Intermolecular Autophosphorylation Regulates Myosin IIIa Activity and Localization in Parallel Actin Bundles

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Myosin IIIa (Myo3A), an unconventional myosin localized to the calyceal processes of photoreceptors and the stereocilia of the inner ear, is unique among members of the myosin superfamily in that its structure includes an N-terminal domain with serine–threonine kinase activity (1, 2). This domain is similar to the catalytic domain of the Pak1 kinases and is capable of autophosphorylating site(s) in vertebrates (3) and is present in the C-terminal domain of the Myo3A motor. The lever arm of Myo3A contains two isoforms of reduced Myo3A tip localization was decreased and leucine-glutamine-rich IQ motifs, which bind calmodulin or myosin light chains. Typically the structure of the tail region for a specific class and subclass of myosin reflects its unique role in the cell, and in Myo3A it includes a third IQ motif, the myosin 3 tail homology I motif (3THDI), and the tail homology II actin-binding motif (3THDII) near its C terminus. Because there is no evidence of a coiled-coil-forming region, Myo3A is presumed to be a single-headed myosin. Human Myo3A has demonstrated both ATPase activity and the ability to move actin filaments in vitro, consistent with the characteristics of a cellular transportor or force transducer (2–5).

Myosin IIIa (Myo3A) transports cargo to the distal end of actin protrusions and contains a kinase domain that is thought to autoregulate its activity. Because Myo3A tends to cluster at the tips of actin protrusions, we investigated whether intermolecular phosphorylation could regulate Myo3A biochemical activity, cellular localization, and cellular function. Inactivation of Myo3A 2IQ kinase domain with the point mutation K50R did not alter maximal ATPase activity, whereas phosphorylation of Myo3A 2IQ resulted in reduced maximal ATPase activity and actin affinity. The rate and degree of Myo3A 2IQ autophosphorylation was unchanged by the presence of actin but was found to be dependent upon Myo3A 2IQ concentration within the range of 0.1 to 1.2 μM, indicating intermolecular autophosphorylation. In cultured cells, we observed that the filopodial tip localization of Myo3A lacking the kinase domain decreased when co-expressed with kinase-active, full-length Myo3A. The cellular consequence of reduced Myo3A 2IQ tip localization was decreased filopodial density along the cell periphery, identifying a novel cellular function for Myo3A in mediating the formation and stability of actin-based protrusions. Our results suggest that Myo3A motor activity is regulated through a mechanism involving concentration-dependent autophosphorylation. We suggest that this regulatory mechanism plays an essential role in mediating the transport and actin bundle formation/stability functions of Myo3A.
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Myo3A kinase. Recently we have reported that Myo3A via its 3THD1 tail motif binds to the WH2 domain of the stereocilia-specific actin-bundling protein espin 1, that Myo3A and espin 1 colocalize at the stereocilia tips of mouse inner ear hair cells and the filopodia tips of COS7 cells, and that their co-transfection results in both stereocilia lengthening and a remarkable 10-fold lengthening of the filopodia (18). Although it is possible that Myo3A may transport essential components of the phototransduction pathway in Drosophila photoreceptors (19–21), parallel functions have not yet been elucidated in the vertebrate eye.

We reported previously the kinetic analyses of Myo3A constructs truncated after the second IQ domain, with and without the kinase domain, designated Myo3 2IQ and Myo3A 2IQ ΔK (4, 5). Notable differences between the two constructs were found in the steady-state and transient kinetics as well as in the degree of filopodia tip localization of the corresponding constructs containing the motor and C-terminal tail. The Myo3A 2IQ ΔK showed a 2-fold higher maximum actin-activated ATPase and 5-fold higher steady-state actin affinity. The rate-limiting step for Myo3A 2IQ was modeled to be a transition between two AM.ADP states, whereas the faster Myo3A 2IQ ΔK exhibited slow, rate-limiting ATP hydrolysis. The Ikebe group (3) reported similar results for a motor-only construct, with differences that may be attributed to their removal of the lever arm and use of lower salt concentrations in assays (5). In addition, it was demonstrated that phosphorylation can reduce the actin affinity of the motor-only construct 100-fold without reducing maximal ATPase activity (3, 12). These results imply that kinase activity may be associated with down-regulation of the myosin motor regulation, we have expressed and purified a kinase-dead construct in which a critical lysine in the kinase catalytic domain has been substituted with arginine to render the kinase domain incapable of autophosphorylating the motor (22). On the basis of our results, we propose a unique form of regulation of a myosin motor allows the cell. This novel form of regulation of a myosin motor allows

**Experimental Procedures**

**Reagents**—ATP and ADP were prepared fresh from powder. Nucleotides were prepared in the presence of equimolar amounts of MgCl₂ before use. [γ³²P]ATP was purchased from GE Healthcare or PerkinElmer Life Sciences Inc.

**Construction of cDNAs**—Previously we generated a construct of human Myo3A containing residues 1–1143 truncated after the second IQ domain (Myo3A 2IQ) and a similar construct without the kinase domain (Myo3A 2IQ ΔK, residues 340–1143) containing a C-terminal FLAG tag (DYKDDDDK) (4, 5). QuickChange site-directed mutagenesis (Agilent Technologies) was used to modify Myo3A 2IQ with a single point mutation, substituting arginine for lysine at residue 50 in the catalytic site of the kinase domain (Myo3A 2IQ K50R or kinase-dead) as described (18, 22). The GFP-tagged Myo3A constructs (Myo3A, Myo3A ΔK, and Myo3A K50R) developed for cell biological analysis (18) were modified to insert the mCherry sequence in place of GFP (see supplemental Fig. S1). The espin 1 construct was described previously (18).

**Protein Expression and Purification**—Recombinant baculoviruses of Myo3A 2IQ, K50R, ΔK, and calmodulin were generated with the FastBac system (Invitrogen). Myo3A 2IQ wild type, ΔK, and K50R were co-expressed with calmodulin. Expression in Sf9 cells and purification with anti-FLAG affinity chromatography were performed as described (4, 5). To prevent autophosphorylation during the purification, the wash and elution buffers did not contain ATP. Protein purity was assessed by Coomassie-stained SDS-polyacrylamide gels. The Bio-Rad microplate Bradford assay was used to determine myosin concentration, with BSA as a standard (4, 5). Absorbance measurements were also performed, with similar results, using a predicted extinction coefficient of 129 500 M⁻¹ cm⁻¹ to calculate the concentration.

Actin was purified from rabbit skeletal muscle using an acetone powder method (23). Concentration was determined by absorbance measured at 290 nm (ε₂₉₀ = 2.66 × 10⁴ M⁻¹ cm⁻¹). A molar equivalent of phalloidin was added to stabilize actin filaments in experiments.

