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Raphaël Etournay[1,*], Aziz El-Amraoui[1,*], Amel Bahloul[1], Stéphane Blanchard[1], Isabelle Roux[1], Guillaume Pézeron[1], Nicolas Michalski[1], Laurent Daviet[2], Jean-Pierre Hardelin[1], Pierre Legrain[3] and Christine Petit[1,‡]

[1]Unité de Génétique des Déficits Sensoriels, INSERM U587, Institut Pasteur, 25 rue du Dr Roux, 75015 Paris, France
[2]Hybrigenics, 3-5 impasse Reille, 75014 Paris, France
[3]Département de Biologie Joliot-Curie, CEA, 91191 Gif-sur-Yvette, France

*These authors contributed equally to this work
‡Author for correspondence (e-mail: cpetit@pasteur.fr)

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Summary

By using the yeast two-hybrid technique, we identified a candidate protein ligand of the myosin 1c tail, PHR1, and found that this protein can also bind to the myosin VIIa tail. PHR1 is an integral membrane protein that contains a pleckstrin homology (PH) domain. Myosin 1c and myosin VIIa are two unconventional myosins present in the inner ear sensory cells. We showed that PHR1 immunoprecipitates with either myosin tail by using protein extracts from cotransfected HEK293 cells. In vitro binding assays confirmed that PHR1 directly interacts with these two myosins. In both cases the binding involves the PH domain. In vitro interactions between PHR1 and the myosin tails were not affected by the presence or absence of Ca^{2+} and calmodulin. Finally, we found that PHR1 is able to dimerise. As PHR1 is expressed in the vestibular and cochlear sensory cells, its direct interactions with the myosin 1c and VIIa tails are likely to play a role in anchoring the actin cytoskeleton to the plasma membrane of these cells. Moreover, as both myosins have been implicated in the mechanotransduction slow adaptation process that takes place in the hair bundles, we propose that PHR1 is also involved in this process.

Key words: Myosin 1c, Myosin VIIa, PHR1, Inner ear, Hair cell

Introduction

Genetic studies have highlighted the crucial roles of unconventional myosins in the inner ear. Indeed, mutations in the genes encoding myosin 1a, myosin IIIa, myosin VI, myosin VIIa, and myosin XVa cause hearing loss in humans and/or mice (reviewed by Petit et al., 2001; Frolenkov et al., 2004) (see also the websites for Hereditary Hearing Loss, http://www.uia.ac.be/dnalab/hhh/ and the Institute of Hearing Research, http://www.ihr.mrc.ac.uk/hereditary/). In deaf mouse mutants lacking myosin VI (Snell's waltzer) (Avraham et al., 1995), myosin VIIa (shaker-1) (Mburu et al., 1997) or myosin XVa (shaker-2) (Probst et al., 1998), the hair bundles of cochlear and vestibular sensory cells (hair cells) are abnormal, thus indicating that these myosins are involved in the development of this mechanoreceptor organelle composed of 20 to 300 actin-filled stiff microvilli, called the stereocilia, arranged in three to four rows of increasing height.

The tails of unconventional myosins are highly divergent and determine the molecules and the structures to which the force of the motor head is applied. The identification of the molecules that bind to myosin tails is thus likely to provide clues about the roles of the various myosins that are present in a given cell type, such as the hair cell. Myosin 1c and myosin VIIa have been detected throughout the cell soma of rodent vestibular and cochlear hair cells. Both myosins are particularly abundant at the cell apical region around the cuticular plate (Hasson et al., 1997; Dumont et al., 2002), a region filled with vesicles that are likely to be involved in exo- and endocytosis exchanges between the cytoplasm and the apical plasma membrane (Kachar et al., 1997). In addition, these two myosins are present in the developing and mature hair bundles (El-Amraoui et al., 1996; Hasson et al., 1997; Dumont et al., 2002). The identification of two myosin VIIa tail ligands that are present in the stereocilia, vezatin and harmonin b, already led us to propose two functions for this motor molecule in the differentiating hair bundle. Myosin VIIa may exert a tension force on a subset of interstereociliar basal links, by directly interacting with the transmembrane protein vezatin (Kussel-Andermann et al., 2000). In addition, myosin VIIa may convey harmonin b, a protein containing a PDZ domain, as the protein is mislocated in myosin VIIa defective mutants (Boëda et al., 2002). No protein ligand of the myosin 1c tail has been identified so far, thus precluding a molecular deciphering of the cell mechanisms in which this myosin is involved. These include the mechanotransduction slow adaptation process that takes place in the stereocilia of mature hair cells (Holt et al., 2002). Here, we show that PHR1, an integral membrane protein expressed in the hair cells (Xu et al., 1999; Xu et al., 2004), directly interacts with the myosin 1c and myosin VIIa tails, and is able to dimerise. We discuss

the functional implications of these findings in the inner ear sensory cells.

Materials and Methods

Yeast two-hybrid screenings

To generate the inner ear two-hybrid cDNA library, the vestibular sensory epithelia from postnatal day 2 to 6 (P2-P6) mice were used as described (Boëda et al., 2002). Random-primed cDNAs were constructed in pP6 derived from the original pGADGH (Bartel et al., 1993) using the two-hybrid system to detect protein-protein interactions (Hartley, 1993). Yeast two-hybrid screenings were carried out as described (Rain et al., 2001; Colland et al., 2004), using three different baits, namely the tail of myosin 1c (myosin 1c IQ_4 tail; amino acids 762-1028), full-length PHR1a (aa 1-243) and PHR1b (aa 1-208) (Fig. 1A,B). Baits were PCR-amplified, then cloned into pB6, a vector derived from pAS2 (Fromont-Racine et al., 1997). The selectivity of the *HIS3* reporter gene was eventually modulated with 3-aminotriazole for each screening to obtain a maximum of 384 histidine-positive clones. The interacting 'prey' fragments of the positive clones were PCR-amplified and sequenced at their 5′ and 3′ junctions on a PE3700 Sequencer (Applied Biosystems). The resulting sequences were used to identify the corresponding gene in the GenBank database (NCBI) using a fully automated procedure.

