin VIIA polyclonal antibody.

To determine whether any proteins specifically associate with myosin VIIA, kidney or cochlear homogenates were applied to an affinity column with the immobilized polyclonal anti-myosin VIIA antibody. These affinity columns were expected to retain not only myosin VIIA but also complexes of myosin VIIA and its interacting partners. The homogenate was applied to anti-myosin VIIA columns, washed extensively and the eluted material analysed by SDS/PAGE and Western blotting. As shown in Figure 2 the immunoreactive material eluted from kidney (Figure 2, lanes 2) and cochlea (Figure 2, lanes 3) consisted of a major band of 240 kDa, representing myosin VIIA, as confirmed by Western blotting with the anti-myosin VIIA antibody (Figure 2, lanes 4 and 5). In addition, bands of approx. 17 and 55 kDa in denaturing conditions (Figure 2A, lanes 2 and 3) and approx. 30 and 55 kDa in non-denaturing conditions (Figure 2B, lanes 2 and 3) were detected. The fact that anti-myosin VIIA antibody did not react with the 17, 30 and 55 kDa species suggests that these proteins were co-purified due to their interactions with myosin VIIA rather than by cross-reacting with the antibody on the column. In addition, a band of around 100 kDa in both denaturing and non-denaturing conditions (Figure 2, lanes 2 and 3) was detected.

N-terminal sequencing and immunoblotting analysis identify the 30 and 17 kDa proteins as calmodulin

Having identified potential myosin VIIA partners, we next subjected the 30 kDa protein to tryptic digestion and N-terminal

Figure 1 Reactivity and specificity of specific polyclonal anti-myosin VIIA antibody

(A) Polyclonal antisera were generated against a peptide from the myosin VIIA tail. The purified polyclonal antibody to the myosin VIIA tail detects a unique band of 240 kDa in mouse lung (lane 1), kidney (lane 2) and testis (lane 3) homogenates as well as in the affinity-purified myosin VIIA from lung (lane 4, this lane is underloaded), kidney (lane 5) and testis (lane 6). A lower-molecular-mass product can be observed in lung (lane 1) and this has been reported previously [15]. A high-molecular-mass cross-reacting band runs at the start of the separating gel and is not blocked by peptide antigen. No specific binding at 240 kDa was detected in a testis homogenate from the mouse mutant *Myo7a*^{sh14626SB}, which carries a stop mutation in the motor domain [19] and expresses less than 1% of the wild-type levels of myosin VIIA [15] (lane 7). A lower-molecular-mass band detected in the mutant may represent cross-reaction between the antibody and truncated protein. (B) Immunolocalization of the myosin VIIA antisera on mid modeolar cochlear section from a 3 day-old mouse. Both outer hair cell (OHC) and inner hair cell (IHC) bodies are stained and there is darker staining at the apical surface of both inner and outer hair cells in the region of the stereocilia hair bundles (indicated by arrows). (C) Immunolocalization of the myosin VIIA antisera on the sensory epithelium of the sacculus. Asterisks indicate two hair-cell nuclei surrounded by staining in the cell body. The overlying otolithic membrane is, as expected, unstained. In both (B) and (C) the scale bars represent 10 μ m. Control sections omitting the primary antibody revealed no staining (results not shown).

sequence analysis (see the Experimental section) and utilized this information to search protein databases. The sequence of one of the fragments from the 30 kDa myosin VIIA-associated protein (EAFSLFDK) revealed complete identity with mouse calmodulin and starts after the expected Lys (amino acid residue 15 in mouse calmodulin, EMBL accession number M19381). Calmodulin is a highly conserved, single polypeptide chain with a molecular mass

Figure 2 Immunoaffinity purification of myosin VIIA-associated proteins

Kidney and cochlear homogenates were applied to the column with immobilized anti-myosin VIIA antibody. The eluted fractions were concentrated and separated by SDS/PAGE (15%, w/v, gel; A) or non-denaturing PAGE (10%, w/v, gel; B). Bands of 240 and 55 kDa in both (A) and (B) and of 17 kDa in (A) and 30 kDa in (B) from both kidney (lanes 2) and cochlea (lanes 3) were visualized after Coomassie Brilliant Blue staining. The immunoblotting analysis of the same fractions using anti-myosin VIIA antibody (lanes 4 and 5 respectively) detected a positive band with a molecular mass of 240 kDa, corresponding to myosin VIIA. Lane 1, molecular-mass markers.

of approx. 17 kDa, which functions as a multi-purpose Ca^{2+} receptor and is known to interact with other myosin molecules [3–6,21,22].

