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Myosin VI and VIIa distribution among inner ear epithelia in diverse fishes

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Abstract

Unconventional myosins are critical motor proteins in the vertebrate inner ear. Mutations in any one of at least six different myosins can lead to human hereditary deafness, but the precise functions of these proteins in the ear are unknown. This study uses a comparative approach to better understand the role of myosins VI and VIIa in vertebrate ears by examining protein distribution for these two myosins in the ears of evolutionarily diverse fishes and the aquatic clawed toad *Xenopus laevis*. Both myosins are expressed in the inner ears of all species examined in this study. Myo7a localizes to hair cells, particularly the actin-rich hair bundle, in all species studied. Myo6 also localizes to hair cells, but its distribution differs between species and end organs. Myo6 is found in hair bundles of most fish and frog epithelia examined here but not in anterior and posterior utricular hair bundles of American shad. These results show that myo7a distribution is highly conserved in diverse vertebrates and suggest functional conservation as well. The finding of myo6 in fish and *Xenopus* hair bundles, however, suggests a novel role for this protein in anamniotic hair cells. The lack of myo6 in specific American shad utricular hair bundles indicates a unique quality of these cells among fishes, perhaps relating to ultrasound detection capability that is found in this species.

Keywords

teleost; hearing; hair cell; saccule; utricle; lagena; myosin

1. Introduction

Congenital deafness affects one out of every 1000 births in the United States, making this the most prevalent of genetic sensory disorders (reviewed in Cryns and Van Camp, 2004). Hereditary deafness is highly heterogeneous, with over 70 nonsyndromic deafness loci mapped

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to date (Cryns and Van Camp, 2004). Mouse models of human deafness are extremely useful for mapping deafness loci and for understanding the expression profiles and function of deafness genes (e.g., Avraham et al., 1995; Littlewood-Evans and Müller, 2000; Karolyi et al., 2003).

Recently, the zebrafish (*Danio rerio*) has been added as a valuable model for hereditary deafness studies, with large-scale mutant screens in progress and many interesting mutations currently under study (Nicolson et al., 1998; reviewed in Whitfield, 2002). Fishes are the largest and most diverse vertebrate group (Nelson, 1994) and show substantial diversity in ear structure across taxa (Popper and Coombs, 1982; Popper and Fay, 1999; Ladich and Popper, 2004). Therefore, restricting the choice of fish models to the zebrafish bypasses the diversity in fish ears (and other structures) that is potentially useful in understanding ear structure and function and in interpreting mutant phenotypes. Comparative studies between diverse fishes provide novel opportunities to understand structure, function, and evolution in vertebrate systems.

While there is great diversity in ear structures among vertebrates, all vertebrate inner ears contain mechanosensory hair cells (see Hudspeth, 1985; Coffin et al., 2004). The apical stereociliary bundles of these cells are actin-rich structures and therefore hair bundles are exceptionally rich in actin-associated proteins (Tilney et al., 1983; Drenckhahn et al., 1991; Belyantseva et al., 2003b; Loomis et al., 2003). One class of important actin-associated proteins is the myosins. Myosins are mechanoenzymes that hydrolyze ATP to move along actin filaments. There are currently 18 classes of myosins; the type II, or “conventional” myosins that form filaments, and the remaining unconventional (non-filament forming) myosins (Sellers, 2000; Berg et al., 2001). At least six distinct myosins are expressed in hair cells (Gillespie et al., 1993; Hasson et al., 1997; Liang et al., 1999; Lalwani et al., 2000; Walsh et al., 2002; Donaudy et al., 2003) and at least three of these (VI, VIIa, and XVa) have been studied because of their suspected involvement in hair bundle maturation and maintenance (Belyantseva et al., 2003a; Self et al., 1998, 1999).

Mice with mutations in any of these three myosins have stereocilia abnormalities, congenital deafness, and vestibular dysfunction, demonstrating the critical nature of these myosins in stereociliary bundle function (Avraham et al., 1995; Gibson et al., 1995; Probst et al., 1998). Moreover, mutations in each of these three myosins have also been shown to be the basis for human congenital deafness underlying Usher’s syndrome type IB (*MYO7A*; Weil et al., 1995) and several forms of non-syndromic deafness including DFNA22 and DFNB37 (*MYO6*; Melchionda et al., 2001; Ahmed et al., 2003), DFNA11 (*MYO7A*; Liu et al., 1997), and DFNB3 (*MYO15*; Wang et al., 1998). Therefore, a better understanding of the role of unconventional myosins in hair cells could lead to treatments for human hereditary deafness.