Expression constructs

Using RACE-PCR, we isolated full-length cDNAs encoding myosin 1c (NM_008659), myosin VIIa (NM_000260) and the four PHR1 transcripts PHR1a (AF000272), PHR1b (AF101053), PHR1c (AF100613) and PHR1d (AF071000) from the mouse inner ear at P2 (see Fig. 1B). PCR-amplified fragments were cloned into pCR2.1-TOPO (Invitrogen). Their sequences were checked prior to transfer into the appropriate expression vector. Inserts were subcloned into pCMV-tag3B (myc tag, Stratagene) and pcDNA3 (No tag, Invitrogen) for in vitro translation and transfection experiments, and into pGEX-4T-1 (GST tag, Amersham) and pEGFP (Clontech) for protein production. The following PHR1 domains were subcloned: PHR1aΔC (aa 1-145), PHR1bΔC (aa 1-145), PHR1a.PH (aa 1-126), PHR1a.mid (aa 70-134), PHR1b.mid (aa 70-178, lacks the E7 domain), PHR1a.AP (aa 131-220) and PHR1.JMD (aa 166-221). The pGEX-PHR1a (aa 1-243) and pGEX-PHR1b (aa 1-208) fusion proteins were produced. The following myosin 1c domains were subcloned: pcDNA3-myo1c (aa 1-1028), pEGFP-myo1c IQ_4 tail (aa 762-1028), pGEX-myo1c IQ_4 tail (aa 762-1028) pCMV-myo1c IQ_4 tail (aa 762-1028); pGEX-myo1c T701 (aa 701-1028). A cDNA encoding the

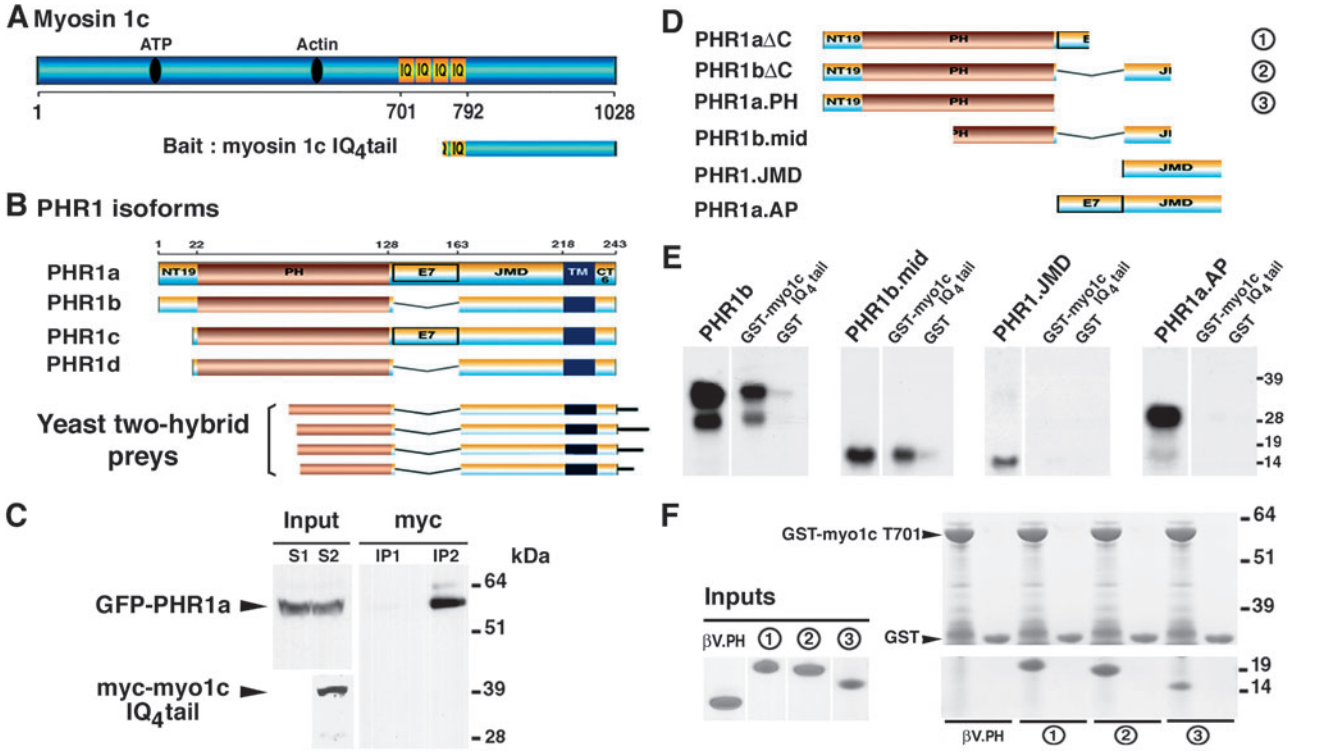

Fig. 1. Myosin 1c binds to PHR1. (A) Predicted structure of myosin 1c. The position of the yeast two-hybrid bait is indicated. (B) Predicted structure of the four PHR1 isoforms. PHR1a, the longest isoform, contains an N-terminal 19 amino acid peptide, a pleckstrin homology (PH) domain, the E7 domain encoded by the alternatively spliced exon 7, a juxtamembrane domain (JMD) that displays no significant similarity to any known protein, a transmembrane (TM) domain, and six putative extracellular residues (CT6). Numbers indicate amino acid positions (1-243) according to the PHR1a sequence. The overlapping cDNAs encoding the PHR1 fragments found to interact with the myosin 1c tail in the yeast two-hybrid system are shown. They all lack the E7 domain. (C) Co-immunoprecipitation of PHR1 and the myosin 1c tail. Extracts from cotransfected HEK293 cells producing both the GFP-tagged PHR1a (lane S1) and the myc-tagged myosin 1c IQ_4 tail (lane S2) were used. The proteins were co-immunoprecipitated by the antibody to myc (lane IP2). Extract from transfected cells producing GFP-PHR1 and the c-myc tag alone (lane S1) was used as a negative control (lane IP1). (D-F) In vitro binding assays. Mapping of the binding site of PHR1. Different fusion proteins shown in D were incubated with immobilised GST-tagged myosin 1c tail fragments (myo1c IQ_4 tail, aa 762-1028; and myo1c T701, aa 701-1028) or with GST. (E) The GST-myosin 1c IQ_4 tail binds to PHR1b and PHR1b.mid, but not to PHR1.JMD and PHR1a.AP. (F) Coomassie stained gel. GST-myosin 1c T701 binds to PHR1aΔC, PHR1bΔC, and PHR1a.PH. By contrast, it does not bind to the PH domain of βV spectrin (βV.PH). Positions of molecular mass markers are indicated in kDa on the right-hand side of blots.

myosin VIIa prey (myo7a prey; aa 934-1071) was subcloned into pCMV-tag3B and pGEX-4T-1. pEGFP-myosin VIIa-tail and pEGFP-myosin VIIa/MyTH4 FERM (aa 1690-2215) were also used (Boëda et al., 2002). The constructs encoding myosin XVa/MyTH4 FERM (aa 3071-3511), and βV spectrin.PH domain (aa 3533-3641) were kindly provided by B. Delprat (Pasteur Institute, Paris, France) and K. Legendre (Pasteur Institute, Paris, France), respectively. Constructs encoding myosin VIIa/SH3 MyTH4 FERM (aa 1605-2215), myosin VIIa/SH3 MyTH4 (aa 1605-1907) were kindly provided by I. Zwaenepoel (Pasteur Institute, Paris, France).