All experiments were performed in KMg50 buffer (50 mM KCl, 1 mM EGTA, 1 mM MgCl₂, 1 mM DTT, and 10 mM Imidazole-HCl, pH 7.0, at 25 °C) in the presence of excess calmodulin to ensure that the IQ domains of Myo3A had bound calmodulin.

**Kinase Activity Assays**—Autophosphorylation of Myo3A 2IQ and Myo3A 2IQ K50R was detected by kinase assay using [γ³²P]ATP or anti-phosphothreonine Western blotting (4, 5). Myosin at specified concentrations was allowed to react with 200 μM [γ³²P]ATP (30 Ci/mmol) at room temperature (~22 °C) for specific time periods ranging from 0 to 60 min. The reaction was stopped at each time point by the addition of SDS loading buffer. Autophosphorylation of Myo3A 2IQ K50R was compared with that of Myo3A 2IQ at 1 μM concentration, as was autophosphorylation of Myo3A 2IQ in the presence of 40 μM actin. Additionally, autophosphorylation rates were compared at a series of myosin concentrations ranging from 0.1 to 1.2 μM. Samples were run on SDS-PAGE, and the incorporation of ³²P into Myo3A was detected by phosphorimaging using the Typhoon 8600 variable mode imager (GE Healthcare). Following phosphorimaging, the gel was Coomassie-stained to assess evenness of loading. Densitometry analysis using NIH ImageJ software was used to determine band intensities and adjust for loading differences.

Autophosphorylation of Myo3A 2IQ ΔK by Myo3A 2IQ WT was measured by Western blotting with anti-phosphothreonine antibodies (4, 5). Wild-type Myo3A 2IQ was incubated for 30 min with 200 μM ATP, then mixed with Myo3A 2IQ ΔK and incubated for 60 min in the presence of 1 mM ATP (final Myo3A 2IQ WT and Myo3A 2IQ ΔK concentrations were 1 and 4 μM, respectively). The reaction was sampled at various time points (0, 2.5, 5, 15, 30, and 60 min) and mixed immediately with SDS-
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PAGE sample buffer to stop the reaction. The chemiluminescent signal was visualized on a Syngene GeneGnome or x-ray film. The blot was stripped and reprobed with anti-FLAG to demonstrate total myosin in each lane.

Steady-state ATPase Activity—Steady-state ATP hydrolysis by Myo3A 2IQ (50–100 nM) in the absence and presence of actin (0–60 μM) was examined by the use of the nicotinamide adenine dinucleotide (NADH)-linked assay (4, 24) with a final MgATP concentration of 1 mM. The assay was performed in an Applied Photophysics stopped-flow spectrophotometer in which the NADH absorbance at 340 nm was monitored continuously for 200 s. The ATPase rate at each actin concentration was determined, and the Michaelis-Menten equation was used to calculate the k_{cat} and K_{ATPase} [V_0 + ((k_{cat} [actin])/(K_{ATPase} + [actin]))], where V_0 is the ATPase rate in the absence of actin, k_{cat} is the maximal ATPase rate, and K_{ATPase} is the actin concentration at which the ATPase activity is half-maximal. Uncertainties are reported as standard errors of the fits unless stated otherwise. The data at each actin concentration represent an average of 3–5 protein preparations.

Steady-state actin-activated ATPase rates for both phosphorylated and unphosphorylated Myo3A 2IQ were obtained by preincubation of Myo3A 2IQ (5 μM) for 60 min (as described above) immediately prior to the assay. Samples were tested in the presence of 0–40 μM actin as described above. Separate incubations were conducted for each ATPase sample using staggered start times to ensure that the lag time between incubation and testing was consistent. A sample of the phosphorylated and unphosphorylated Myo3A 2IQ, taken immediately after the 60-min incubation period, was prepared for Western blot analysis to determine the phosphothreonine content.

Actin Co-sedimentation Assays—Steady-state actin affinity of Myo3A 2IQ K50R as well as that of phosphorylated and unphosphorylated Myo3A 2IQ was determined by actin cosedimentation. The pyruvate kinase-phosphoenolpyruvate ATP regeneration system was used to eliminate the accumulation of ADP. Myo3A 2IQ (5 μM) was treated with ATP (200 μM) for 60 min at room temperature to achieve maximal phosphorylation. An unphosphorylated control was also treated for 60 min in the absence of ATP. Myo3A 2IQ (diluted to 0.1 μM) was mixed with actin (0–40 μM) in the presence of the regeneration system and 2 mM ATP and incubated for 10 min at room temperature. The samples were immediately subjected to ultracentrifugation in a TLA 100.2 Beckman centrifuge at 95,000 rpm for 15 min at 4°C. The supernatant and pellet were separated, the pellet was resuspended in 8 M urea, and samples were run on SDS-PAGE. The fraction of actin-bound myosin was determined by measuring the relative amount of myosin in the pellet sample normalized to the total (supernatant + pellet). Quantification of bands and densitometry analysis were performed using ImageJ software. KaleidaGraph software was used to fit the data to the quadratic binding equation.

Cell Culture and Transient Transfection—COS7 cells were grown as described previously (25). Briefly, cell cultures were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (HyClone), 1 mM sodium pyruvate (Invitrogen), 2 mM GlutaMAX (Invitrogen), and 100 units of penicillin-streptomycin (Invitrogen). Cells were passaged using 0.25% trypsin-EDTA (Invitrogen). Prior to transfection, the cells were plated onto acid-washed, 22-mm-square, No. 1.5 coverslips at 35,000–45,000 cells/coverslip (one coverslip/well of a 6-well dish) and allowed to adhere overnight. Cells were transiently transfected using polyethylenimine (PEI) as described previously (26). Briefly, 0.3 μg of plasmid DNA (0.6 μg total for co-transfection experiments) was diluted into 100 μl of Opti-MEM media (Invitrogen) without serum or antibiotics and mixed with 3 μl of PEI (1 mg/ml in water). After gentle mixing, the transfection complexes were allowed to form by incubation at room temperature for 15 min and then added dropwise to the well containing the cells to be transfected (3 ml of medium/well).