To confirm further that calmodulin interacts specifically with myosin VIIA we used anti-calmodulin antibody for immunodetection of the affinity-purified material from kidney homogenate. As shown in Figure 3, the anti-calmodulin antibody reacts with both the 17 kDa- (Figure 3, lane 1) and 30 kDa- (Figure 3, lane 2) associated proteins, identified in denaturing and non-denaturing conditions, respectively, from affinity-purified material in kidney homogenate. This indicated that the 17 kDa myosin VIIA-associated protein is calmodulin. The anomalous electrophoretic mobility of 30 kDa found under non-denaturing conditions probably reflects the fact that calmodulin is a highly acidic protein and migrates with an anomalously large apparent Stokes radius [23]. Taken together these results support the conclusion that calmodulin associates specifically with myosin VIIA.

Immunoprecipitation with anti-calmodulin antibodies co-purifies myosin VIIA

In order to verify the association of calmodulin with myosin VIIA, we used anti-calmodulin antibody to immunoprecipitate material from mouse kidney homogenate (Figure 4A). The anti-calmodulin antibody immunoprecipitated a band of 17 kDa,

Figure 3 Western-blot analysis of myosin VIIA-associated proteins using anti-calmodulin antibody

Kidney homogenate was affinity purified on a column with immobilized anti-myosin VIIA antibody. The fractions eluted from the column were separated by SDS/PAGE (15% gel, lane 1) or non-denaturing PAGE (lane 2) and transferred to nitrocellulose membrane. The membranes were analysed with anti-calmodulin antibody, which specifically detects a protein of 17 kDa in denaturing conditions and of 30 kDa in non-denaturing conditions.

Figure 4 Immunoprecipitation with anti-calmodulin antibody co-precipitates myosin VIIA

Anti-calmodulin immunoprecipitates were prepared from kidney homogenate, resolved by SDS/PAGE (15% gel) and analysed by Western blotting. **(A)** Immunoprecipitations carried out in the presence of Ca^{2+} . Coomassie Brilliant Blue staining shows two species of 240 and 17 kDa in the immunoprecipitated material (lane 2). As a control, Protein A-Sepharose with anti-calmodulin antibody were incubated with lysis buffer rather than kidney homogenate (lane 3), confirming that the bands at 60 and 25 kDa represent the heavy and light chains of the antibody used in the immunoprecipitation. Immunoblotting analysis with anti-calmodulin antibody (lane 4), detects a band of 17 kDa, while the anti-myosin VIIA antibody detects a band of 240 kDa (lane 5). **(B)** As for **(A)**, but immunoprecipitations were carried out without Ca^{2+} .

Figure 5 Western-blot analysis of myosin VIIA-associated proteins using anti-55p and anti-MAP2 antibodies

Kidney homogenate was affinity-purified on a column with immobilized anti-myosin VIIA antibody. The fractions eluted from the column were separated by SDS/PAGE (15% gel in **A** and 8% gel in **B**) and transferred to nitrocellulose membrane. The membranes were analysed with anti-55p (lanes 1) and anti-MAP2 (lanes 2) antibodies. Both anti-55p and anti-MAP2 antibodies specifically recognize a protein of 55 kDa on 15% gel and bands of 280 and 55 kDa on 8% gel.