Immunocytofluorescence analysis

HeLa cells were transfected using Effectene reagent (Qiagen), following the manufacturer's instructions. Immunocytofluorescence analysis was carried out on fixed cells as described (Kussel-Andermann et al., 2000). We used an anti-myc mouse monoclonal antibody (clone 9E10, Santa Cruz) and secondary antibodies coupled with Alexa 488 or Alexa 568 (Amersham, Molecular Probes). Cells were analysed with a laser-scanning confocal microscope (LSM-540, Zeiss).

Co-immunoprecipitation experiments

Human embryonic kidney HEK293 cells were transfected using Lipofectamine Plus Reagent (Invitrogen), according to the manufacturer's instructions. The cell pellets were solubilised by sonication in PBS, complemented with EDTA-free cocktail of protease inhibitors (Roche). Cell extracts were prepared using 0.1% Triton X-100, and 0.1% sodium deoxycholate in PBS, pH 7.4. Immunoprecipitations were done using either polyclonal anti-GFP antibodies (Invitrogen) or monoclonal anti-c-myc antibodies preincubated with protein G-agarose (Pharmacia). The immunoprecipitates were electrotransferred to nitrocellulose sheets and probed with anti-c-myc (1:500 dilution), anti-myosin VIIa (1:3000), or anti-GFP (1:5000) antibodies. Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies (Amersham) and the ECL chemiluminescence system (Pierce) were used for detection.

Production and purification of GST-tagged proteins

GST-tagged proteins were produced in BL21 bacteria. For all PHR1 proteins and the PH domain of βV spectrin, the production was triggered with 0.5 mM IPTG at an OD_{600nm} of 0.8. After 2 hours incubation at 30°C, cells were collected and centrifuged 20 minutes at 4°C. The pellet was washed once in PBS and frozen in liquid nitrogen. After cell lysis in PBS with 1% Triton X-100, 1 mM DTT, 300 mM NaCl, and protease inhibitor cocktail, supernatants were incubated with glutathione–Sepharose beads (Pharmacia) for 2 hours at 4°C, then washed three times with a saline buffer (0.1% Triton X-100, 1 mM DTT, in PBS supplemented with 50 mM NaCl). The proteins of interest were retrieved from GST-fusion proteins by thrombin cleavage (Sigma). Thrombin was then eliminated by using benzamidine-agarose beads (Sigma). For myosin 1c tail production, 0.1 mM IPTG was added at an OD_{600} of 0.6, and cells were incubated overnight at 18°C. Soluble proteins were treated as described above.

In vitro binding experiments

The in vitro binding assays were carried out using purified GST-tagged fusion proteins. Each binding experiment was done at least in triplicate. In one series of experiments, ^{35}S-labelled proteins were translated in vitro with the T3/T7-coupled transcription–translation system (Promega) according to the manufacturer's instructions. To test the myosin 1c tail-PHR1b interaction, the same amount of GST-tagged myosin 1c IQ_4 tail or GST alone (2 μg), was incubated with pre-equilibrated glutathione-Sepharose beads for 90 minutes at 4°C. The beads were washed three times with binding buffer (5% glycerol, 5 mM $MgCl_2$, 0.1% Triton X-100, in PBS, pH 7.4) supplemented with EDTA-free protease inhibitor cocktail, and then incubated with the in vitro translated ^{35}S-labelled PHR1b for 3 hours, at 4°C on a rotating wheel. The beads were then washed four times with binding buffer supplemented with 150 mM NaCl and bound proteins were resuspended in 30 μl of 2× SDS sample buffer. Samples were then analysed on a 4-12% SDS-polyacrylamide gel. The gel was fixed, dried and exposed to a Biomax film (Kodak).

In a second series of experiments GST-tagged fusion proteins were purified on glutathione-Sepharose beads and then either eluted with 20 mM reduced glutathione at pH 8 or cleaved by thrombin. To test the interaction between the myosin 1c tail and different PH-containing domains of PHR1b, the same amount (3 μg) of purified GST-tagged myosin 1c tail (T701) or GST alone was incubated with pre-equilibrated glutathione-Sepharose beads for 90 minutes at 4°C. Purified and cleaved PHR1b domains (3 μg) were incubated overnight with either GST-tagged myosin 1c T701 or GST alone, in a saline buffer (PBS containing 5% glycerol, 0.1% Triton X-100, and complete protease inhibitor cocktail). The beads were then washed five times with binding buffer supplemented with 150 mM NaCl, and bound proteins were resuspended in 30 μl of 2× SDS sample buffer. Samples were then analysed on a 4-12% SDS-polyacrylamide gel stained with Coomassie Brilliant Blue R250.

For the PHR1 dimerisation test, the full-length GST-tagged PHR1a protein was produced as described above, then purified on glutathione-Sepharose beads and eluted in 20 mM reduced glutathione. The purified PHR1a extract was loaded on a gel filtration column Superdex™ 75 prep grade (Amersham). The following molecular mass standards were also applied to the column for calibration: bovine serum albumin (66 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa), ribonuclease A (13.7 kDa). Confidence intervals for the molecular mass values were calculated from the regression curve. In order to check the integrity of the purified PHR1a extract, we carried out mass spectroscopy and Edman sequencing. ESI-MS measurements were performed on ESI-triple quadrupole API365 (MDS-Sciex, Toronto, Canada). The N-terminal sequencing was performed on an ABI 494 (USA) machine.

Results and Discussion

PHR1, an integral membrane protein, directly interacts with the myosin 1c tail

Myosin I isoforms are widely expressed in invertebrates and vertebrates. They can be grouped into two phylogenetically distinct subclasses. Subclass 1 isoforms have long tails that contain basic (TH1), proline-rich (TH2) and Src homology-3 (SH3) domains, whereas the subclass 2 isoforms contain only a short TH1 tail domain. Myosin 1c belongs to subclass 2. Myosin 1c consists of a motor head domain with actin- and ATP-binding sites, a neck region containing three IQ motifs, and a short basic tail domain (Fig. 1A). The three IQ domains bind to Ca^{2+}-free calmodulin with high affinity. A fourth IQ motif that seems to bind to calmodulin with a very low affinity has also been reported (Gillespie and Cyr, 2002). In order to identify proteins that interact with myosin 1c in the inner ear, we used a fragment encompassing the fourth IQ motif and the entire C-terminal basic tail domain (i.e. amino acids 762-1028, Fig. 1A), hereafter referred to as myosin 1c IQ_4 tail, to screen a P2-P6 mouse inner ear library (see Materials and Methods), in the yeast two-hybrid system.