Here, we look at distribution of two important hair cell proteins, *myo6* and *myo7a*, in the inner ears of phylogenetically diverse fishes. In normal mammalian hair cells, *Myo6* localizes to the cuticular plate region (apical cell surface at the base of the stereocilia) and is thought to function as a membrane anchor around individual stereocilia (Hasson et al., 1997). In the *Myo6* mouse mutant *Snell’s waltzer*, stereocilia develop normally but fuse shortly after birth (Avraham et al., 1995; Self et al., 1999), and a similar phenotype is seen in the zebrafish *myo6b* mutant, *satellite* (Kappler et al., 2004; Seiler et al., 2004). It is hypothesized that *Myo6* functions to anchor the plasma membrane in between individual stereocilia and that in the absence of normal *Myo6*, the membrane “zips up,” forming giant stereocilia which then degenerate (Hasson et al., 1997; Self et al., 1999). *Myo6* is uniquely qualified to perform this anchoring function because it is one of the few myosins known to move backwards (toward the minus end) along actin filaments (Wells et al., 1999). It could therefore move toward the base of the stereocilia and exert constant tension on the membrane.

Myo7a is also expressed in mammalian hair cells, where it is found in the cytoplasm and throughout the hair bundle (Hasson et al., 1997). Mutations in this myosin in both mouse and zebrafish lead to short, splayed stereocilia, suggesting a role in linkage of stereocilia and maintenance of bundle structure (Gibson et al., 1995; Self et al., 1998; Ernest et al., 2000). Myo7a also plays a functional role in hair cells. Abnormally large hair bundle deflections (beyond the physiological range) are required to open transduction channels in mouse mutants, implying that Myo7a operates in series with the transduction channel (Kros et al., 2002).

In the present study, we examine myo6 and myo7a distribution in the ears of fishes in order to better understand myosin distribution, and therefore function, in vertebrate hair cells. As fishes are the largest and most diverse group (Nelson 1994), we use a phylogenetic “cross-section” approach by selecting species separated by wide stretches of evolutionary time. Species included the jawless sea lamprey (*Petromyzon marinus*) and the “primitive” bony lake sturgeon (*Acipenser fulvescens*) to represent specific points in early fish evolution. We also looked at zebrafish, American shad (*Alosa sapidissima*), and oscar (*Astronotus ocellatus*) since they are taxonomically diverse teleost fishes in which the structural characteristics of the inner ear end organs vary (Ladich and Popper, 2004). We then extend the comparison by using the aquatic African clawed frog *Xenopus laevis* as a representative anamniotic tetrapod. Ancestors of the sea lamprey were probably among the earliest vertebrates, arising over 500 million years ago, while teleost fishes are a relatively derived group that is approximately 200 million years old (Nelson, 1994; Hedges and Kumar, 2002). Therefore, the species studied here span a wide range of evolutionary branch points. Phylogenetic relationships between the study species are shown in Figure 1.

2. Materials and Methods

2.1. Animals

Sea lamprey were donated by Dr. Avis Cohen at the University of Maryland. Lake sturgeon were provided by the Wisconsin Department of Natural Resources, and American shad by the Pepco Chalk Point Generating Station. *Xenopus* were a gift from Dr. Eric Haag, University of Maryland. Zebrafish and oscars were purchased from local commercial suppliers. Sea lamprey were studied in larval (ammocete) form while all other animals were juveniles or adults. All animals were sacrificed with an overdose of buffered MS-222 (Sigma-Aldrich, St. Louis, MO) followed by decapitation under an animal care protocol approved by the University of Maryland Institutional Animal Care and Use Committee.

2.2. Immunohistochemistry

Antibodies—Primary Myo6 and Myo7a antibodies were provided by Dr. Tama Hasson at the University of California, San Diego (these antibodies are now commercially available from Proteus Biosciences, catalog # 25-6791 (myo6) and 25-6790 (myo7a)). Anti-Myo6 is a rabbit polyclonal antibody raised to amino acids 1049–1054 of porcine Myo6 (Hasson and Mooseker, 1994). Anti-Myo7a is a rabbit polyclonal antibody raised to amino acids 880–1077 of human MYO7A (Hasson et al., 1995). Both antibodies exhibit consistent labeling in a variety of species (Hasson et al., 1997). These antigens are highly conserved across vertebrate groups, with 83% and 81% identity between zebrafish and mouse for myo6 and myo7a, respectively.