Live Cell Imaging of Cells Expressing Fluorescent Proteins—Transfected cells were imaged for ∼18–30 h following transfection. The coverslips were assembled into Rose chambers containing imaging medium (Opti-MEM without phenol red supplemented with 5% fetal bovine serum and 100 units of penicillin-streptomycin). During imaging, a Nevtek ASI400 airstream incubator was used to maintain the cellular environment at ∼35°C. Single images and time lapse image sequences were collected using a Nikon TE2000-PFS fluorescence microscope with a 60 × 1.4 NA. phase objective. Images were obtained using a CoolSnap HQ2 cooled charge-coupled device digital camera (Photometrics) and NIS-Elements AR software (Nikon). For single images, exposure times were held constant at 400 ms for all fluorescence channels (GFP and mCherry) and 60 ms for the bright field channel (phase contrast). Images were collected for all three channels for all treatment groups. Time lapse image sequences were collected at 5- or 10-s intervals, and exposure times were optimized to maximize signal acquisition while minimizing photo-fading due to imaging. Images were captured only for the channels where a signal was expected. Typical exposure times for fluorescence channels during time lapse acquisition ranged from 150 to 500 ms.

Fixed Cell Imaging of COS7 Cells Expressing Fluorescent Proteins—COS7 cells (ATCC, Manassas, VA) were plated on coverslips and maintained at 37°C in DMEM with 10% FBS. Cultures were transfected using the protocol described above or GeneLuce transfection reagent (Novagen, San Diego, CA) and then incubated for 24 h. Samples were fixed for 20 min in 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized for 30 min in 0.5% Triton X-100 in PBS, and then counterstained with 0.001 unit/μl Alexa Fluor-647 phalloidin (Molecular Probes) and mounted using ProLong Antifade (Molecular Probes). Fluorescence confocal images were obtained with a Nikon microscope equipped with a 100 × 1.40 N.A. objective and a spinning disk confocal unit (PerkinElmer).

Cultures and Transfections of Inner Ear Hair Cells—The organ of Corti and vestibular tissue were dissected from postnatal day 0–4 rats, attached to coverslips coated previously with 150 μg/μl Cell-Tak (BD Biosciences), and maintained at 37°C and 5% CO2 in DMEM/F12 (Invitrogen) with 5–7% FBS and 1.5 μg/ml ampicillin (Sigma). For transfections, 50 μg of DNA was precipitated onto 25 μg of 1-μm gold particles and loaded into Helios Gene Gun cartridges (Bio-Rad). Tissue explants were transfected with the gene gun set at 95 psi of helium and maintained in culture for 18–48 h. Samples were
then fixed by immersion in 4% paraformaldehyde in PBS, pH 7.4, for 2 h at room temperature, permeabilized with 0.5% Triton X-100 for 30 min, counterstained with 0.001 unit/μl Alexa Fluor-568 phalloidin (Molecular Probes), and then mounted using ProLong Antifade (Molecular Probes). Fluorescence confocal images were obtained with a Nikon microscope equipped with a 100 × 1.40 N.A. objective and a spinning disk confocal unit (PerkinElmer).

Data Analysis, Statistical Analysis, and Software Used—Both NIS-Elements AR (Nikon) and Metamorph 6.0 (Universal Imaging) were used for image analysis. To calculate the ratio of tip intensity to cell body intensity \( r_{i/c} \), integrated intensity was measured for a 4 × 4-pixel region of the background \( b_n \), the filopodial tip \( b_i \), and the cell body \( b_c \) and calculated using the following equation.

\[
    r_{i/c} = \frac{b_i - b_n}{b_c - b_n} \quad \text{(Eq. 1)}
\]

To calculate the relative intensity at a point along the filopodium \( P_n \), where \( n \) is the distance along the filopodium between 0 and 2.1 μm, the intensities at 0.1-μm intervals along the most distal 2.1 μm of the filopodium \( (i_0 \text{ to } i_{2.1}) \) were measured, and the relative intensity was calculated by dividing \( i_n \) by the sum of all points along the filopodium.

\[
    P_n = \frac{i_n}{\sum_{n=0}^{2.1} i_n} \quad \text{(Eq. 2)}
\]

The velocities of filopodial extensions and rearward movements were calculated using the kymograph tool in Metamorph. Briefly, a region consisting of the path followed by the particle was selected. The kymograph tool generated a new image in which the intensity along the selected path was represented by a 1-pixel-wide line for each image plane of the time lapse. For example, a kymograph for a 20-pixel-long path consisting of 50 image planes would generate a 20 × 50-pixel kymograph file. Distance traveled and time interval were measured from the kymograph and used to calculate velocity. Filopodial lengths were measured using the “length” tool in NIS-Elements, and filopodial density was calculated by dividing the number of thin protrusions extending from a cell margin (not contacting another cell) by the length of that cell margin. Data were compared by Tukey analysis using Minitab 12 software. Data are expressed as means ± S.E. of the mean. All images were prepared for publication using NIS-Elements AR, Metamorph 6.0, Photoshop CS, or some combination of these software packages.

RESULTS

The expression yields and purity of Myo3A 2IQ K50R co-expressed with calmodulin in the baculovirus insect cell (S9) system were comparable with those for Myo3A 2IQ in this and our previous studies (4, 5). Stoichiometry of calmodulin to Myo3A 2IQ was determined by mixing Myo3A 2IQ (1 μM) with actin (5 μM) and varying concentrations of calmodulin (0–10 μM) and subjecting the sample to ultracentrifugation. The acto-

Myo3A 2IQ calmodulin pellet was subjected to SDS-PAGE and the amount of calmodulin determined by running a series of known calmodulin concentrations in the same gel (supplemental Fig. S2). The stoichiometry of calmodulin to Myo3A 2IQ was found to be 2:1 in the samples with at least 2.5 μM excess calmodulin added.

All experiments were performed in Kmg50 buffer in the presence of 10 μM calmodulin to ensure that all IQ motifs were calmodulin-bound. The conditions and methods for the experiments were identical to those in our previous kinetic characterizations of Myo3A 2IQ and Myo3A 2IQ ΔK (4, 5) unless otherwise indicated, which allowed direct comparison with the current kinase-dead and fully phosphorylated Myo3A 2IQ analyses. We investigated the properties of the phosphorylation process in addition to the steady-state activity of a fully phosphorylated Myo3A 2IQ. Full phosphorylation was accomplished by incubating the myosin with 200 μM ATP in the presence of 10 μM calmodulin (at room temperature for 60 min) as described previously (4) and then using it immediately in the assay.

Myo3A K50R Mutant Does Not Undergo Autophosphorylation—The elimination of kinase activity by the K50R point mutation was evaluated by Western blot of samples taken at specific time points during 60 min of incubation with 200 μM ATP using antiphosphothreonine as the primary antibody (Fig. 1A). No phosphothreonine increase was detected at a myosin concentration of 0.5–5 μM, whereas the Myo3A 2IQ wild-type protein showed rates of increase in phosphorylation similar to our previous reports (4). Nitrocellulose membranes were stripped and reprobed with anti-FLAG to evaluate the evenness of protein loading for each sample.