Figure 6 Immunoprecipitation with anti-55p and anti-MAP2 antibodies co-precipitates myosin VIIA

Anti-55p and anti-MAP2 immunoprecipitates from kidney homogenate were resolved by SDS/PAGE (15% gel) and analysed by Western blotting. Coomassie Brilliant Blue staining identified two species of 240 and 55 kDa in both anti-55p- (lane 2) and anti-MAP2- (lane 3) immunoprecipitated materials. As a control, Protein A-Sepharose with anti-55p (lane 4) and anti-MAP2 (lane 5) antibodies were incubated with lysis buffer rather than kidney homogenate, confirming that the bands at 60 and 25 kDa represent the heavy and light chains of the antibody used in the immunoprecipitation. Immunoblotting analysis with anti-55p (lane 6) and anti-MAP2 (lane 7) antibodies detect a band of 55 kDa, while anti-myosin VIIA antibody detects a band of 240 kDa in both anti-55p (lane 8) and anti-MAP2 (lane 9) immunoprecipitates. Lane 1, molecular-mass markers.

together with a band of 240 kDa in the presence of Ca^{2+} (Figure 4A, lane 2), as revealed by total protein staining. The additional bands of 60 and 25 kDa (Figure 4A, lane 3) represent the heavy and light chains of the antibody used for immunoprecipitation. Immunoblotting experiments were performed using anti-calmodulin antibody, which detected only the expected 17 kDa band (Figure 4A, lane 4). Western-blot analysis with anti-myosin VIIA antibody detected the expected band of 240 kDa corresponding to myosin VIIA, but not the 17 kDa calmodulin band (Figure 4A, lane 5). Identical results were obtained when immunoprecipitation with anti-calmodulin antibody was performed in the absence of Ca^{2+} (Figure 4B). These results suggest a Ca^{2+} -independent binding of calmodulin to myosin VIIA. The immunoprecipitation experiments with anti-calmodulin antibody confirmed the specificity of the interaction between calmodulin and myosin VIIA.

Figure 7 Determination of the apparent dissociation constant for myosin VIIA binding to calmodulin and MAP-2B

Calmodulin (**A**) and MAP-2B (**B**) were incubated with increasing concentrations of ^{125}I -myosin VIIA in blocking buffer for 2 h at room temperature (see the Experimental section). Blots were washed, air-dried and processed for autoradiography. The pieces were counted directly for radioactivity. Scatchard analysis of the binding reaction to calmodulin gave a K_d value of 10^{-9} M (**C**). Scatchard analysis of the binding reaction to MAP-2B provided evidence for two binding sites with K_d values of approx. 10^{-10} and 10^{-9} M (**D**). B, bound protein; F, free protein.

MS and immunoblotting analysis identify the 55 kDa protein as MAP-2B

Peptide mass fingerprinting of 55p followed by database searches (see the Experimental section) was utilized to identify the 55p protein. Trypsin digestion of 55p material electroeluted from gels

followed by mass analysis revealed 13 peptides in total with matches to human or mouse MAP-2B (MOWSE scores of 1.33 e^5 and 2.64 e^4 respectively). To further confirm the identity of 55p and its interaction with myosin VIIA, we used anti-55p and anti-MAP2 antibodies for immunodetection of the affinity-purified material from kidney homogenate. As shown in Figure 5(A), both anti-55p (Figure 5A, lane 1) and anti-MAP2 (Figure 5A, lane 2) antibodies reacted with the 55 kDa protein from affinity-purified material in kidney homogenate. These results confirm that the 55 kDa myosin VIIA-associated protein is derived from MAP-2B. The 55 kDa protein appears to be either an isoform with a lower molecular mass, or a degradation fragment of the whole MAP-2B molecule occurring during its affinity purification and gel-electrophoresis separation, since MAP-2B has a molecular mass of more than 200 kDa. When high-molecular-mass proteins from the affinity-purified kidney homogenate were separated on 8% (w/v) gel (Figure 5B) and blotted with anti-55p (Figure 5B, lane 1) and anti-MAP2 (Figure 5B, lane 2) antibodies, proteins of around 280 and 55 kDa positively reacted with both antibodies. Taken together, these results support the conclusion that MAP-2B associates specifically with myosin VIIA.