Whole-mount epithelia—Both right and left ears from four animals of each species were used for each antibody. Myo6 and myo7a were immunolabeled using polyclonal antibodies. Tissue was fixed in 4% paraformaldehyde (PFA) for 1 hour at 4° C and sensory epithelia were dissected out of the head and rinsed in 0.1 M phosphate buffer (PB). All solutions for tissue processing were made using PB. Tissue was then briefly digested with type XI collagenase and permeated with 1% Triton-X, blocked in 10% normal goat serum (all from Sigma-Aldrich),

and exposed overnight to primary antibody at a concentration of 1:150 (myo6) or 1:250 (myo7a) in 1% goat serum.

Following incubation in primary antibody, tissue was rinsed in 1% goat serum and exposed to either Alexa 594 or Alexa 633 goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR). Tissue was then rinsed in PB, double labeled with Alexa 488 phalloidin to label actin-rich stereocilia, and coverslipped with Prolong-Antifade (phalloidin and Antifade are from Molecular Probes). Negative controls included omission of primary antibody, secondary antibody, or phalloidin.

Cryosections—Ears were fixed and dissected as described for whole mounts. Epithelia were saturated in increasing sucrose solutions (5%–25%), and embedded in Tissue Tek® OCT embedding medium (Sakura, Torrance, CA). Sections of 12 μ m thickness were cut using a Leica CM3050S cryostat. Post-embedding immunocytochemistry was the same as used for whole epithelia except that the collagenase/triton step was omitted, and all PB rinses included 0.5% Tween-20 (Sigma-Aldrich).

2.3. Image acquisition and processing

A Zeiss LSM 510 confocal microscope was used to image all samples. Optical sections were taken at varying intervals (0.1–2 μ m) and analyzed with Adobe Photoshop (Macintosh, v. 7.0) software. Adobe Photoshop was used to adjust brightness and contrast but not to alter results. Three-dimensional reconstructions were performed with LSM software 5.0 from Zeiss.

2.4. Western blotting

Ears and brains from each species (minimum two animals per species, more for small animals such as zebrafish) were dissected in protease inhibitors (Sigma-Aldrich) and homogenized in Laemlli sample buffer (Bio-Rad, Hercules, CA) with 5% β -mercaptoethanol (Calbiochem, San Diego, CA). While sufficient tissue was not available from all species, the use of zebrafish, American shad, and *Xenopus* represents sufficient phylogenetic diversity that differences in protein weight or antibody binding affinity should be detected. Proteins were separated on a 10% denaturing tris-glycine gel (Invitrogen, Carlsbad, CA), transferred to a PVDF transfer membrane (Amersham Biosciences, Piscataway, NJ), and probed with primary antibody (the same antibodies as for immunocytochemistry). Membranes were then exposed to donkey anti-rabbit horseradish peroxidase and developed using an ECL+Plus detection kit (all from Amersham Biosciences). As both Myo6 and Myo7a are present in mouse inner ear (Avraham et al., 1995; Gibson et al., 1995), mouse cochlear tissue was used for positive control protein. Mouse cochlear protein was a gift from Dr. Mireille Montcouquiol. Primary antibody was omitted for negative controls.

2.5. Epitope expression

Both myosin antibodies were raised to mammalian antigens (Hasson and Mooseker, 1994; Hasson et al., 1995) and thus these antibodies do not necessarily properly cross-react with fish tissue. Additionally, recent work shows that zebrafish possess two myosin VI genes, *myo6a* and *myo6b* (Kappler et al., 2004; Seiler et al., 2004). It was not known whether the myo6 antibody used here binds to zebrafish myo6a, myo6b, or to both proteins. Therefore, zebrafish myosins were expressed in cultured mammalian cells to verify that the existing antibodies recognize myo6 and myo7a in fish. In addition, myo6a and myo6b were expressed in separate cell lines to determine which proteins were recognized by the myo6 antibody. PCR primers were designed to amplify the cDNA region corresponding to the antigenic epitope for myo6a, myo6b, or myo7a. Forward primers included an EcoR1 (*myo6a* and *myo6b*) or XhoI (*myo7a*) restriction site followed by a start codon. Reverse primers included a stop codon and

SalI (*myo6a* and *myo6b*) or BamHI (*myo7a*) site. All PCR products were confirmed by sequencing. Primer sequences are given in Table 1.