Myo3A 2IQ Is Capable of Intermolecular Phosphorylation of Myo3A 2IQ ΔK—The ability of prephosphorylated wild-type Myo3A 2IQ to phosphorylate Myo3A 2IQ ΔK was evaluated by
Western blot of samples taken at specific time points during 60 min of incubation with 1 mM ATP, using anti-phosphothreonine primary antibodies (Fig. 1B). Phosphothreonine signal from the Myo3A 2IQ ΔK band increased over time when incubated with prephosphorylated Myo3A 2IQ WT. In the absence of Myo3A 2IQ, Myo3A 2IQ ΔK was incapable of autophosphorylation under similar assay conditions. Our results estimate enhanced phosphorylation of Myo3A 2IQ when it is co-transfected with Myo3A compared with Myo3A ΔK transfected alone (supplemental Fig. S3). The anti-phosphothreonine Western blot from the immunoprecipitated samples displayed a high background level, which made the results difficult to examine. Future experiments will examine in vivo intermolecular phosphorylation with alternative methods such as mass spectrometry or phosphosppecific antibodies.

Autophosphorylation of Myo3A Is Not Influenced by Presence of Actin—An early study predicted that the autophosphorylated residue(s) of the human Myo3A motor is within a 20-kDa segment at its C terminus (2). To determine whether actin influences the rate or degree of Myo3A 2IQ autophosphorylation we performed parallel time course kinase assays incubating Myo3A 2IQ in the absence and presence of 40 μM actin with 200 μM [γ32P]ATP. Kinase assays were performed as described under “Experimental Procedures,” and the results were visualized by SDS-PAGE and phosphorimaging, as well as Coomassie staining (Fig. 2A). By densitometry analysis we determined that the presence of actin had no appreciable influence on the relative degree and rate of autophosphorylation (Myo3A 2IQ = 0.11 ± 0.02 min−1, ActoMyo3A 2IQ = 0.14 ± 0.02 min−1) (Fig. 2B). The results are averaged from 5–6 separate experiments on three different protein preparations.

Myo3A Autophosphorylation Is Dependent on Myo3A Concentration—To determine whether autophosphorylation of Myo3A is an intermolecular or intramolecular process, we performed time course kinase assays in the presence of 200 μM [γ32P]ATP at a series of myosin concentrations between 0.1 and 1.2 μM (Fig. 3A). After phosphorimaging, gels were Coomassie-stained to determine total protein levels. Densitometry analysis was used to determine the time course of phosphate incorporation at each concentration based on the phosphorylated band intensity relative to total protein levels. Results are averaged from 3–4 separate experiments on three different protein preparations.

We found that autophosphorylation rates were nearly linearly dependent on myosin concentration within the range tested (Fig. 3B). The rates obtained were as follows: at 0.1 μM, the rate was 0.05 ± 0.03 min−1; at 0.3 μM, 0.12 ± 0.03 min−1; at 0.6 μM, 0.21 ± 0.06 min−1; and at 1.2 μM, 0.29 ± 0.06 min−1. The data were fit to a hyperbolic equation to estimate the maximum rate of kinase activity. Error bars indicate S.D. from four assays on three different protein preparations.

Myo3A Steady-state ATPase Activity Is Dependent on Both the Presence of an Active Kinase and the Phosphorylation State—We evaluated the steady-state enzymatic activity of Myo3A 2IQ K50R as compared with that of the wild-type Myo3A 2IQ construct in its phosphorylated state (5 μM treated with 200 μM

![Graph](image-url)
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TABLE 1

Steady-state enzymatic and actin binding properties of Myo3A 2IQ

The basal ATPase rate ($V_0$) was measured in the absence of actin. The actin-activated ATPase results were fit to the Michaelis-Menten equation to determine the maximum ATPase rate ($k_{cat}$) and actin concentration at which the ATPase rate was half-maximal ($K_{ATPase}$). Actin co-sedimentation assays were performed to determine the steady-state actin affinity ($K_{act}$) for each construct or condition. The values for Myo3A 2IQ $\Delta K$ were taken from Dosé et al. (5). Phos, phosphorylation; dePhos, dephosphorylation.

| Myo3A construct | $V_0$ | $k_{cat}$ | $K_{ATPase}$ | $K_{act}$ |
|-----------------|------|---------|-------------|----------|
|                 | $s^{-1}$ | $s^{-1}$ | $\mu M$ | $\mu M$ |
| Myo3A 2IQ Phos  | 0.09 ± 0.01 | 0.51 ± 0.04 | 18.9 ± 3.9 | 21.6 ± 1.9 |
| Myo3A 2IQ dePhos| 0.07 ± 0.04 | 0.77 ± 0.08 | 9.4 ± 2.9 | 11.3 ± 0.5 |
| Myo3A 2IQ K50R  | 0.09 ± 0.04 | 0.79 ± 0.03 | 9.4 ± 1.7 | 10.9 ± 1.2 |
| Myo3A 2IQ $\Delta K$ | 0.08 ± 0.02 | 1.47 ± 0.08 | 6.0 ± 1.4 | 1.4 ± 0.4 |

whereas the actin concentration at half-maximal ATPase activity ($K_{ATPase}$) was 9 ± 2 µM (18) (Fig. 4A). $k_{cat}$ for the kinase-dead construct was comparable with our previous results on Myo3A 2IQ (4), but it was 2-fold lower than the Myo3A 2IQ $\Delta K$ (1.47 ± 0.08 s$^{-1}$) (Table 1) (4, 18).

We measured the steady-state actin-activated ATPase activity of fully phosphorylated Myo3A 2IQ with a range of actin concentrations (0–40 µM) and compared it with unphosphorylated controls. Our results indicate that the maximum ATPase rate ($k_{cat}$) was reduced 35% by phosphorylation (0.51 ± 0.04 s$^{-1}$ compared with 0.77 ± 0.08 s$^{-1}$) and the $K_{ATPase}$ increased 2-fold (19 ± 4 µM compared with 9 ± 3 µM) (Fig. 4A).

We performed Western blots of the phosphorylated and dephosphorylated samples that were examined in the ATPase assay and demonstrated that phosphothreonine content was enhanced to similar levels compared with our time course experiments (Fig. 4B). We obtained $k_{cat}$ and $K_{ATPase}$ values for the Myo3A 2IQ unphosphorylated control that were similar to Myo3A 2IQ K50R. Results for the $k_{cat}$ of the controls were similar to those obtained for our previous work (4); however the $K_{ATPase}$ values were lower than our previous report. An important difference that may account for the $K_{ATPase}$ values is that the wash and elution buffers did not contain ATP, which perhaps prevented autophosphorylation during the purification. The data are a summary of five experiments from five separate protein preparations.