Immunoprecipitation with anti-55p or with anti-MAP2 antibodies co-purifies myosin VIIA

We used the anti-55p and anti-MAP2 antibodies to immunoprecipitate material from mouse kidney homogenate (Figure 6). Both anti-55p (Figure 6, lane 2) and anti-MAP2 (Figure 6, lane 3) immunoprecipitated a band of 55 kDa, together with a band of 240 kDa, as revealed by total protein staining on SDS/PAGE resolving gel (15%, w/v). The additional band of 60 kDa represents the heavy chains of the anti-55p (Figure 6, lane 4) and anti-MAP2 (Figure 6, lane 5) antibodies used for immunoprecipitation. Immunoblotting analysis with both anti-55p (Figure 6, lane 6) and anti-MAP2 (Figure 6, lane 7) antibodies

Figure 8 Mapping of MAP-2B binding sites on myosin VIIA tail domain

(**A**) Fusion proteins corresponding to defined regions of the myosin VIIA tail were overexpressed in bacteria (see the Experimental section). The tail region of myosin VIIA contains a number of domains, including regions of identity with myosin IV, a region sharing identity with the Band 4.1 family of membrane-associated proteins [18], and regions with very significant sequence similarity to the tail domain of a calmodulin-binding kinesin protein found in plants [19]; these regions are indicated. In addition, a putative SH3 region has been identified [19]. A. thaliana CBP, *Arabidopsis thaliana* calmodulin-binding kinesin protein; S. tuberosum, *Solanum tuberosum*; N. tabacum, *Nicotiana tabacum*. (**B**) Affinity-purified kidney homogenate from anti-myosin VIIA antibody columns was separated by SDS/PAGE (15% gels) and blotted with the purified fusion peptides. Amino acid regions corresponding to each lane are shown on the right.

detected only the 55 kDa band, whereas the anti-myosin VIIA antibody detected the expected band of 240 kDa corresponding to myosin VIIA, but not the 55 kDa protein (Figure 6, lanes 8 and 9). These results confirmed the specificity of the interaction between MAP-2B and myosin VIIA.

Binding studies

Ligand-binding studies (Figures 7A and 7B) were performed using ^{125}I -myosin VIIA prepared from affinity-purified material and calmodulin or MAP-2B (see the Experimental section). In experiments with calmodulin, Scatchard analysis of the binding reaction provided evidence for one binding site with a K_d value of approx. 10^{-9} M (Figure 7C). For MAP-2B, the analysis indicated at least two binding sites with K_d values of approx. 10^{-10} and 10^{-9} M (Figure 7D).

Furthermore, we mapped the sites for binding of myosin VIIA to MAP-2B by examining the binding of MAP-2B to fusion proteins corresponding to five different regions of the myosin VIIA tail domain (Figure 8). Strong binding was observed on the medial tail region (Figure 8B, lane 3) corresponding to a putative SH3-binding domain [19]. Weaker, but clearly detectable, binding was observed for two peptides (Figure 8B, lanes 4 and 5) from C-terminal tail domains of myosin VIIA. No binding was observed to the first two peptides (Figure 8B, lanes 1 and 2) of the N-terminal tail domain of myosin VIIA.

DISCUSSION

Myosin VIIA plays an important role in sensory hair-cell function. Identification of the proteins that interact with myosin VIIA is essential to understanding the role of myosin VIIA in neuroepithelial development and function. However, the cargo or other proteins that interact with this unconventional myosin are unknown. In this study, we demonstrate that myosin VIIA interacts specifically with calmodulin and MAP-2B in mouse kidney and cochlea. The specificity of the interactions was confirmed using two approaches; first by co-purification on an affinity column with immobilized anti-myosin VIIA antibodies and secondly by co-immunoprecipitation with anti-calmodulin and anti-MAP2 antibodies. Also, the binding study and ligand blotting using myosin VIIA tail fusion proteins provide evidence for specific, high-affinity binding sites for myosin VIIA to calmodulin and MAP-2B. A Scatchard plot of binding of myosin VIIA to calmodulin was linear, indicating a single binding site. For MAP-2B the plot was non-linear, indicating either two or more discrete sites, or alternatively co-operative interactions between the binding sites. Analysis of the binding of MAP-2B to fusion proteins from the tail domain of myosin VIIA provided evidence for the location of these sites within medial and C-terminal regions of the myosin VIIA tail. Strongest binding appeared to occur to a putative SH3-binding domain that had previously been identified for the myosin VIIA mouse sequence [19].