Myo6a or *6b* RT-PCR products were directionally cloned into the pIRES2-EGFP vector (BD Biosciences, San Jose, CA) and used to transfect NIH 3T3 cells. *Myo7a* products were cloned into the same vector and transfected into HEK293 cells. Cells that expressed EGFP were successfully transfected, while EGFP-negative cells served as internal negative controls. Cells were plated on coverslips, fixed in 4% paraformaldehyde, and processed for immunocytochemistry as described for tissue sections (above). Following secondary antibody labeling, cells were rinsed and double-labeled with GFP antibody conjugated to Alexa 488 (Molecular Probes).

3. Results

3.1. Antibody specificity and controls

Western blots show that the myo6 antibody binds to a single approximately 150 kDa band from zebrafish ear, *Xenopus* brain, and mouse cochlea (Fig. 2A). A single band of the same molecular weight was also observed in western blots using protein extracted from the ears of American shad (data not shown). Anti-myo7a binds to two bands in mouse cochlea, a predominant band at 200 kDa and a faint band at 250 kDa. Similarly, the myo7a antibody binds to a single 200 kDa band in protein extracts from zebrafish and American shad ears (Fig. 2B).

Although the antibodies localized to protein bands of the same size in samples obtained from mice and multiple fish species, we wanted to confirm that these antibodies were binding specifically to fish myosins. Therefore, we generated cDNA vectors to express the zebrafish specific regions of *myo6a*, *myo6b* and *myo7a* that correlate with the regions of these proteins that were used to generate the antibodies (Hasson and Mooseker, 1994; Hasson et al., 1995). To identify transfected cells, each vector also expressed green fluorescent protein (GFP) as a separate transcript. NIH3T3 cells, which do not express endogenous Myo6, were labeled by the anti-myo6 antibody following transfection with the *myo6a* vector (Fig. 2C–E). Similar results were observed for *myo6b* (Fig. 2F) and for *myo7a* (Fig. 2G). Because NIH3T3 cells express Myo7a, the *myo7a* vector was transfected in HEK293 cells. Since the myo6 antibody recognizes both *myo6a* and *myo6b*, it cannot be determined which protein is being labeled in the experiments described here. However, *in situ* hybridization in zebrafish with *myo6a* and *myo6b*-specific riboprobes indicates that only *myo6b* is expressed in the inner ear (Seiler et al., 2004; Coffin, 2005). Therefore, myo6 immunolocalization data in teleost fishes (zebrafish, American shad, and oscar) presented here is probably specific for *myo6b*.

To confirm that antibody labeling was specific, we carried out control experiments with fish inner ear epithelia in which the primary or secondary antibody was omitted (Fig. 2I, J). Results indicate no antibody labeling (red channel) in the absence of primary or secondary antibody. In addition, specific labeling of myo6 in stereociliary bundles was confirmed by omission of phalloidin labeling (Fig. 2K), demonstrating no bleed-through from the green channel. Immunocytochemistry results shown here and in the next section were verified using both secondary antibodies (Alexa 594 and 633) with no detectable difference in labeling patterns.

3.2. Myosins VI and VIIa in fish and *Xenopus* hair cells

Hair cells in each of the species studied express both myosins, while other cells in the inner ear do not express either myo6 or myo7a (Figs. 3–7). Myo7a is present in the cytoplasm and stereocilia of all hair cells examined in this study (Fig. 3 and data not shown). Labeling appears evenly distributed along the length of the stereocilia. This pattern is consistent in all species

examined here, although there is some variability in fluorescence intensity that was not quantified.

Myo6 distribution, however, differs between epithelia within a single organism and also between species. Myo6 is present in the cytoplasm and cuticular plate of all hair cells examined (Figs. 4–7). Increased labeling is observed in the cuticular plate at the apical surface of the cell. These findings are consistent with other studies in frogs and mammals (Hasson et al., 1997). However, myo6 labeling in the stereocilia is not identical across species. Myo6 is present throughout the length of the stereocilia in hair cells located in the macula communis (the single otolithic epithelium) and canal cristae of the sea lamprey (Fig. 4). Myo6 is also found in stereocilia of every end organ in the lake sturgeon, zebrafish, oscar, and *Xenopus*, although the label in the oscar utricle is very weak as compared to labeling in other end organs (labeling in the utricle is shown in Fig. 5). No apparent differences in labeling were detected between different epithelial regions (e.g., striola vs. extrastriola) within a single end organ in any of these species. In the American shad, however, myo6 is present in hair bundles of most epithelia (see Fig. 6 for examples in the saccule, lagena, and cristae) but the utricular labeling pattern is more complex. Myo6 is present in hair bundles of the central utricular macula in the shad but not in the anterior and posterior utricular maculae (Fig. 7).