The Steady-state Actin Affinity of Myo3A 2IQ Is Altered by the Phosphorylation State—The actin co-sedimentation assay was performed in the presence of ATP and an ATP regeneration system on fully phosphorylated Myo3A 2IQ in parallel with buffer-treated controls. Samples of phosphorylated and unphosphorylated control Myo3A 2IQ were prepared as described above (60-min incubation at room temperature in the presence and absence of 200 µM ATP) and immediately run in the assay at 0.1 µM concentration. The fraction of myosin that bound to actin was plotted as a function of actin concentration (0–40 µM) and fit to a quadratic equation to determine the steady-state actin affinity ($K_{act}$). As seen in Fig. 4B, the steady-state actin affinity of phosphorylated Myo3A 2IQ was weaker than the dephosphorylated control, with a $K_{act}$ of 21.6 ± 1.9 µM as compared with 11.3 ± 0.5 µM for the control.

We also determined the steady-state actin affinity of Myo3A 2IQ K50R (10.9 ± 1.2 µM). This value is similar to that obtained for the unphosphorylated wild-type Myo3A 2IQ, whereas it is ~8-fold weaker than our previous measurements of Myo3A.
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2IQ ΔK (see Table 1). The results represent the average from 3–5 experiments on 3–5 different protein preparations.

Expression of Myo3A Constructs in COS7 Reveals Differences in Localization Related to Presence of an Active Kinase Domain—Previous work has shown that expression of Myo3A constructs in the presence of exogenous espin 1 in COS7 cells leads to tip localization of labeled Myo3A but with differences in distribution (18). To determine whether this localization was independent of the presence of espin 1 and dependent on the presence of an active kinase, we expressed tagged Myo3A constructs without exogenous espin 1 expression. To measure the enrichment of the constructs at the filopodial tip, we measured the integrated intensity of a 4 × 4-pixel region of the filopodial tip and corrected for cell-to-cell variation in expression levels by dividing the tip intensity by the intensity of a 4 × 4-pixel region within the cell body. As reported previously with espin co-expression (18), the wild type (GFP-Myo3A) labeled the entire filopodial structure without enrichment at the filopodial tip (Fig. 5C, 1.1 ± 0.2, n = 52 filopodia from 12 cells). GFP-Myo3A K50R (Fig. 5D) showed a 3-fold enhancement of tip localization (Fig. 5A, 3.1 ± 0.4, n = 52 filopodia from 11 cells, p < 0.03, Tukey test), and mchr-Myo3A ΔK (Fig. 5E) showed a 10-fold enhancement of label at the filopodial tip when compared with GFP-Myo3A (Fig. 5A, 10.4 ± 1.5, n = 27 filopodia from 15 cells, p < 0.0001, Tukey test). The less pronounced tip enrichment of Myo3A K50R compared with that of Myo3A ΔK indicated that the presence of an inactive kinase domain still impacts Myo3A localization. To determine whether tip localization was sensitive to the presence of an active kinase domain, we co-expressed mchr-Myo3A ΔK with either GFP-Myo3A, GFP-Myo3A ΔK, or GFP-Myo3A K50R (Fig. 5F) and analyzed the amount of mchr-Myo3A ΔK present at the tips of filopodia from these cells. The presence of active kinase in the form of GFP-Myo3A reduced the tip localization of mchr-Myo3A ΔK (6.8 ± 1.0, n = 76 filopodia from 15 cells) compared with mchr-Myo3A ΔK expressed alone (12.8 ± 1.5, n = 52 filopodia from 15 cells, p < 0.02, Tukey test). However, co-expression with either kinase-deleted GFP-Myo3A ΔK (11.3 ± 1.9, n = 77 filopodia from 15 cells) or a kinase-inactive GFP-Myo3A K50R (10.8 ± 1.0, n = 74 filopodia from 11 cells) did not result in decreased mchr-Myo3A ΔK localization when compared with mchr-Myo3A ΔK expressed alone.

We used whole cell fluorescence measurements for the difference in brightness between eGFP (quantum yield = 0.60) and mCherry (quantum yield = 0.22) to determine the relative expression levels of each pair of constructs in the co-transfected cells (27). The green fluorescence:red fluorescence ratio was 0.79 ± 0.10 for wild-type GFP-Myo3A: mchr-Myo3A ΔK (n = 34 cells), 0.67 ± 0.12 for GFP-Myo3A K50R:mchr-Myo3A ΔK (n = 17 cells), and 0.61 ± 0.12 for GFP-Myo3A ΔK:mchr-Myo3A ΔK (n = 20 cells). The values were not significantly different between conditions (Tukey analysis).

Measurement of the distribution of labeled Myo3A constructs along the length of the filopodia also supported the hypothesis that Myo3A kinase activity regulates the localization of Myo3A. The distribution of wild-type GFP-Myo3A along the most distal 2.1 μm of filopodia indicated that Myo3A was not concentrated at the filopodial tip (Fig. 6, A and D). GFP-Myo3A K50R also displayed a minimal concentration at the filopodial tip (Fig. 6B), whereas mchr-Myo3A ΔK showed a strong enhancement of label at the filopodial tip (Fig. 6C). The
**FIGURE 6.** Co-expression of GFP-Myo3A with mchr-Myo3A ΔK results in redistribution of mchr-Myo3A ΔK labeling along the filopodium. COS7 cells expressing GFP-Myo3A (A) or GFP-Myo3A K50R (B) display labeling of both the filopodial tip and shaft, whereas cells expressing mchr-Myo3A ΔK (C) have increased localization of label at the filopodial tip as compared with the shaft. D, when displayed as the relative intensity at points along the most distal 2 μm of the filopodium, differences between mchr-Myo3A ΔK and GFP-Myo3A are statistically significant for the points within the orange boxes (p < 0.05, Tukey test, nGFP-Myo3A = 77 filopodia from 24 cells, nGFP-Myo3A K50R = 77 filopodia from 28 cells, nmchr-Myo3A ΔK = 30 filopodia from 19 cells). E, when co-expressed with GFP-Myo3A, the amount of mchr-Myo3A ΔK at the filopodial tip decreases with a corresponding increase along the shaft. Co-expression with GFP-Myo3A K50R (F) or GFP-Myo3A ΔK (G) does not result in a redistribution of mchr-Myo3A ΔK label as seen by displaying the data as the relative intensity at points along the most distal 2 μm of the filopodium (H). Yellow boxes indicate the points at which mchr-Myo3A ΔK levels are significantly different when compared with mchr-Myo3A ΔK alone (p < 0.05, Tukey test, nmchr-Myo3A ΔK with GFP-Myo3A = 58 filopodia from 19 cells, nmchr-Myo3A ΔK with GFP-Myo3A K50R = 97 filopodia from 38 cells, nmchr-Myo3A ΔK with GFP-Myo3A ΔK = 96 filopodia from 27 cells). A–C and E–G, scale bar = 2 μm; image intensities are scaled to best represent the relative brightness along the filopodium.
Kinase-dependent Regulation of Myo3A