We identified 71 clones by histidine nutritional selection and β-galactosidase (β-gal) activity. The inserts from the isolated clones were sequenced, leading to the identification of four

overlapping clones that encode the C-terminal region of a protein named PHR1 (Krappa et al., 1999; Xu et al., 1999) (Fig. 1B). *PHR1* is abundantly expressed in mature photoreceptor cells, cochlear and vestibular hair cells, and olfactory receptor neurons. In the cochlea *PHR1* is also expressed in a subset of interdental cells at the surface of the spiral limbus (Xu et al., 2004). Immunohistochemistry and chemical extraction studies on the photoreceptor outer segment have shown that PHR1 is an integral membrane protein (Xu et al., 1999). PHR1 contains an N-terminal pleckstrin homology domain (Krappa et al., 1999; Xu et al., 1999). Pleckstrin homology (PH) domains are 100- to 120-amino acid modules best known for their ability to bind to phosphoinositides (Lemmon et al., 2002). Unlike other PH domains (Rebecchi and Scarlata, 1998), that of PHR1 lacks most of the eight conserved residues known to coordinate binding to phosphoinositides (Xu et al., 1999). The use of two promoters and alternative splicing of exon 7 lead to four different *PHR1* transcripts (Krappa et al., 1999; Xu et al., 1999), hereafter referred to as *PHR1a-d*. PHR1a and PHR1b both result from the use of the more upstream promoter. They contain a 19-amino acid N-terminal peptide (NT19), which is absent from PHR1c and PHR1d. In addition, PHR1a and PHR1c contain a 36-amino acid segment encoded by exon 7, hereafter referred to as E7 (aa 131-166), whereas PHR1b and PHR1d do not (Fig. 1B). Thus, the PHR1 sequence can be subdivided in six different regions, namely NT19 (in PHR1a and PHR1b), PH domain, E7 (in PHR1a and PHR1c), JMD, TM domain, and a short six amino acid C-terminal peptide CT6 (Fig. 1B). The overlapping sequences of the yeast two-hybrid PHR1 preys code for a peptide that extends between residues 70-208 of PHR1b (aa 51-189 of PHR1d), that is the C-terminal part of the PH domain and the entire JMD, TM and CT6 domains (Fig. 1B).

To confirm the interaction of PHR1 with myosin 1c, HEK293 cells were cotransfected with plasmids encoding the GFP-tagged PHR1a and c-myc-tagged myosin 1c IQ_4tail (see Materials and Methods). Cell extracts were incubated with an anti-c-myc antibody. PHR1a co-immunoprecipitated with the myosin 1c IQ_4 tail. Co-immunoprecipitation was not observed when the c-myc-tagged myosin 1c IQ_4 tail was substituted by c-myc tag alone (Fig. 1C).

To assess a possible direct interaction between the two proteins, we carried out in vitro binding assays. Using in vitro translated ^{35}S-labelled proteins, we found that full length PHR1a, PHR1b and PHR1d bound to the GST-tagged myosin 1c IQ_4 tail (Fig. 1E and data not shown). In the reciprocal binding experiment, either full-length PHR1a or PHR1b fused to GST did bind to ^{35}S-labelled full-length myosin 1c (Fig. 2 and data not shown). To define the myosin 1c-interacting region of PHR1, three different PHR1 fragments, namely the C-terminal part of the PH domain (PHR1b.mid; aa 70-178) and the downstream region containing (PHR1a.AP; aa 128-220) or lacking (PHR1.JMD; aa 166-221) the E7 fragment (Fig. 1D), were incubated with the GST-myosin 1c IQ_4 tail or with GST alone. PHR1b and PHR1b.mid, but not PHR1a.AP or PHR1.JMD, bound to the GST-myosin 1c IQ_4 tail (Fig. 1E). Based on these results, bacterially expressed untagged PHR1a fragments (Fig. 1D) obtained by cleavage of the GST-fusion protein with thrombin, were incubated either with the GST-tagged myosin 1c T701 (aa 701-1028) corresponding to the entire neck and tail part of myosin 1c or with GST alone. PHR1aΔC (aa 1-145), PHR1bΔC (aa 1-145) and PHR1a.PH (aa 1-126) fragments did interact with GST-tagged myosin 1c T701, but not with GST alone. Under the same experimental conditions, the PH domain of βV spectrin (aa 3533-3641) did not bind to GST-myosin 1c T701 (Fig. 1F). We conclude that the myosin 1c tail interacts with PHR1 via its PH domain.

All myosin I TH1 tail domains have net positive charges and many, including that of myosin 1c, have been shown to interact with phospholipids in vitro (Titus, 2000; Tang et al., 2002). In addition, myosin 1c directly interacts with PtdIns(4,5)P_2 (PIP_2) and other anionic phospholipids via its IQ domains (Hirono et al., 2004). Both interactions have only been detected in the presence of high Ca^{2+} concentrations (Tang et al., 2002; Hirono et al., 2004), however, and they may not fully account for the subcellular targeting of myosin 1c. Therefore, the proposed anchoring of myosin 1c to the plasma membrane (Cyr et al., 2002) may also involve integral membrane protein(s). Based on the present results, we suggest that PHR1 is one of these. In order to test whether the free Ca^{2+} concentration can affect the interaction between myosin 1c tail and PHR1, we carried out the in vitro binding experiments in the presence of either 100 μM EGTA (a calcium-chelating agent) or a 50 μM free Ca^{2+} concentration, two conditions designed to mimic low- and high-Ca^{2+} environments that may be encountered by myosin 1c in the hair cell bundle (Gillespie and Cyr, 2002). The PHR1-myosin 1c interaction was detected under both conditions (Fig. 2A). It has been found that calmodulin completely blocks the targeting of the myosin 1c neck-tail