3.3. Unusual hair cells in sea lamprey

Lamprey hair cells are unusual in that they contain a unique cytoplasmic organelle described previously in electron microscopy studies (Löwenstein and Osborne, 1964; Hoshino, 1975; Popper and Hoxter, 1987). The present study shows that this unusual organelle labels distinctly with phalloidin (Fig. 4E), demonstrating that it contains actin. This actin organelle begins near the apical surface of the cell and extends through the cytoplasm to the base of the cell, appearing to curve around the nucleus. Myosin labeling (both myo6 and myo7a) is not higher in this structure than in the surrounding cytoplasm, suggesting that these myosins do not specifically associate with the lamprey actin organelle.

4. Discussion

All vertebrate hair cells investigated in this study express both myosin VI and myosin VIIa. The presence of both proteins in evolutionarily distant taxa such as the primitive sea lamprey and the more recently evolved teleost fishes suggests that these proteins were present in ears of early vertebrate. This result is expected, as these proteins are considered critical for normal hair cell function (reviewed in Friedman et al., 1999). However, myo6 distribution differs between species and end organs, suggesting possible diverse roles for this protein in hair cells of some vertebrate species and highlighting a potentially important feature of American shad utricular hair cells.

4.1. Conservation of myosin VIIa distribution

Myo7a was observed to be located throughout the stereocilia in all fishes included in this study. The same distribution is also seen in both cochlear and vestibular hair cells of mammals (Hasson et al., 1995, 1997). However, this distribution differs from that of bullfrogs where myo7a is concentrated in the proximal third of the hair bundle near the basal tapers (Hasson et al., 1997). Hasson et al. (1997) suggested that Myo7a associates with lateral stereociliary links, which are located throughout mammalian hair bundles but are concentrated near the basal tapers of bullfrog hair bundles (Furness and Hackney, 1985; Jacobs and Hudspeth, 1990). In contrast, electron micrographs of hair bundles from cypriniform fishes (minnows and relatives, including the zebrafish) reveal both ankle links and lateral links, with lateral links distributed throughout the hair bundle (Neugebauer and Thurm, 1984; Söllner et al., 2004). Therefore, myo7a distribution in fish hair bundles is consistent with an association with lateral

links. Future studies using transmission electron microscopy (TEM) to visualize lateral links, coupled to immunogold labeling of myo7a within fish stereocilia, would better examine this putative myo7a-lateral link relationship.

The importance of Myo7a for proper hair bundle formation is illustrated in the *Shaker1* mouse mutant and the *mariner* zebrafish mutant (Gibson et al., 1995; Ernest et al., 2000). In these *Myo7a* mutants, hair bundles are greatly disorganized and do not respond properly to stimulation. Recent electrophysiological studies in *Shaker1* mice show that the stimulus magnitude necessary to open the transduction channels is altered in *Myo7a* mutants (Kros et al., 2002). This finding supports the hypothesis that *Myo7a* is associated with the transduction complex, perhaps by acting in series with the extracellular tip links that are believed to gate the transduction channel. Conservation of myo7a distribution in hair cells of fishes and mammals suggests conservation of function as well.

4.2. Differences in hair cell myosin VI distribution

Myo6 distribution in fishes and *Xenopus* differs in a species and end organ-specific manner. Myo6 is found throughout the length of the stereocilia in all end organs of the sea lamprey, lake sturgeon, zebrafish, and *Xenopus*. However, myo6 labeling is very faint in the oscar utricle in all specimens examined and is lacking in stereocilia of the anterior and posterior utricular maculae in the American shad. This latter distribution pattern, where myo6 is seen in hair cell bodies and the cuticular plate region but not in stereocilia, is similar to the known Myo6 distribution in mammalian hair cells and is consistent with a membrane anchoring function for this protein (Hasson et al., 1997; Self et al., 1999). A prior study in larval zebrafish did show myo6 labeling in the hair bundle but labeling was restricted to the basal portion of the bundle (Kappler et al., 2004). In contrast, myo6 labeling in adult zebrafish in the present study was apparent throughout the length of the bundle. This difference could result from the different life-history stages (larval vs. adult) used in each study.