FIGURE 7. Phosphorylation state of mchr-Myo3A ΔK results in differences in filopodial length and filopodial density but not in filopodia extension velocities or movements toward the cell body. A, filopodial extension velocity in mchr-Myo3A ΔK-expressing COS7 cells was not sensitive to co-expression with either GFP-Myo3A or GFP-Myo3A K50R. Velocities of movement toward the cell body (gray bars) were ~20 nm/s and not significantly different between groups. B, the average lengths of filopodia in GFP-Myo3A-expressing cells were not different from lengths of filopodia in mchr-Myo3A ΔK-expressing cells. C, expression of these constructs also resulted in a change in filopodial density, as measured by the number of filopodia/micron of cell edge that was not contacting another cell. Cells expressing mchr-Myo3A ΔK displayed increased filopodial density compared with cells expressing GFP-Myo3A (p < 0.0002, Tukey test) or cells expressing mchr-Myo3A ΔK in combination with GFP-Myo3A (p < 0.002, Tukey test) but not when compared with cells expressing mchr-Myo3A ΔK in combination with GFP-Myo3A K50R. D–G, representative phase-contrast images illustrating the filopodial density in transfected COS7 cells under the conditions examined in C. (Scale bars = 5 μm).
Kinase-dependent Regulation of Myo3A

In this study, we investigated the role of the kinase domain in the regulation of Myo3A motor activity and the cellular consequences resulting from such regulation. Our previous work revealed that without the 34-kDa kinase domain the Myo3A motor demonstrates a 2-fold higher maximal ATPase rate and activity would result in regulation of the cellular function(s) of Myo3A, which we observed in our cultured cell model system as a decrease in filopodial protrusions and decreased localization to the tips of protrusions.

Phosphorylation Reduces Myo3A Motor Activity—Our in vitro assays indicate that the kinase domain is capable of phosphorylating the Myo3A motor and thereby changing its activity. The K50R mutation abolished kinase activity as indicated by phosphothreonine Western blot (Fig. 1). The catalytic lysine is well conserved in the active site of protein kinases, and the mutation to arginine is commonly used to create a catalytically inactive protein kinase (22). In the ATPase assay we found the kinase-dead construct to be similar to the dephosphorylated (buffer-treated) wild-type Myo3A 2IQ in its actin binding properties (K_{ATPase} and K_{actin}) and maximum actin-activated ATPase rate (k_{cat}). Phosphorylation of wild-type Myo3A 2IQ resulted in a 2-fold weaker actin binding affinity and a 33% reduction in maximal ATPase activity (see Table 1). Our previous results suggest that the rate-limiting step in the Myo3A 2IQ ATPase cycle is a transition between actomyosin ADP states. It is possible that phosphorylation reduces this rate-limiting transition. The maximal ATPase rate of myosins typically correlates well with the in vitro motility rate. These data indicate that phosphorylated Myo3A likely has a reduced rate of motility.

The Ikebe group (3, 12) has reported the kinetic analysis of a motor-only human Myo3A construct with both the kinase and neck domains removed. They report that the motor-only construct exhibits an extremely high affinity for actin, suggesting that this construct behaves very differently than the construct in our studies. The kinetic analysis of this motor-only construct following phosphorylation with an external kinase domain

similar to those from experiments in which espin 1 was not present (Fig. 8A). The presence of active kinase in the form of mchr-Myo3A reduced the tip localization of GFP-Myo3A ΔK (14.4 ± 1.3, n = 51 filopodia from 12 cells) compared with GFP-Myo3A ΔK expressed alone (45.8 ± 2.8, n = 22 filopodia from 10 cells, *p < 0.0001, Tukey test). However, co-expression with either kinase-deleted mchr-Myo3A ΔK (38.1 ± 5.0, n = 22 filopodia from 10 cells) or kinase-inactive mchr-Myo3A K50R (33.4 ± 3.6, n = 48 filopodia from 15 cells) did not result in decreased GFP-Myo3A ΔK localization when compared with GFP-Myo3A ΔK expressed alone, suggesting that the sensitivity of GFP-Myo3A ΔK localization to an active kinase domain is independent of the presence of espin 1. We also transfected rat neonatal inner ear tissue with GFP-Myo3A and GFP-Myo3A K50R constructs (Fig. 8, B and C). The GFP-Myo3A K50R construct localized to stereocilia tips more efficiently than the GFP-Myo3A. The large variability in transfected hair cell stereocilia orientation (i.e., ranging between ~0 and 90° relative to the coverslip) made it impossible to quantify differences in tip-to-base distributions, but in general we observed a shorter decay length for the K50R construct than for the full-length protein (data not shown), as predicted by previous theoretical studies (31). However, it should be noted that hair cells contain endogenous Myo3A capable of phosphorylating GFP-Myo3A K50R intermolecularly and reducing its tip localization.

DISCUSSION

In this study, we investigated the role of the kinase domain in the regulation of Myo3A motor activity and the cellular consequences resulting from such regulation. Our previous work revealed that without the 34-kDa kinase domain the Myo3A motor demonstrates a 2-fold higher maximal ATPase rate and

FIGURE 8. Fluorescently tagged Myo3A K50R does not alter filopodial tip labeling of Myo3A ΔK in the presence of espin 1 and shows stereocilia tip localization when expressed in hair cells. A, in the presence of espin 1, expression of active kinase in the form of mchr-Myo3A decreased the tip labeling of GFP-Myo3A ΔK by ~3-fold when compared with GFP-Myo3A ΔK alone (p < 0.0001, Tukey test). Expression of mchr-Myo3A ΔK or mchr-Myo3A K50R did not show statistically significant decreases in the tip labeling of GFP-Myo3A ΔK. To test whether GFP-Myo3A K50R displayed altered tip localization in stereocilia, we expressed GFP-Myo3A (green) (B) and GFP-Myo3A K50R (green) (C) in hair cells. The cells were fixed and counterstained with Alexa Fluor-568 phalloidin (red).