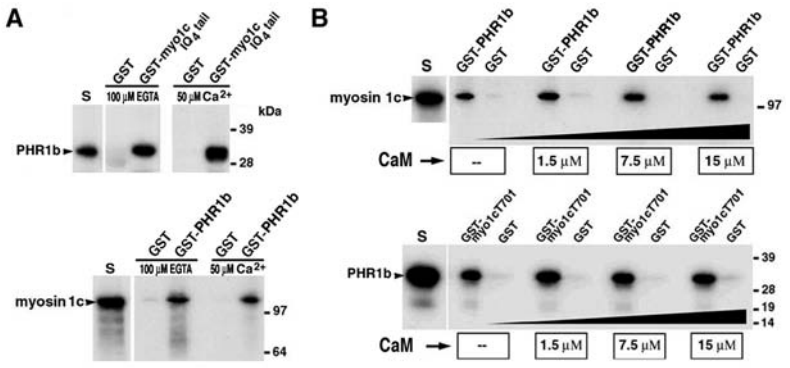

Fig. 2. Effect of Ca^{2+} and calmodulin on the myosin 1c-PHR1 interaction. (A) Effect of the Ca^{2+} concentration. Myosin 1c IQ_4 tail or GST alone was incubated with [^{35}S]PHR1b in the presence of 100 μM EGTA or 50 μM Ca^{2+}. PHR1b interacts with the tail of myosin 1c in both cases (upper panel). In the reciprocal experiment, full-length myosin 1c binds to GST-PHR1b in the presence of 100 μM EGTA or 50 μM Ca^{2+} (lower panel). (B) Effect calmodulin (CaM). GST-PHR1b binds to ^{35}S-labelled full-length myosin 1c in the absence (–) or presence of calmodulin (1.5, 7.5 or 15 μM) (upper panel). In the reciprocal experiment, GST-myo1c T701 or GST was incubated with ^{35}S-PHR1b in the absence or presence of calmodulin (1.5, 7.5 or 15 μM). The myosin 1c IQ_4 tail-PHR1b interaction is not affected by any of the calmodulin concentrations tested (lower panel). Positions of molecular mass markers are indicated in kDa on the right-hand side of blots.

fragment (myosin 1c T701) to a putative 'membrane receptor' in the hair bundle (Cyr et al., 2002), possibly PtdIns(4,5)P_2 (Hirono et al., 2004). In addition, increasing the Ca^{2+} concentration has been shown to weaken calmodulin association to the myosin 1c IQ motifs (Gillespie and Cyr, 2002). This would result in conformational changes of the motor head-neck region that would in turn prevent the coupling between energy released by ATP hydrolysis and the creation of a tension force exerted on the membrane. We tested whether the binding of PHR1 to myosin 1c is modulated by calmodulin in the presence of low or high free-Ca^{2+} concentrations. Calmodulin (1.5-15.0 μM) failed to prevent the PHR1-myosin 1c interaction in the presence of 100 μM EGTA (Fig. 2B) or 50 μM Ca^{2+} (data not shown). Together, these results suggest that myosin 1c associates to the plasma membrane both in Ca^{2+}/calmodulin-dependent (Cyr et al., 2002; Hirono et al., 2004) and Ca^{2+}/calmodulin-independent (this study) ways that could involve lipid and protein ligands respectively.

PHR1 interacts with the myosin VIIa tail

In order to find additional PHR1-interacting partners in the hair cell, we used full-length PHR1b as the bait in a yeast two-hybrid screening of the same mouse inner ear library. Sequence analysis of the clones positive for *HIS3* and *LacZ* revealed a single myosin VIIa clone (myo7a prey; aa 934-1071). This clone encodes a short tail fragment that partially overlaps with the first 54 amino acids of the first MyTH4 domain (see Fig. 3A). Extracts from HEK293 cotransfected cells producing the entire myosin VIIa tail and GFP-tagged PHR1a were immunoprecipitated by an antibody to GFP and the myosin VIIa tail was detected in the immunoprecipitate (Fig. 3B).

In vitro binding assays demonstrated direct interaction between PHR1b and the myosin VIIa original prey (138 aa tail fragment), the entire myosin VIIa tail or the full-length myosin VIIa (Fig. 3C,D). Both the absence of Ca^{2+} and increasing the Ca^{2+} concentration up to 500 μM did not affect the PHR1b-myosin VIIa tail interaction (Fig. 3C).