Fish otolithic epithelia are used to detect both auditory and vestibular signals (Popper and Fay, 1993, 1999; Popper et al., 2003). While the contributions to hearing of each of the three otolithic epithelia are not well understood, there is a growing body of evidence to suggest that the involvement of each end organ varies by species and, to a great degree, is correlated with the hearing capabilities of a species. For example, there is evidence to suggest that the primary auditory end organ in the oscar is the saccule, and behavioral studies have shown that this species hears sounds to no higher than 800 Hz (Yan and Popper, 1992). In contrast, otophysan fishes such as zebrafish (and related goldfish) are highly specialized for hearing, and can detect sounds to 4 kHz or above (reviewed in Fay, 1988; Lanford et al., 2000; Higgs et al., 2003). Hearing in these species involves a modified chain of vertebrae, the Weberian ossicles, which acoustically couples the pressure-detecting swim bladder to the saccule (Weber, 1820; Fay and Popper, 1985). As a result of this coupling, the bandwidth and sensitivity is much broader than in any of the other species studied here. In contrast, there is evidence that the lagena in otophysans, which is not directly connected to the swim bladder, only detects sounds to a few hundred Hz, and recent evidence suggests that the lagena in otophysans contributes to sound source localization for low-frequency sounds (Meyer et al., 2004). While research on sturgeon hearing is just beginning, auditory brainstem response (ABR) recordings indicate responses up to 500 Hz (Lovell et al., 2005), while preliminary results by Meyer et al. (2005) using electrophysiological recordings from eighth nerve afferents showed phase locking up to 800 Hz. While the earliest literature suggested that the utricle is strictly a vestibular end organ (see Popper and Fay, 1999), there is a growing body of evidence suggesting that the utricle, at least in some species, is also involved in hearing (Popper and Tavalga, 1981; Lu et al., 2004).

Clupeiform fishes such as shads and herrings present an unusual case among teleost ears. These fishes, unlike any other vertebrate studied to date, have a specialized three-part utricle that is

used, at least in part, for detecting auditory stimuli (Denton et al., 1979; Blaxter et al., 1981). While some Clupeiformes have auditory sensitivities and bandwidths similar to the fishes discussed above (Mann et al., 2001; 2005), others, including the American shad, are unique in that they can respond to ultrasound signals up to 180 kHz (Mann et al. 1997; 1998; 2001). The specific mechanism of ultrasound detection in this animal is unknown.

The presence of myo6 in the majority of fish and frog hair bundles suggests a novel role for this protein, one not seen in mammalian hair bundles that lack Myo6. The upregulation of myo6 throughout the bundle in some fish and frog end organs implies that it may have an additional role in these hair cells. Myo6 plays multiple roles in endocytosis in some cells (Hasson, 2003) and may have distinct structural and functional roles in the Golgi complex of fibroblasts (Warner et al., 2003). It is therefore reasonable to suggest that myo6 may have a functional as well as a structural role, possibly membrane trafficking or receptor-mediated endocytosis, in most fish hair bundles.

It is noteworthy that in fishes, myo6-positive hair bundles are found in epithelia with relatively low frequency responses (i.e., below 4 kHz) while the only fish hair bundles completely lacking myo6 are found in a putative ultrasound detector in fishes, the utricle of the American shad. Myo6 labeling in the oscar utricle was faint, however, and it is possible that this labeling was artifact. While the function of the oscar utricle has not been specifically studied, it is likely a low-frequency detector similar to that found in other fishes without accessory auditory specializations (Popper and Fay, 1999).

The possible role of the utricle in ultrasound detection is not understood, nor is the contribution of each of the three utricular maculae to auditory detection. The absence of myo6 in the bundles of hair cells in the shad anterior and posterior utricle may highlight unique qualities of these hair cells among fishes and is an interesting avenue for future study. An ABR study demonstrated that gulf menhaden (*Brevoortia patronus*) are also sensitive to ultrasound up to 180 kHz, while other clupeiform fishes such as bay anchovies (*Anchoa mitchilli*) do not respond to auditory stimuli above 4 kHz (Mann et al., 2001). Examination of myo6 distribution in the utricles of clupeiforms with differing auditory responses are necessary to substantiate the hypothesis that myo6 distribution in shad utricular hair cells is correlated with ultrasound detection.