5-fold tighter actin affinity (4, 5), suggesting regulation by the kinase domain. Rather than eliminating the kinase domain, in our current study we chose to inactivate the intact kinase domain by mutating a key catalytic lysine residue (K50R) and, conversely, by in vitro phosphorylation of the motor prior to examination in biochemical assays. Additionally, we also chose to investigate the mechanism by which autophosphorylation occurs in vitro and in live cells. These investigations provided a direct evaluation of the properties and effects of Myo3A motor autophosphorylation. Our studies suggest that Myo3A undergoes concentration-dependent regulation by autophosphorylation (CD-RAP), in which high local Myo3A concentrations at the tips of bundled actin structures would lead to intermolecular autophosphorylation and reduced Myo3A motor activity (Fig. 9). Reduced motor
Kinase-dependent Regulation of Myo3A

In hair cell stereocilia, the actin core is bundled by espin 1, which may be transported to the stereocilia tip by ATP hydrolysis-powered Myo3A movements (left). At the barbed end of actin bundles (top middle) high concentrations of Myo3A leads to Myo3A kinase-mediated intermolecular autophosphorylation. Phosphorylated motor is removed from the tip compartment by retrograde flow, which could be coupled by tail:actin interactions, tail:espin 1 interactions, motor:actin interactions, or diffusion back to the cell body (right). The end result would be modulation of Myo3A concentrations in the tip compartment leading to regulation of bundled actin protrusion formation/stability.

FIGURE 9. Model for concentration-dependent regulation by autophosphorylation (CD-RAP) for Myo3A. In hair cell stereocilia, the actin core is bundled by espin 1, which may be transported to the stereocilia tip by ATP hydrolysis-powered Myo3A movements (left). At the barbed end of actin bundles (top middle) high concentrations of Myo3A leads to Myo3A kinase-mediated intermolecular autophosphorylation. Phosphorylated motor is removed from the tip compartment by retrograde flow, which could be coupled by tail:actin interactions, tail:espin 1 interactions, motor:actin interactions, or diffusion back to the cell body (right). The end result would be modulation of Myo3A concentrations in the tip compartment leading to regulation of bundled actin protrusion formation/stability.

resulted in a 100-fold reduction in actin affinity of the motor (3, 12). The maximal ATPase results of two reports from the same group (2, 3, 12) are contradictory, as the earlier study reported a 3-fold lower maximal rate in the dephosphorylated state, whereas the more recent report suggests no change. The differences between this work and our studies likely arise from differences in the constructs analyzed.

A key component of our proposed model would be that phosphorylation in the presence of high actin concentrations and high Myo3A concentrations would serve to reduce motor activity. As phosphorylation rate increased linearly with increasing Myo3A concentration, our results suggest that autophosphorylation occurs intermolecularly. High actin concentrations did not interfere with autophosphorylation, demonstrating that phosphorylation could occur in bundled actin structures, such as at the tips of filopodia or stereocilia. Therefore, as Myo3A concentrations increase in the tip compartment, phosphorylation becomes more likely. Down-regulation of the motor ATPase activity and actin affinity could lead to removal of Myo3A from the tip compartment by either diffusion or retrograde movement tied to actin retrograde flow.

Inactivation of the Myo3A Motor May Contribute to Lower Myo3A Levels at the Tips of Actin-based Protrusions—Our biochemical analysis of Myo3A 2IQ in the phosphorylated and dephosphorylated states predicts that active Myo3A kinase activity could regulate Myo3A localization in parallel actin-bundled structures in living cells. We chose to examine CD-RAP of Myo3A in COS7 cells, as it allowed for the examination of kinase activity in a relatively less complicated model system.

Hair cells are difficult to culture and express Myo3A, Myo3B, and espin 1 endogenously. COS7 cells have few filopodial extensions and do not express Myo3A, Myo3B, or espin 1. Additionally, exogenous expression of Myo3A ΔK results in easily identifiable filopodia with striking tip localization of the fluorescently tagged Myo3A ΔK constructs. Previous work has indicated a difference in filopodial localization between fluorescently tagged wild-type Myo3A and tagged Myo3A ΔK (5, 16, 32). Our approach was to examine changes in the localization and activity of mchr-Myo3A ΔK in response to the exogenous expression of Myo3A containing an active kinase domain by co-transfecting mchr-Myo3A ΔK with other GFP-Myo3A constructs.

Our current results suggest that intermolecular phosphorylation of Myo3A mediates its localization in parallel actin bundles. The most pronounced localization to filopodia and stereocilia was found with the fluorescently tagged Myo3A ΔK construct (Fig. 5A). We predicted that Myo3A ΔK tip localization would be reduced with phosphorylation by GFP-Myo3A. Tip localization of mchr-Myo3A ΔK was reduced in the presence of active kinase-containing Myo3A compared with samples co-transfected with constructs lacking kinase activity. The distribution of mchr-Myo3A ΔK was also shifted from the filopodial tip to the shaft in the presence of Myo3A. These results are not due to a lack of Myo3A binding sites in the tip compartment, as increased expression of Myo3A ΔK (in the form of GFP-Myo3A ΔK) did not change the distribution and localization of mchr-Myo3A ΔK. Additionally, our images show co-localization of mchr-Myo3A ΔK and GFP-Myo3A in the same tip compartment, which is consistent with our model of intermolecular phosphorylation occurring at the filopodia or stereocilia tips. We were able to show that the Myo3A 2IQ ΔK construct could be phosphorylated in vitro by a construct containing the kinase domain. Taken together, these data suggest that CD-RAP of mchr-Myo3A ΔK occurs in filopodia.

There are multiple possible mechanisms mediating the tip localization of actin bundle-based motors. First, the rate of tipward movement is a function of the net velocity (tipward motor-based movement minus the actin retrograde flow rate). Because the retrograde flow rate is relatively constant, a faster motor will accumulate more rapidly at the tips. A motor that is readily inactivated at the tip of the actin bundle may not accumulate there if inactivation leads to removal from the tip compartment. Also, the interactions of other components of the tip compartment with the motor may be regulated by other factors.
Because Myo3A \( \Delta K \) has a 2-fold higher maximal ATPase activity compared with Myo3A K50R and both are constitutively active, the 4-fold difference in tip localization highlights the importance of motor velocity. Although the filopodial extension velocities were similar for each condition observed, we were not able to visualize intrafilopodial tipward movements of Myo3A. However, we did observe indirect evidence of Myo3A traffic that was below our threshold of detection as we could see increases and decreases in the intensity of filopodial tips without detectable Myo3A traffic along the filopodial shaft, as reported for Myo15 (33). If Myo3A localization at the tip of the actin bundle is impacted by the accumulation of Myo3A due to intrafilopodial motility, then differences in velocity could lead to the observed differences between Myo3A \( \Delta K \) and Myo3A K50R. The role of inactivation through phosphorylation is highlighted by the 2-fold difference in tip localization when comparing Myo3A K50R and Myo3A, as both have similar maximal ATPase rates in the dephosphorylated state, but phosphorylated Myo3A would be predicted to be slower. Our results suggest that both motor velocity and tip inactivation may impact the tip localization of actin bundle-based motors.