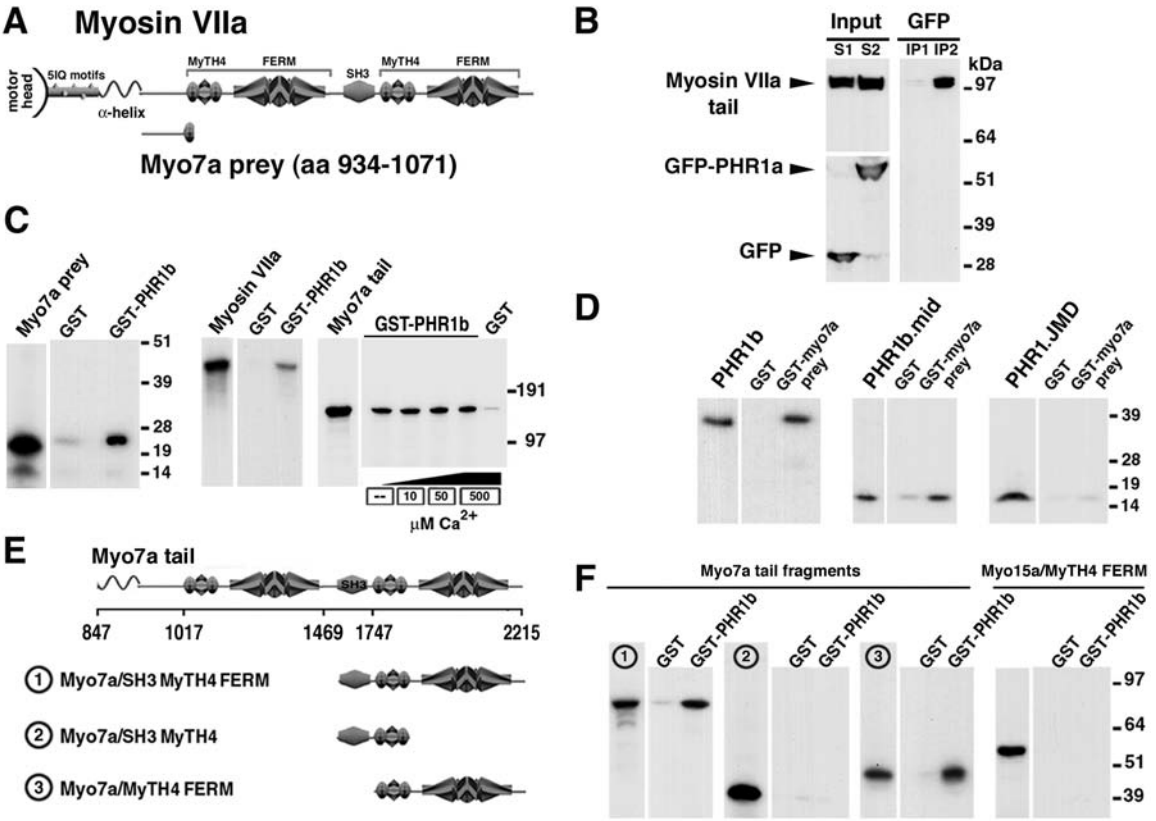

Fig. 3. Myosin VIIa binds to PHR1. (A) Predicted structure of myosin VIIa. The yeast two-hybrid myosin VIIa prey obtained using PHR1b as the bait is indicated. (B) Binding of the myosin VIIa tail to GFP-tagged PHR1a in cotransfected HEK293 cells. Protein extract from cotransfected HEK293 cells producing both the GFP-tagged PHR1a and the untagged-myosin VIIa tail (lane S2) was used for immunoprecipitation. The myosin VIIa tail and PHR1a are co-immunoprecipitated by the anti-GFP antibody (lane IP2). Extract from cotransfected cells expressing the myosin VIIa tail and GFP alone (lane S1) was used as a negative control (lane IP1). (C) GST-PHR1b binds to the ^{35}S-labelled myosin VIIa prey fragment, entire myosin VIIa tail, and full-length myosin VIIa. The PHR1-myosin VIIa tail interaction is affected by neither the absence of Ca^{2+} (–), nor high free Ca^{2+} concentrations (10, 50 or 500 μM). (D) In the reciprocal experiment, GST-tagged myosin VIIa prey fragment or GST alone was incubated with in vitro translated [^{35}S]PHR1b, [^{35}S]PHR1b.mid or [^{35}S]PHR1.JMD (see Fig. 1D). GST-myo7a prey binds to PHR1b and PHR1b.mid, but not to PHR1.JMD. (E,F) The myosin VIIa tail constructs (E) used for the in vitro binding assays in F are given a number (circled). GST-PHR1b or GST was incubated with different ^{35}S-labelled myosin VIIa protein fragments. GST-PHR1b binds to Myo7a/SH3 MyTH4 FERM and Myo7a/MyTH4 FERM, but not to Myo7a/SH3 MyTH4. Binding is not detected with the MyTH4 FERM C-terminal tail fragment of myosin XVa (Myo15a/MyTH4 FERM). MyTH4, myosin tail homology 4; FERM, 4.1, ezrin, radixin, moesin; SH3, src homology 3. Positions of molecular mass markers are indicated in kDa on the right-hand side.

To define the myosin VIIa-interacting region of PHR1, we carried out binding assays with different PHR1 fragments (see Fig. 1D). The GST-myosin VIIa prey bound to PHR1b.mid, but not to PHR1.JMD (Fig. 3D), thus indicating that PHR1 interacts with myosin VIIa via the PH domain.

Myosin VIIa consists of a motor head domain, five IQ motifs and a long tail (aa 847-2215). The tail contains an α-helix dimerisation domain, followed by two MyTH4 FERM repeats separated by an SH3 domain (Fig. 3A). In cotransfected HeLa cells producing c-myc-tagged PHR1b and a GFP-tagged myosin VIIa tail fragment (myosin VIIa/MyTH4 FERM; aa 1690-2215) lacking the myosin VIIa prey region, the two proteins colocalised in the cytoplasm (data not shown), suggesting the presence of an additional binding site in the C-terminal region of myosin VIIa. To define the PHR1-interacting regions of myosin VIIa, we carried out binding assays with different myosin VIIa tail fragments (Fig. 3E). GST-PHR1b bound to SH3 MyTH4 FERM (aa 1605-2215) and MyTH4 FERM (aa 1690-2215) C-terminal tail fragments of myosin VIIa. In contrast, GST-PHR1b did not bind to myosin VIIa/SH3 MyTH4 (aa 1605-1907), or to the FERM domain (aa 1896-2215) and MyTH4 domain (aa 1752-1890) alone. Finally, GST-PHR1b did not bind to the MyTH4 FERM C-terminal tail fragment (aa 3071-3511) of myosin XVa (Fig. 3F and data not shown). Together, these results indicate the presence of at least two PHR1-interaction sites within the myosin VIIa tail, namely one in the interdomain separating the dimerisation domain from the first MyTH4 and another in the C-terminal MyTH4 FERM.

PHR1 can form homodimers

We used full-length PHR1a as the bait in a third yeast two-hybrid screening of the inner ear library. We identified four clones that encode two overlapping regions of PHR1b/d, i.e. from amino acid number 70 to the C-terminal end (Fig. 4A). In in vitro binding assays, GST-tagged PHR1a interacted with the four isoforms, PHR1a-d (Fig. 4B and data not shown). Moreover, by using different PHR1 fragments, we were able to show that the JMD domain is sufficient for PHR1 homomerisation (Fig. 4B, lower panel). To confirm the ability of PHR1 to form homodimers we ran a gel filtration column with full length PHR1a purified from a GST-PHR1a fusion protein by thrombin cleavage (see Materials and Methods). In