4.3. Actin organelle in sea lamprey hair cells

All hair cells in the sea lamprey inner ear contain an unusual actin-rich cytoplasmic organelle. This organelle was first noted in *Lampetra fluviatilis* by Löwenstein and Osborne (1964) but was suggested to be endoplasmic reticulum based on its striated appearance. As described by Popper and Hoxter (1987), this organelle extends basally from just below the cuticular plate at the apical surface of the cell to the basal region of the cell body. Interestingly, a similar actin-rich structure is seen in cochlear inner hair cells and vestibular hair cells of the *Shaker2* mutant mouse (Probst et al., 1998). *Shaker2* is a *Myo15a* mutant with extremely short stereocilia and congenital deafness. Myo15a localizes to the tips of normal hair bundles and is proposed to function in organization of the hair bundle staircase (Belyantseva et al., 2003a). *Myo15a* mutant hair cells do not form a normal staircase, suggesting that actin filaments form improperly in these mutants (Probst et al., 1998; Anderson et al., 2000). While sea lamprey hair bundles appear to have a normal staircase shape, the presence of this unusual actin structure suggests that the hair cells in this species may lack myo15a, or that it may function differently in normal lamprey hair cells as compared to hair cells of jawed vertebrates. If myo15a is present in lamprey hair cells, characterizing this protein in lamprey may help clarify its function(s). Such information may shed light on pathological mechanisms in mammalian *Myo15a* mutants.

4.4. Conclusions

Myosin VI and myosin VIIa are present in all vertebrate hair cells studied to date, underscoring the importance of these proteins in auditory and vestibular function and suggesting ancient evolution of this hair cell feature. Future studies should focus on myosin distribution in other chordate mechanoreceptors in order to better understand the evolution of these complex cells. The recent discovery of hair cell-like structures in the coronal organ of urochordates (Burighel et al., 2003) provides a useful platform for further comparative work.

As all fish hair cells contain these critical proteins, fishes may serve as useful models for future studies of human hereditary deafness. Furthermore, studies in fishes such as the American shad that have end organ-specific differences in myo6 distribution might help parse out the range of functions of this important hair cell protein. Finally, comparative cellular and molecular studies between American shad and other clupeiform fishes may help to unravel the mechanism of ultrasound detection in this species.

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Fig. 1. Simplified phylogenetic relationships between the species used in this study. The lineage leading to *Xenopus* also contains all other tetrapods as well as the sarcopterygian (lobe-finned) fishes. Based on Nelson (1994). Taxonomic names are given in the text.

Fig. 2.

Antibody characterization and controls. (A) Myo6 western blot. Myo6 antibody labels one band of the expected molecular weight in mouse, *Xenopus* and zebrafish (~150 kDa). (B) Myo7a western blot. Anti-myo7a labels a single band of ~200 kDa in zebrafish and American shad. This band corresponds to the smaller of two labeled bands obtained from mouse cochlea. (C–H) Epitope expression in cultured cells demonstrating antibody binding to zebrafish myosins. GFP-labeled cells are positively transfected with the specific construct (myo6a, myo6b, or myo7a, see below), while GFP-negative cells were not transfected and therefore serve as negative controls for immunolabeling. (C–E) myo6a, (F) myo6b, (G) myo7a, (H) control. (C) Myo6a-transfected NIH-3T3 cell, shown here expressing GFP. (D) The myo6a-transfected cell from panel C, immunolabeled for myo6. This demonstrates that the myo6 antibody binds to zebrafish myo6a. (E) Triple-labeled myo6a-transfected cell showing GFP expression from panel C (green), myo6 immunocytochemistry from panel D (red) and DAPI labeling (blue), which shows the successfully transfected cell surrounded by non-transfected cells. (F) Triple-labeled (anti-GFP, anti-myo6, DAPI) NIH3T3 cell expressing myo6b. This cell demonstrates that the myo6 antibody also binds zebrafish myo6b. (G) Triple-labeled (anti-GFP, anti-myo7a, DAPI) HEK293 cells transfected with myo7a, showing that the myo7a antibody binds zebrafish myo7a. (H) NIH3T3 cell transfected with myo6b but not labeled with anti-myo6, showing that GFP fluorescence does not bleed-through to red fluorescent channels. (I–K) Immunocytochemistry controls in whole-mount inner ear epithelia. Each panel is a merged image of phalloidin (green) and anti-myo6 (red) images. (I) Zebrafish utricle processed for myo6 immunocytochemistry but with primary antibody omitted, demonstrating no bleed-through of the phalloidin fluorescence to the red channel. (J) American shad utricle labeled