We also completed an analysis of full-length fluorescently tagged Myo3A K50R transfected into rat inner ear hair cells. This is a follow-up to our immunolocalization studies in which the GFP-tagged full-length versions of our Myo3A constructs (WT, K50R, and \( \Delta K \)) were transfected into COS7 cells (18) so that their localization characteristics in an endogenous environment could be investigated. As seen in the filopodia of COS7 cells, Myo3A localized primarily to the tips of actin bundles in stereocilia, with more pronounced localization for the Myo3A K50R construct and especially so for Myo3A \( \Delta K \). The differences in the actin treadmilling rate of stereocilia, where treadmilling is slow, and of filopodia, where treadmilling is more rapid, are expected to affect the net velocity of Myo3A toward the tips and thus account for the more efficient wild-type Myo3A tip localization in stereocilia (18).

**Autophosphorylation of the Myo3A Motor Provides One Mechanism by Which the Cellular Function(s) of Myo3A Can Be Regulated**—Based on the hypothesis that Myo3A is regulated by autophosphorylation, and on the in vitro data indicating that phosphorylated Myo3A has decreased motor ATPase activity, it may seem inconsistent that we did not observe a difference in filopodial extension velocities in the presence of Myo3A containing an active kinase domain but did observe a change in filopodial density. One possible explanation is that the Myo3A molecules actively localized to the filopodial tip during the extension event were not yet phosphorylated and that phosphorylated molecules do not remain at the tip but instead are shed from the compartment. This would result in decreased levels of Myo3A \( \Delta K \) at the filopodial tip and possibly a redistribution of Myo3A along the filopodial shaft (as we observed). Additionally, with Myo3A kinase activity, the number of Myo3A \( \Delta K \) molecules in the tip compartment might drop below the level required to maintain a filopodial extension, leading to decreased numbers of filopodia overall.

Another possibility is that high concentrations of wild-type Myo3A protein in the cell body lead to phosphorylation of mchr-Myo3A \( \Delta K \) in the cytoplasm, thereby decreasing the pool of mchr-Myo3A \( \Delta K \) molecules available for filopodial formation and localization. The molecules that eventually make it into the filopodia are in the active, unphosphorylated state. The mechanism for the enhancement of filopodia formation is espin 1-independent, as this result was observed in cells not expressing espin 1. It is possible that Myo3A serves to collect and cross-link the barbed ends of growing actin filaments into A-structures (34) capable of extending filopodia, similar to the mechanism proposed for Myo10 (35). However, rather than dimerization serving as a mechanism for cross-linking filaments as proposed for Myo10, the combination of the actin-binding domain in the tail of Myo3A and the actin interactions of the motor would allow a Myo3A molecule to traverse along one filament as it interacts with another. This mechanism would bring the ends of the actin filaments close to the filopodial tip to support filopodial extension, just as microtubule-based motors play critical nontransport roles in the organization of the mitotic spindle (reviewed in Ref. 36). In support, the tail actin-binding domain has been shown to be necessary for the filopodial localization of Myo3A (32). It is interesting to note that although Myo3A, Myo10, and Myo15A all localize to bundled actin structures and to filopodial tips when expressed in COS7 cells, only Myo3A and Myo10 seem to have an impact on filopodial density (28, 37). Our data suggest that Myo3A and Myo15A may play different roles in the formation and maintenance of stereocilia. If Myo3A plays a role in the initiation of actin protrusions, CD-RAP could provide a mechanism for regulating the number of actin filaments that are initially incorporated into such a bundle. Whether phosphorylation occurs at the tip or in the cell body, these mechanisms are not mutually exclusive, and both fit our model as both are dependent on the local concentration of Myo3A.

Although both our in vitro data and our transfection data support CD-RAP of the Myo3A motor as a mechanism for regulation, other possibilities exist for phosphorylation-dependent regulation. Phosphorylation of the tail domain of Myo3A may also play a regulatory role. Indeed, phosphorylation of the C terminus of Myo5 results in decreased cargo binding ability (38, 39), and a similar mechanism could result in decreased Myo3A interaction with either espin 1 or actin. Additionally, it has yet to be determined whether Myo3A is also regulated via intramolecular folding as demonstrated for smooth muscle myosin II (40, 41) and Myo5 (42, 43). It is likely that multiple mechanisms of Myo3A regulation contribute to the observed differences in mchr-Myo3A \( \Delta K \) distribution in filopodia.

The biological events modeled for Myo3A activity require a regulation strategy not only for phosphorylation via the Myo3A kinase domain but also for its dephosphorylation. However, the cellular phosphatase responsible for dephosphorylating the target residues on Myo3A in vivo has not yet been identified. Future studies on the dephosphorylation processes involved will be required for clarification. The bulk of serine-threonine dephosphorylations are catalyzed by phosphatases that contain catalytic and regulatory subunits (44, 45). Previously, we successfully dephosphorylated purified Myo3A using catalytic subunits of both calf intestinal and protein phosphatase type 1 (data not shown). The determination of specific targeting subunits of the serine-threonine phosphatases...
involved and their localization in stereocilia will be important to building an understanding of the reversibility of this signaling pathway (44, 46).

In summary, our results provide strong support for the hypothesis that the activity of Myo3A is modulated by concentration-dependent autophosphorylation, reducing steady-state actin affinity and altering motor ATPase. As a result of down-regulation of the motor, the cellular activity of Myo3A may be down-regulated as well. The CD-RAP model of Myo3A function in inner ear hair cells (Fig. 9) proposes that unphosphorylated myosin molecules translocate to actin bundle tips carrying espin 1 as cargo. Upon arrival, the activity of the kinase domain may lead to phosphorylation of other Myo3A molecules, a process for which the likelihood increases with increasing Myo3A (and thus espin 1) concentration. A decrease in Myo3A function results in detachment from the actin bundle and subsequent removal from the area by diffusion or actin treadmilling, either utilizing maintenance of the espin 1 interaction or through actin–Myo3A interaction via the actin-binding domain in the tail of Myo3A. Currently it is difficult to determine whether the rearward movement of Myo3A involves the tail domain, as the tail actin-binding domain has been shown necessary for filopodial localization of GFP-Myo3A constructs in HeLa cells (32). The possible consequences of rapid phosphorylation at high myosin concentrations might be determination of the number of actin filaments incorporated into a nascent bundle or that espin 1 cannot become incorporated into the actin bundle before Myo3A is removed from the tip compartment. Our studies provide a basis for understanding the role of Myo3A in transport within and formation/maintenance of actin-bundled structures including the stereocilia of inner ear hair cells.

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