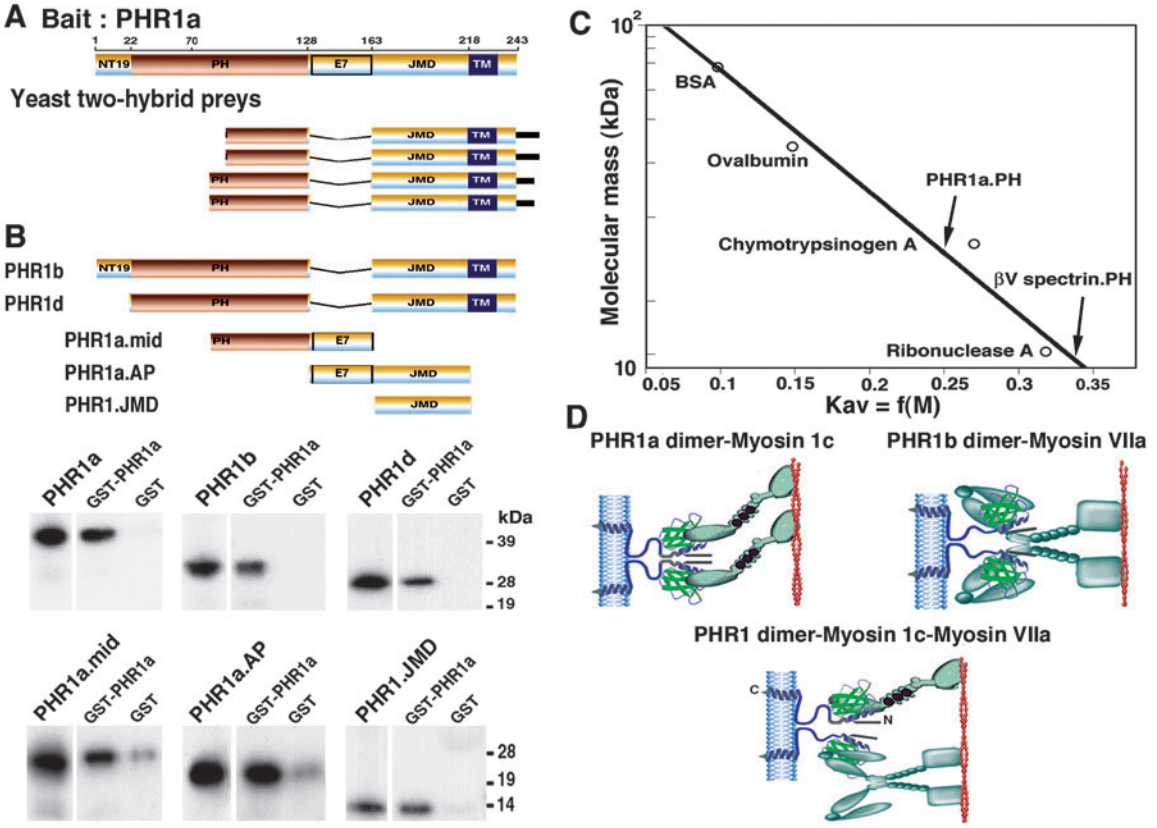

Fig. 4. PHR1 can form dimers. (A) Yeast two-hybrid PHR1 preys obtained using PHR1a as bait. (B) PHR1 isoforms can form heteromers. Top panel, PHR1 constructs used for the in vitro binding assay. Middle panel, GST-PHR1a or GST alone was incubated with full length [^{35}S]PHR1a, [^{35}S]PHR1b, or [^{35}S]PHR1d. GST-PHR1a interacts with every PHR1 isoform. Lower panel, PHR1.JMD is sufficient for PHR1 homomerisation. (C) Gel filtration analysis of the purified PH domains of PHR1a (PHR1a.PH) and βV spectrin (βV spectrin.PH). The figure shows the regression curve between the molecular mass and the partition coefficient (K_{av}) defined by $K_{av} = (Ve - Vo)/(Vt - Vo)$, where *Ve* is elution volume of the protein, *Vo*, column void volume and *Vt*, total bed volume. The molecular masses of eluted fractions are estimated according to the fractions corresponding to four molecular mass standards: bovine serum albumin (BSA), ovalbumin, ribonuclease A and chymotrypsinogen A. PHR1a.PH is eluted as a dimer, whereas βV spectrin.PH is eluted as a monomer. (D) Schematic diagram illustrating how a PHR1 dimer can recruit two myosin 1c, two myosin VIIa, or one myosin 1c and one myosin VIIa molecule to the plasma membrane.

parallel, we analysed the integrity of the PHR1a fragment by Edman sequencing and mass spectrometry in denaturing conditions and found that the C-terminus of the protein was degraded. The residual fragment has a predicted molecular mass of 15878±1 Da. It contains the N-terminal extension (NT19), the entire PH domain and ten amino acid residues of the E7 domain. The estimated molecular mass of 28±5 kDa that we obtained by gel filtration corresponds to a PHR1a dimer. In contrast, the elution fraction of the βV spectrin PH domain (predicted molecular mass, 12 kDa), that we used as a control, corresponds to an estimated molecular mass of 13±5 kDa, i.e. to monomers (Fig. 4C). These results indicate that PHR1 can form dimers in vitro.

As myosin 1c and myosin VIIa bind to the same region of PHR1, namely the PH domain, a given PHR1 molecule may not interact with both myosins at the same time. However, PHR1 dimers could interact simultaneously with two myosin 1c, two myosin VIIa, or one myosin 1c and one myosin VIIa molecule (see Fig. 4D). Myosin VIIa and myosin 1c are actin-based motors moving to the plus end of the filaments (Zhu et al., 1996; Inoue and Ikebe, 2003). Myosin VIIa is a two-headed motor protein that has been proposed to be processive (Inoue and Ikebe, 2003). Myosin VIIa has a much lower affinity for ATP than for ADP, which makes this myosin a good candidate to maintain a tension force at a given site (Inoue and Ikebe, 2003). Myosin 1c is a single-headed, non-processive motor protein. It is thus predicted to create only transient tension forces on its ligands, unless several myosin 1c molecules bind to the same ligand molecule (see Gillespie and Cyr, 2004). As a result of the PHR1 dimerisation, the bridging of several myosin 1c molecules is expected to increase the probability of creating a tension at a given plasma membrane location (see Fig. 5B). Interestingly, a cooperative role of two unconventional myosins has already been reported. Studies on *Dictyostelium* mutants have demonstrated that different myosins I play a cooperative role in the production of the resting cortical tension that is required for fluid macropinocytosis and cell motility (reviewed by Titus, 2000). Indeed, although *Dictyostelium* mutants lacking either a subclass-2 (*myoA*$^-$) or a subclass-1 (*myoB*$^-$) myosin I have normal levels of cortical tension, double myosin I mutants (*myoA*$^-$/*B*$^-$) display a 50% reduction in cortical tension (Dai et al., 1999; Titus, 2000).

PHR1 and hair cell vesicular trafficking

In the retina, pulse-labelling experiments in isolated frog photoreceptors, led Krappa and colleagues (Krappa et al., 1999) to suggest that PHR1 functions as a mediator of post-Golgi protein trafficking. By studying ciliogenesis in rat trachea epithelial cells, it has also been shown that PHR1 transcripts are upregulated upon stimulation of ciliary differentiation (Andrews et al., 2000). As myosin VIIa and