with anti-myo6 but with the secondary antibody omitted. The plane of section is at the cuticular plate level, showing no myo6 staining in this region. (K) American shad utricle with clear myosin labeling in the cuticular plate region. Phalloidin was omitted, showing that myo6 staining is genuine and not caused by bleed-through from the green channel. Scale bar in C is 25 μm and applies to panels C–H. Scale bars in I–K are 2 μm .

Fig. 3. Myosin VIIa immunofluorescence in hair cells of (A–C, whole mount epithelium) oscar lagena and (D–F, confocal cross-section) lake sturgeon utricle. The left column is actin labeled with phalloidin (green), the right column in myo7a immunofluorescence (red), and the middle column is the merged image. Arrows in C and F indicate hair bundles immunolabeled for myo7a. Panel F also shows cytoplasmic labeling, which is consistent across species and end organs. Scale bars are 2 μ m.

Fig. 4.

Myosin distribution in sea lamprey hair cells. (A–B) myo6 and (C–D) myo7a labeling in the macula communis. Only the merged phalloidin/myosin images (panels A and C) and myosin labeling (panels B and D) are shown. Both myo6 and myo7a are present in all hair bundles of the sea lamprey inner ear, which is indicated by arrows in panels B and D. (E) Intracellular actin-rich projection (arrows) in lamprey hair cells, labeled with phalloidin (green). Arrowheads point to the stereocilia at the apical cell surface. Brightness and contrast were adjusted to highlight stereocilia and the actin organelle, the actin cytoskeleton is not apparent in this image. Cell bodies are labeled with anti-myosin (red), which labels the cytoplasm but not the nuclei. All scale bars are 2 μm.

Arrowheads point to the stereocilia at the apical cell surface. Brightness and contrast were adjusted to highlight stereocilia and the actin organelle, the actin cytoskeleton is not apparent in this image. Cell bodies are labeled with anti-myosin (red), which labels the cytoplasm but not the nuclei. All scale bars are 2 μm.

Fig. 5. Myosin VI distribution in fish and *Xenopus* utricular hair cells. (A–C) Lake sturgeon, (D–F) zebrafish, (G–I) oscar, (J–L) *Xenopus*. Arrows in C, F, I, and L indicate hair bundles that are immunolabeled for myo6. In the oscar utricle (G–I), this staining is very faint and may be artifact. All images are from whole-mount epithelia but cryosectioned tissue shows identical labeling patterns (data not shown). All scale bars are 2 μ m.

Fig 6. Myo6 immunolabeling in American shad inner ear epithelia. (A–B) saccule, (C–D), lagena, and (E–F) lateral canal crista. Labeling in these epithelia was consistent across species, where myo6 was seen in all hair bundles (arrows indicate some examples). All shad images were obtained using identical laser output (here and in Fig. 7). Scale bars in A and E are 2 μ m, scale bar in C is 1 μ m.

Fig. 7. Hair cells from the tri-partite shad utricle labeled with phalloidin and anti-myosin VI. (A–B) Anterior macula, where myosin VI is present in the cuticular plate region but not in hair bundles. (C–D) Middle macula, where hair bundles do contain myosin VI (arrows). (E–F) Posterior macula, which shows an identical myosin VI distribution to the anterior macula. Arrowheads in (B and F) point to bundles that are not labeled with anti-myosin VI. Scale bars in A and C are 2 μm , scale bar in E is 1 μm .

Table 1

Primers for epitope expression in cultured cells. Start codons in forward primers and stop codons in reverse primers are indicated in bold. Restriction sites are in gray.

Gene	Primer
myo6a	For: GATCGAATTC AT GGTTGCTCCACCACAAAAGCTCAAGAGCTT Rev: GATCGTCG ACT CAGTTCCTCAGGTACTGGATTCCC
myo6b	For: GATCGAATTC AT GGCCCAGAACGAGGCAGAACT Rev: GATCGTCG ACT CAAGCATTCTTCAAGTATTGGAT
myo7a	For: CTCGAG AT GTACAAACGACTCAAAGGAGAGTAC Rev: GGATCCT CAG TAGGTTTCTTTCCGAGTG