The present results, while demonstrating that calmodulin and myosin VIIA interact, do not shed any light on the nature of the molecular interaction. However, it seems likely that calmodulin interacts at IQ motif sequences in the neck domain of myosin VIIA (see the Introduction). In all of the myosins studied so far, the head and tail domains are connected by a neck domain, consisting of repeats of about 24 amino acids, known as the IQ motif [3–6]. The number of IQ motifs can be regulated by alternative splicing [6]. To date, several myosin classes have been

shown to bind calmodulin. These include members of the class I, III and V myosins [3–6,21,22,24,25]. The class VII myosins can now be added to this list. Whereas we have demonstrated a specific interaction between calmodulin and myosin VIIA in kidney and hair cells in the inner ear, it cannot be assumed that similar interactions occur in other cell types. Nevertheless, it seems likely that calmodulin will interact with myosin VIIA in all tissues in which it is present, where it may act to regulate myosin VIIA activity.

Our goal was to identify molecules that interact with myosin VIIA and which may play a critical role with myosin VIIA in hair cell or photoreceptor cell function, cell types where studies on mouse myosin VIIA mutants indicate that this particular motor plays a critical functional role. The interaction with calmodulin suggests that it is possible that myosin VIIA activity is regulated by calcium, as is the case for members of the myosin I and V classes, which bind calmodulin light chains [3–6,21,22]. The process of mechanotransduction in inner-ear hair cells leads to calcium influx, stimulating afferent cochlear neurons and activating calcium-gated channels in the baso-lateral membrane that repolarize the hair cell [26,27]. The physiological role of calmodulin bound to myosin VIIA and its relationship, if any, with the calcium fluxes accompanying mechanotransduction will require further investigation. However, calmodulin is known to be expressed in hair cells [28]. Furthermore, calmodulin antagonists abolish adaptation in hair cells, suggesting that calmodulin binds to myosin I, the putative adaptation motor in the hair cell, and plays a role in regulating the activity of this myosin class in this cell type [29].

Myosin VIIA mutations in the human population can lead to retinitis pigmentosa, and myosin VIIA is known to be expressed in mammalian photoreceptor cells (see the Introduction). In *Drosophila* the *ninaC* gene encodes a class III myosin that binds calmodulin and is expressed in photoreceptor cells. Mutations in *ninaC* demonstrate that proper localization of calmodulin within the photoreceptor cells is dependent on this class III myosin [24,25]. Again, however, any physiological role of calmodulin binding to myosin VIIA in mammalian photoreceptor cells is unknown and it is unclear how this would relate to myosin VIIA's role in opsin transport between inner and outer segments [17]. There are no known mutations in the IQ domain of myosin VIIA in mouse. However, systematic mutation screening of Usher syndrome type 1b patients that carry severe congenital hearing impairment along with retinitis pigmentosa has revealed a number of affected individuals carrying a missense mutation (Ala⁸²⁶ → Thr) in the fourth IQ motif of myosin VIIA [30]. The Ala⁸²⁶ → Thr change might be expected to impair calmodulin binding but this has not been investigated directly.

MAP-2B proteins have two domains, one of which binds to microtubules [31]. The other domain is thought to be involved in binding to other molecules. Staining with antibodies to MAP-2 shows that these microtubule-associated proteins bind along the entire length of cytoplasmic microtubules. However, it is clear that some microtubule-associated proteins are not only associated with microtubules but also bind to other proteins independent of tubulin. For example, it has been shown that the MAP-2B is associated with class C L-type calcium channels in neurons [32]. These channels are localized in dendritic spines, the postsynaptic sites of excitatory synapses [33]. MAP-2B is a major microtubule-associated protein but microtubules are not detectable in postsynaptic spines [34]. Nevertheless, the association of MAP-2B with myosin VIIA suggests that myosin VIIA may be part of a complex involved in a dual-track system of cell transport involving kinesin and actin motors in the manner already observed for myosin V [35], which has been shown to

interact directly with conventional kinesin. Moreover, it has been shown that myosin VIIA is localized to the connecting cilia in photoreceptor cells and myosin VIIA mutations affect opsin transport through the connecting cilia [17]. In conclusion, it is apparent that it will be important to explore further the potential roles and molecular mechanisms for myosin VIIA in both actin- and microtubule-based transport.

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