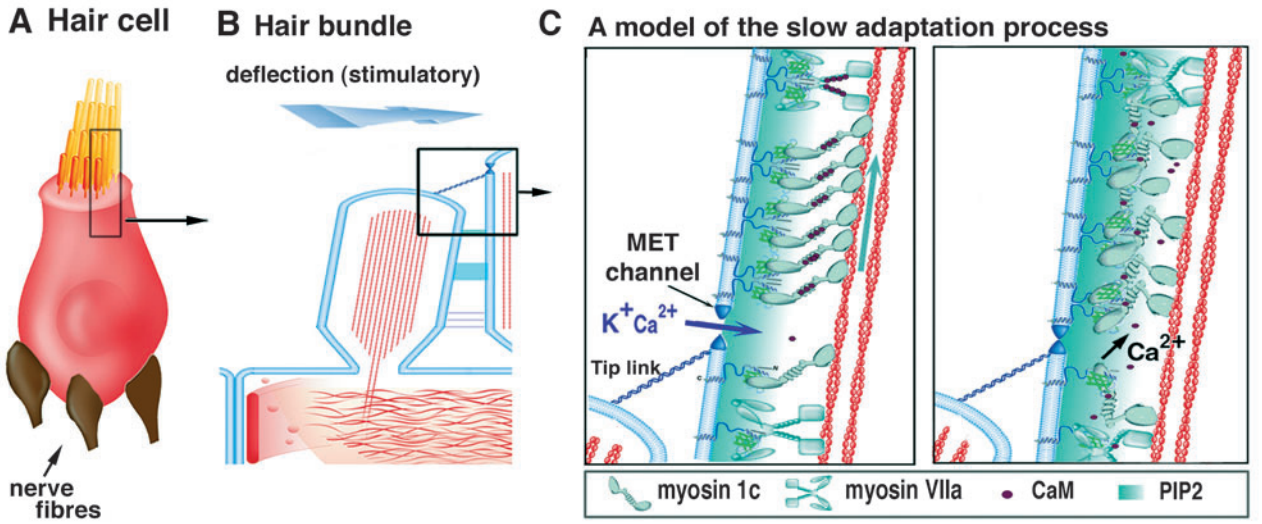

Fig. 5. Schematic representation of an inner ear sensory cell and proposed molecular model of the slow adaptation process. (A) The hair bundle is composed of 20-300 actin-filled stiff microvilli, called the stereocilia, arranged in three to four rows of increasing height. Stereocilia are held together by different types of lateral links. In addition, a single tip link joins the tip of each stereocilium to the lateral side of its taller neighbour in the adjacent row. (B) According to the gating spring hypothesis (Howard and Hudspeth, 1987), deflection of the hair bundle in the excitatory direction exerts a tension force on the tip links. The force is transmitted to the mechanoelectrical transduction (MET) channels (believed to be located close to the tip link insertion in the membrane), increasing their probability of being open. An influx of cations (mainly K^+ ions, but also Ca^{2+} ions) through the open MET channels depolarises the hair cell leading to neurotransmitter release and signalling to the central nervous system via afferent nerve fibres. (C) A model of the slow adaptation process evoked by sustained deflection of the hair bundle. The Ca^{2+} influx through the open MET channels triggers the adaptation process (see Fettiplace and Ricci, 2003; Gillespie and Cyr, 2004). The increase of the stereociliar Ca^{2+} concentration weakens calmodulin (CaM) binding to the myosin 1c IQ motifs, which in turn interact with anionic phospholipids [such as PtdIns(4,5)P_2 (PIP_2)] in the membrane. This also leads to the dissociation of myosin molecules from actin filaments. The resulting reduction in the tension exerted on the stereocilia membrane is thought to underlie the slow adaptation process, i.e. a decrease of the MET channel open probability while the mechanical stimulus is persisting. The PHR1-myosin complexes are expected to function as elastic molecular crosslinkers that contribute to and modulate the membrane tension. Unlike myosin 1c-phospholipids interactions, they may not be dependent on the local Ca^{2+} concentration.

myosin 1c are abundant in the hair cell apical region around the cuticular plate, a region characterised by a dense vesicular traffic (Kachar et al., 1997), these myosins, together with PHR1, might be involved in the sorting of proteins en route to the hair bundle.

Is PHR1 involved in the mechanotransduction slow adaptation process?

Owing to its location at the stereociliary tip, i.e. at the mechano-electrical transduction (MET) channel putative area, in amphibian hair cells (Metcalf, 1998; Steyger et al., 1998), myosin 1c has long been regarded as a good candidate for the adaptation motor of the hair cell (Hudspeth and Gillespie, 1994; Gillespie and Cyr, 2004). Indeed, a role of myosin 1c in the slow adaptation process has been demonstrated in postnatal day 1 to 8 (P1-P8) mouse vestibular hair cells, by using a chemical/genetic approach (Holt et al., 2002). Because cochlear hair cells from P2 shaker-1 mice adapt more rapidly than those from control mice, myosin VIIa may also play a role in the slow adaptation process (Kros et al., 2002). It is presently unknown whether the two myosins actually cooperate in all types of hair cell or whether myosin VIIa in cochlear hair cells plays a role equivalent to that of myosin 1c in vestibular hair cells (Gillespie and Cyr, 2004). Inhibitors of PtdIns(4,5)P_2 synthesis lead to defective hair cell mechanotransduction, and reduce the rates of fast as well as slow adaptation. Because myosin 1c directly binds to PtdIns(4,5)P_2 through its IQ domains, it has been proposed that PtdIns(4,5)P_2 is a component of the molecular complex underlying the slow adaptation process (Hirono et al., 2004). Upon deflection of the hair bundle by an excitatory stimulus, MET channels open and Ca^{2+} ion influx increases the Ca^{2+} concentration in the stereocilia, leading to calmodulin-myosin dissociation. The free IQ domains would then bind to anionic phospholipids such as PtdIns(4,5)P_2, thereby strengthening the interaction between myosin 1c and the membrane, whereas myosin head domains may detach from the core of actin filaments (Fig. 5B). At rest, when myosin 1c IQ domains are occupied by calmodulins, myosin molecules would bind to the stereocilia membrane through their tail domain (Hirono et al., 2004). Assuming that PHR1 is present at the tip of the stereocilia, this integral membrane protein presumably anchors myosin 1c and myosin VIIa to the membrane (Fig. 5B), in a way that may not be dependent on the local Ca^{2+} concentration (see Fig. 2 and Fig. 3C).

Despite the fact that the murine PHR1 is abundantly expressed in several types of sensory cells (i.e. olfactory, retinal, vestibular and cochlear cells) and various other cell types, an abnormal phenotype was not detected in *PHR1* null mouse mutants up to 1 year of age (Xu et al., 2004). In particular, these mice do not have an overt hearing impairment or balance defect. The apparent normal phenotype of *PHR1* null mice may reflect functional redundancy between PHR1 and other protein(s). PHR2, which has 57% amino acid identity with PHR1 in the PH domain and is expressed in many tissues (Krappa et al., 1999; Xu et al., 2004), is an obvious candidate, although it remains to be shown whether it is expressed in the same cell types as PHR1. However, refined analyses of the *PHR1* mutant mice in different genetic backgrounds and/or environmental conditions (for instance, chronic exposure to high sound levels), could help reveal subtle deficiencies of the balance and auditory functions. In conclusion, the *PHR1* null mutant is a valuable model to determine whether PHR1 is required for the proper targeting of myosin 1c and myosin VIIa in the hair bundle.

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